ORIGINAL ARTICLE

Light affects ascorbate content and ascorbate-related gene expression in tomato leaves more than in fruits

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Abstract Little is known about the light regulation of vitamin C synthesis in fruits. In contrast, previous studies in leaves revealed that *VTC2* (coding for GDP-L-galactose phosphorylase) was one of the key genes up-regulated by light in leaves. Our objective was to determine how the expression of ascorbate (AsA) synthesis genes in tomato (*Solanum lycopersicum*) was modified according to light irradiance in both leaves and fruits. Seven days of shading strongly decreased total ascorbate (reduced and oxidized form) content in leaves (50%) and to a lesser extent in fruits (10%). Among the last six steps of AsA biosynthesis, only two genes, *VTC2* and *GPP1* (one of the two unigenes coding for L-galactose-1-P phosphatase in tomato), were down-regulated by long-term shading in red ripe fruits, compared to seven genes regulated in leaves. This underlines that light affects AsA-related gene expression more in leaves than in ripening fruits. Moreover, this study reveals strong daily changes in transcript levels of enzymes of the AsA biosynthetic pathway in leaves (11 of the 12 studied genes showed significant changes in their expression pattern). Among those genes, we found that diurnal variation in transcript levels of *VTC2* and *GME1* correlated to leaf AsA content measured 8 h later. This study provides a new hypothesis on the role of *GME1* in addition to *VTC2* in light-regulated AsA biosynthesis.

Keywords Biosynthesis · Gene expression · Light · *Solanum* · Tomato · Vitamin C

Abbreviations

AsA	Ascorbate
DHA	Dehydroascorbate
Fv/Fm	Maximum quantum efficiency of PSII
GMP	GDP-D-mannose pyrophosphorylase
GME	GDP-D-mannose 3′,5′-epimerase
GGP	GDP-L-galactose phosphorylase
GPP	L-galactose-1-P phosphatase
L-GalDH	L-galactose dehydrogenase
GLDH	L-galactono-1,4-lactone dehydrogenase
PAR	Photosynthetically active radiation
PSII	Photosystem II
RT-QPCR	Real-time quantitative polymerase chain reaction
T-AsA	Total ascorbate (AsA + DHA)
UT	Universal time
WVA106	Tomato cultivar West Virginia 106

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Introduction

Plant vitamin C content is regulated by environmental factors and especially the light environment (Gatzek et al. 2002; Tabata et al. 2002; Tamaoki et al. 2003; Chen and Gallie 2004; Yabuta et al. 2008; Bartoli et al. 2009). The major biosynthetic pathway of AsA in plants (L-galactose pathway, Wheeler et al. 1998) involves ten enzymatic steps

(Fig. 1). The first six steps of the L-galactose pathway are used to synthesize activated nucleotide sugars that are also precursors of cell wall polysaccharides and glycoproteins. The committed pathway starts from step 7 with GDP-L-galactose phosphorylase coded by the two genes *VTC2* and *VTC5*, which have been recently identified (Laing et al. 2007; Dowdle et al. 2007; Linster et al. 2008). This enzyme converts GDP-L-galactose to L-galactose-1-P. Finally, the next three steps: GPP (L-galactose-1-P phosphatase), L-GalDH (L-galactose dehydrogenase) and GLDH (L-galactono-1,4-lactone dehydrogenase) convert L-galactose-1-P to L-ascorbate. Genes have been identified for all of the reactions involved in the L-galactose pathway, so that it is possible to study the mechanisms that regulate the expression of genes involved in AsA synthesis.

Light regulation of AsA-related gene expression has been principally studied in leaves. Maruta et al. (2008) reported good correlations between AsA content and phosphomannose isomerase expression and activity (PMI, step 3, Fig. 1) in *Arabidopsis* leaves during the day and in response to the light environment. Several steps of the AsA biosynthetic pathway have also been reported to be regulated by light. Indeed, expression of *GMP* (coding for GDP-D-mannose pyrophosphorylase), *VTC2* (coding for GDP-L-galactose phosphorylase) and *GPP* (coding for L-galactose-1-P phosphatase) increased in leaves of *Arabidopsis* under continuous light and decreased when plants were moved to the dark (Yabuta et al. 2007). *VTC2* seemed to be a key gene regulated by light, as its expression and the activity of the corresponding enzyme GGP increase rapidly and strongly on transfer to high light (Dowdle et al. 2007). For *GME*, *GalDH* and *GLDH*, contradictory results have been reported in terms of their response to light. According to Gatzek et al. (2002), high light did not increase GalDH expression. Gene expression coding for GLDH (step 10) and the corresponding enzymatic activity were found to decrease when leaves were shaded or moved to the dark in *Arabidopsis* (Tamaoki et al. 2003), tobacco (Tabata et al. 2002), chinese cabbage (Li et al. 2008) or apple trees (Li et al. 2009), but did not systematically increase with increased light intensity (Pignocchi et al. 2003; Yabuta et al. 2007). The impact of light on *GME* gene expression has been little studied: Yabuta et al. (2007) reported that transcript levels did not vary within the 3 days following plant transfer to light or dark, while Dowdle et al. (2007) showed a twofold increase in the number of transcripts after 24 h of high light. In addition to light intensity-triggered regulation, Bartoli et al. (2009)

Fig. 1 Major ascorbate biosynthetic pathway in higher plants (L-galactose pathway). Enzymes catalyzing the numbered reactions are in bold type for each step: *1* hexokinase (HXK), *2* phosphoglucose isomerase (PGI), *3* phosphomannose isomerase (PMI), *4* phosphomannomutase (PMM), *5* GDP-D-mannose pyrophosphorylase (GMP), *6* GDP-D-mannose 3',5'-epimerase (GME), *7* GDP-L-galactose phosphorylase (GGP), *8* L-galactose-1-P phosphatase (GPP), *9* L-galactose dehydrogenase (L-GalDH), *10* L-galactono-1,4-lactone dehydrogenase (GLDH). The first committed step for L-ascorbate synthesis GGP (step 7) is catalyzed by VTC2 and VTC5. *Encircled number* indicate the copies of the genes found in *S. lycopersicum*
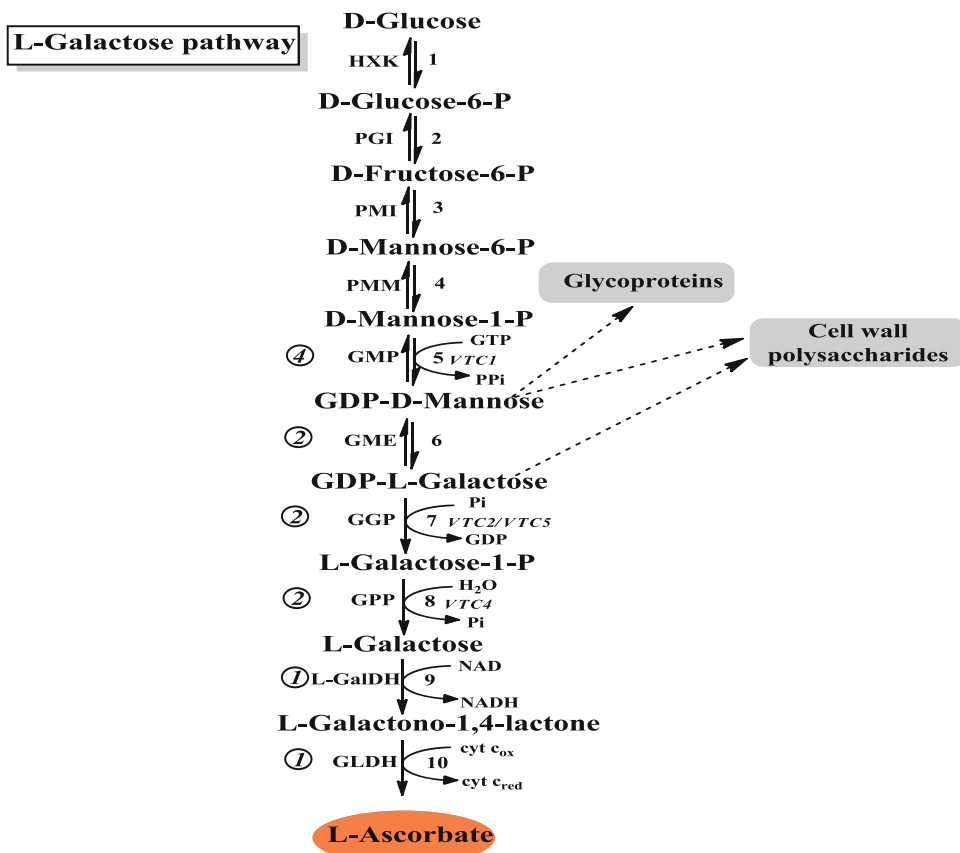
also reported that AsA content and GLDH activity decreased under low red/far-red light ratio, a key shade signal.

Less is known about light-regulated AsA synthesis in fruits. In contrast to leaves, light regulation of AsA synthesis may be more complex: changes of AsA fruit content could be due to light regulation of AsA-related gene expression or enzyme activities at the fruit level, and also to light regulation of AsA (or precursor) intake from leaves to the fruit. Indeed, it was previously shown that such transport exists in zucchini (Hancock et al. 2003), but can be negligible compared to fruit AsA synthesis in blackcurrant (Hancock et al. 2007). In tomato, the importance of fruit AsA synthesis over AsA transport from leaves has already been suggested by using shading experiments (Gautier et al. 2009). Moreover, sugar content seemed not to be limiting for AsA synthesis in tomato fruit as demonstrate recently by Massot et al. (2010). In fruit peel, only one study reported the role of light in the regulation of the AsA-related gene expression (Li et al. 2009). Therefore, regulation of fruit AsA biosynthesis by light is still unclear.

To get a better understanding of light regulation of AsA synthesis, we compared the expression of the genes coding for the six last steps of the AsA pathway (Fig. 1, steps 5–10) in tomato leaves and red ripe fruits for shaded and control plants after 7 days of shading, or in plants submitted to diurnal variations of light. We studied, in tomato, four unigenes corresponding to the plant amino acid sequences of GMP (*GMP1–GMP4*), two unigenes for GME (*GME1*, *GME2*) and two unigenes for GPP (*GPP1*, *GPP2*). We showed that light affects AsA content and AsA-related gene expression in leaves more than in fruits. Moreover, strong temporal changes in T-AsA content and in the expression of the genes of AsA synthesis were found in leaves. Studying the correlation between transcript levels and AsA content revealed the hypothetical role of GME1 in addition to VTC2 to regulate leaf AsA content.

Materials and methods

The study was performed on a cherry tomato *Solanum lycopersicum* L. 'West Virginia 106' ('WVa106'), kindly provided by West Virginia University Agricultural Experiment Station (Morgantown, WV, USA).

Long-term shading experiment

Plant growth

Plants were grown in a glasshouse in Avignon (southern France, 44°N). Plants with five growing leaves were transplanted on 23 March into 5-L pots containing potting soil in a multispan Venlo-type greenhouse, N–S oriented. Plant nutrition, chemical pest and disease control were in accordance with commercial practices. Water was supplied to the plants using a drip irrigation system to maintain 20–30% drainage. Flowers were mechanically pollinated three times a week. All plant side shoots were removed as they appeared.

Shading treatment and sampling

On 11 June 2009, fruits at breaker stage (start of yellow coloration of the fruit at the locular position) on trusses no. 5 and 6 were tagged. On the same day, young fully developed leaves that were sun exposed were marked. Half of the plants were then totally shaded at 12–13 h universal time (UT) with a perforated silver net, which reflected 71% of incident radiation (Lee Filter: no. 271; Andover, Hampshire, UK). These shading treatments mostly affected irradiance and had a low impact on temperature due to the reflective properties and the perforations of the film (supplemental Fig. S1, Gautier et al. 2009). Seven days later, on 18 June 2009, previously tagged fruits and leaves were harvested at midday. Fruits had reached the red ripe stage. Five replicates were collected; a replicate corresponds to a batch of three tomatoes for fruit samples or the five larger leaflets of a leaf for leaf samples. Pericarp tissue and leaflets were immediately frozen in liquid nitrogen and stored at –80°C before grinding to powder in liquid nitrogen.

Diurnal experiment

Plant growth

Plants with five growing leaves were transplanted on 4 March 2008 into soil, in a glasshouse, N–S oriented. Plant management was similar to the other experiment.

Sampling

Green, mature fruits (29 days after pollination), red ripe fruits (45 days after pollination) and young fully expanded leaves were harvested every 4 h after sunrise (0430) to 2000 hours (UT, 1 h following sunset) on a sunny day that started being cloudy in the afternoon (14 May), and after sunrise and midday on a cloudy day (15 May). Five replicates were collected; a replicate corresponds to a batch of three tomatoes or the five larger leaflets of a leaf. Following harvest, fruit pericarp and leaflets were frozen in liquid nitrogen and stored at −80°C before grinding to powder in liquid nitrogen.

Photosynthesis, respiration and stomatal conductance measurements

Measurements were performed on five young, fully expanded leaves, every 4 h from sunrise to 20 h (UT) on the sunny day and at sunrise and midday on the cloudy day. Net CO_2 assimilation rates, respiration and stomatal conductance of leaves were measured using PP System CIRAS-1 (Amesbury, MA, USA) apparatus with the following parameters: 26°C, 600 µmol m^{-2} s^{-1}, 370 ppm CO_2, 60% relative humidity and an air flow of 200 mL s^{-1}.

Fluorescence measurements

Fluorescence measurements were performed using a portable Fluorescence Monitoring System (FMS 1, Hansatech®, Kings Lynn, Norfolk, UK), a leaf clip holder for adapting leaves to darkness conditions and a PAR/temperature leaf clip for ambient light environment. The variable to maximum fluorescence ratio (Fv/Fm) was calculated. This ratio, measured in the non-energized state after darkness adaptation for about 30 min, is a reliable measure of the maximal (potential) efficiency of excitation capture by open PSII in dark-adapted conditions. Fv/Fm is used as an estimate of the functional state of the photosynthetic apparatus at a given environmental situation (Krause and Weis 1991). A decrease in Fv/Fm indicates photo-inhibition of PSII.

Light intensity measurements

Photosynthetically active radiation (PAR) was measured above plants with a JYP 1000 sensor every minute, averaged and stored every 15 min on a delta logger (Delta-T DL2e, Delta-T Devices Ltd., Cambridge, UK). Light intensity measured during long shading and diurnal experiments are presented in Supplemental Fig. S1.

Ascorbate assay and gene expression

Assays of AsA and DHA (dehydroascorbate) content were carried out as previously described by Stevens et al. (2006), on ground powder conserved at −80°C. Briefly, 0.5–1 g of powder was homogenized with 600 µL of ice-cold 6% trichloroacetic acid. Samples were centrifuged for 15 min at 16,000g at 4°C; 20 µL of supernatant was used in each assay. Two assays were carried out on each sample, one to measure the total AsA content (T-AsA = AsA+DHA, including addition of 5 mM dithiothreitol) and one to quantify the AsA content (omission of dithiothreitol from the assay). DHA content was obtained from the difference between total AsA and AsA content. Fruit dry

matter content was determined on a subsample after lyophilization.

Total RNA was isolated from ground powder conserved at −80°C from leaves (200 mg) and fruits (250 mg) by phenol–chloroform extraction. Samples were homogenized with 1 mL of TRI Reagent® Solution (Ambion). After 10 min at room temperature, 500 µL of chloroform was added and samples were homogenized. They were incubated for 5 min at room temperature and then centrifuged for 15 min at 12,000g. The aqueous phase was collected and mixed with 0.7 volume of isopropanol. Samples were homogenized by inversion and after incubation at room temperature for 10 min and were centrifuged for 10 min at 12,000g and 4°C. The supernatants were discarded and pellets were washed with 70% ethanol. After centrifugation, ethanol was removed and pellets were dissolved in 50 µL of RNAse-free water and stocked at −20°C before reverse transcription. To eliminate DNA contamination, 15 µg of total RNA was treated with RQ1 RNAse-free DNAse (Promega). Then, reverse transcription was performed with 5 µg of DNA-free RNA, and treated with Rnasin (Promega) to inhibit RNAse activity, using oligo (dT)$_{21}$ (10 µM) and SuperScript® II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA obtained was diluted fivefold in water and 2 µL aliquots were stored before use. Quantitative real-time PCR (RT-QPCR) analyses were performed with the Stratagene Mx3005P® thermocycler (Stratagene, Cedar Creek, TX, USA) using the Gotaq® qPCR Master Mix (Promega) according to the manufacturer's instructions in a reaction volume of 24.5 µL. Expression analyses of genes were performed for the six last steps of AsA biosynthesis (Fig. 1). We analyzed four GDP-D-mannose pyrophosphorylase genes [SlGMP1 (SGN-U315592), SlGMP2 (SGN-U313112), SlGMP3(SGN-U313111) and SlGMP4 (SGN-U329408)], two GDP-L-galactose phosphorylase genes [SlVTC2 (SGN-U312646), SlVTC5 (SGN-U316430)], two L-galactose-1-phosphate phosphatase genes [SlGPP1 (SGN-U345930), SlGPP2(SGN-U317967)], an L-galactose dehydrogenase gene [SlGalDH (SGN-U319047)] and an L-galactono-1,4-lactone dehydrogenase gene [SlGLDH (SGN-U317657)]. For each reaction, three technical replicates were run. Relative gene expression was calculated by the $2_T^{-\Delta\Delta C}$ method (Livak and Schmittgen 2001) with *Actin1* [SlActin (SGN-U314757)] as an internal control. Primer sequences are detailed in Supplemental Table S1).

Results

We analyzed the regulation by light of AsA synthesis-related gene expression comparing the response of leaves

and fruits to 7 days of shading. Then, to study the short-term regulation of T-AsA content in leaves and fruits, we further subjected the plants to diurnal fluctuations.

T-AsA content and AsA-related gene expression in leaves and red fruits respond differently to shading

Plants were shaded for 7 days to study the impact of a severe (−71% of incident radiation) and long-term decrease of light irradiance, on fruit and leaf T-AsA content. Shading reduced the levels of T-AsA in leaves (66% reduction) more than fruits (only 10% reduction, which was not significant at 5%, Fig. 2).

To determine the impact of shading treatment on AsA synthesis-related gene expression, we compared the relative abundance of transcripts of biosynthetic pathway genes in non-shaded and shaded leaves and fruits. In tomato, we found for GMP, GME and GPP more than one unigene corresponding to the plant amino acid sequences coding for ascorbate-related genes (Fig. 1). Shade significantly reduced the transcripts of GMP1, GMP3, GME1, GME2, VTC2, GPP1 and GPP2 in leaves (Fig. 3a). This down-regulation was very marked for GME1, where the transcript number was reduced 13-fold after 7 days of shade. Thus, from step 5 of the AsA biosynthetic pathway, all steps in terms of gene expression appeared to be regulated by light in the leaves, except for the last two genes, GalDH and GLDH (steps 9 and 10).

In fruits, transcripts of genes of the AsA biosynthetic pathway were less affected by shade than in leaves, as only transcripts of GPP1 and VTC2 were significantly down-regulated by shade (Fig. 3b).
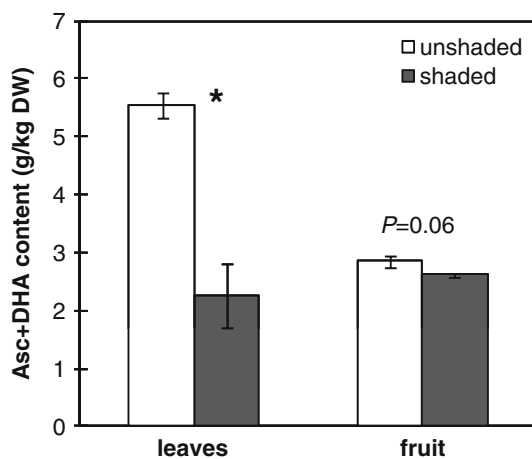
Fig. 2 Total ascorbate (T-AsA) content in S. lycopersicum cv. 'WVa 106' young fully developed leaves and red ripe fruits harvested at midday on plant grown under unshaded (open bars) or shaded (gray bars) conditions after 7 days of shading. Data are means of five replicates (±SE). Asterisks indicate a significant difference between treatments else P value is noted (Tukey test, $P < 0.05$)

Vitamin C shows daily fluctuations in leaves but not in fruits

To study the short-term regulation of T-AsA content in both leaves and fruits, we subjected plants to natural fluctuations in radiation (over a period of 1 day) and compared a sunny day with a cloudy day. Variations in T-AsA levels in leaves followed a bell curve with a maximum at noon and were as strong as for long-term shading (reduction of up to 50% between 1200 and 2000 hours, Fig. 4). Leaves sampled during a cloudy day displayed lower levels of T-AsA. This important regulation of T-AsA may be linked to its role in different processes at the leaf level (photosynthesis, respiration, etc.), protecting the plant against photo-oxidative stress. For this reason, we monitored how radiation modified the functioning of leaves over a period of 1 day. Stomatal conductance, leaf respiration and CO_2 assimilation followed radiation level variations during the day (Fig. 5, Fig. S1). As previously shown with T-AsA content, stomatal conductance, leaf respiration and CO_2 assimilation were lower during a cloudy day. The maximum quantum yield of PSII (Fv/Fm), used to estimate the functional state of the photosynthetic apparatus, showed a decrease at midday (<0.85, Fig. 4d) during the sunny day, which may indicate a stress during the day (photoinhibition). This decrease was less marked during a cloudy day in accordance with lower light stress. These measurements demonstrated a state of light stress (variations in Fv/Fm) during the day in leaves coupled with changes in photosynthesis and stomatal conductance that could explain AsA daily variations. Indeed, T-AsA content was positively correlated with Fv/Fm ratio ($R^2 = 0.47$, Supplemental Fig. S2).

In green mature or red fruits, however, there were no significant diurnal variations in T-AsA levels (Supplemental Fig. S3) or between a sunny day and a cloudy day, so T-AsA buffering was more marked in fruits than in leaves.

Two additional genes GalDH and GLDH respond to daily light fluctuations in leaves

We further studied daily modifications in the expression of genes of the AsA biosynthetic pathway in leaves, as only leaves showed daily fluctuations of T-AsA content. Two different types of transcript accumulation kinetics were thus observed (Fig. 6). The transcripts of GME1, GME2, VTC2 and GPP2 were very abundant at sunrise and then declined rapidly during the day to reach a minimum at between 1200 and 2000 hours. More specifically, we noted that VTC2 and GME1 showed very strong variations during the day (respectively, 16- and 6-fold higher at sunrise than at 2000 hours). For other genes (VTC5, GalDH), transcripts
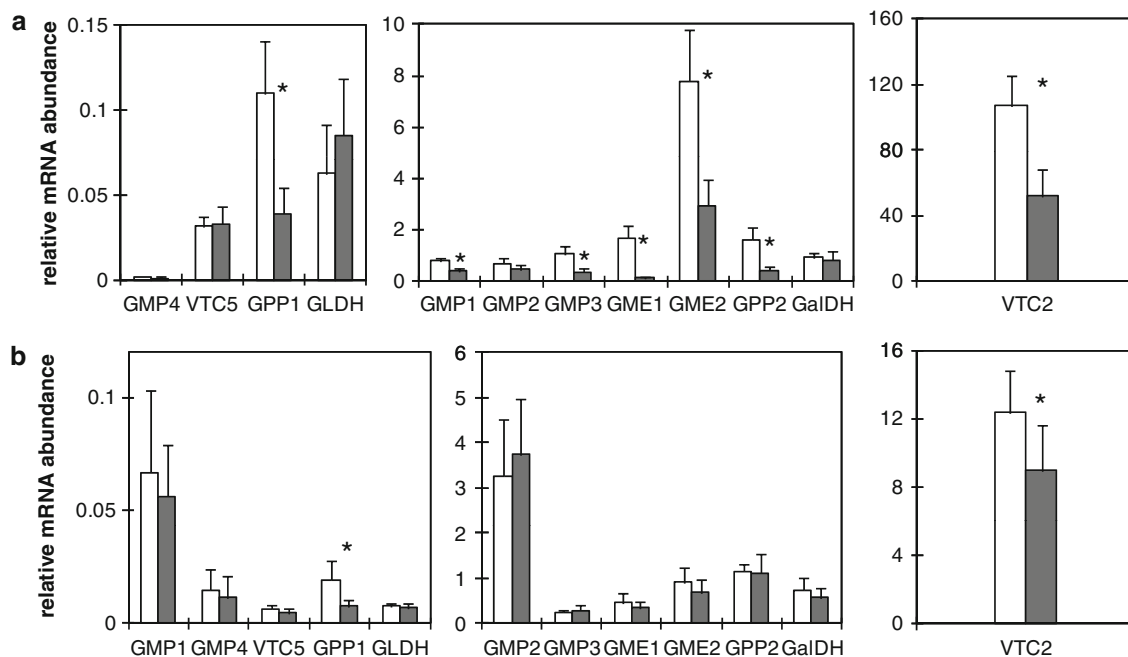
Fig. 3 Relative abundances of AsA-related mRNAs in *S. lycopersicum* 'WVa 106' leaves (**a**) and red ripe fruits (**b**) harvested at midday on plant grown under unshaded (*open bars*) or shaded (*gray bars*) conditions after 7 days of shading. Data were obtained by quantitative real-time PCR normalized against Actin1. Data are means of four replicates (±SD) and *asterisks* indicate significant differences between treatments (Tukey test, $P < 0.05$)
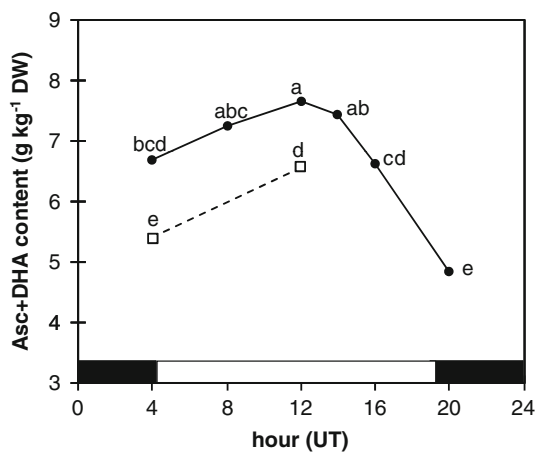
Fig. 4 Total ascorbate (T-AsA) content in *S. lycopersicum* 'WVa 106' leaves during a sunny (*filled circles*) or a cloudy day (*open squares*). Values are means of five replicates and *different letters* indicate significant differences (LSD test, $P < 0.05$). The *bar* below the graph indicates dark and light periods: sunrise took place at 0415 and sunset at 1900 hours

were low in the morning and in the evening and peaked at around 1600 hours. When a sunny day was compared with a cloudy day, light-regulated genes, that responded during long-term shading all showed a reduction in the number of transcripts, starting from sunrise. Moreover, two additional genes that did not respond to long-term shade, *GalDH* and *GLDH*, were also reduced at shorter term during a cloudy day (Fig. 7). At midday, radiation levels did not differ markedly between the 2 days (Supplemental Fig. S1) and a comparison of relative transcript levels between the 2 days (sunny and cloudy) did not highlight any significant differences (Supplemental Fig. S4).

Discussion

Light regulation of vitamin C synthesis in leaves

Our results confirm the importance of radiation in the regulation of T-AsA content in leaves during long-term shading. The diminution of T-AsA content was related to a reduction in *GMP1*, *GMP3*, *VTC2*, *GPP1* and *GPP2* gene transcripts as had previously been observed in *Arabidopsis* (Yabuta et al. 2007). However, supplementary genes (step 6, Fig. 1) were markedly regulated by light with the two genes *GME1* and *GME2* that were down-regulated under shade (a 13-fold and 3-fold reduction in the number of transcripts, respectively). The step controlled by GME constitutes an important control point in AsA synthesis, because it lies at the intersection of AsA synthesis and that of cell wall polysaccharides. In tomato, regulation of AsA synthesis-related gene expression may be more complex than in *Arabidopsis*, as several copies exist coding for the same enzyme. Thus, two of the four copies of the *GMP* genes (*GMP2*, *GMP4*) did not respond to light. Moreover,
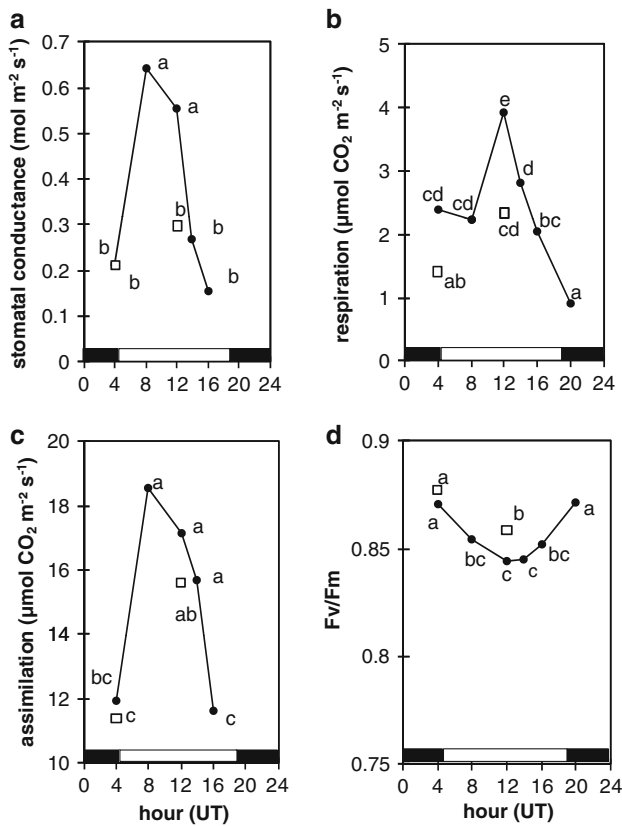
Fig. 5 Impact of irradiance on stomatal conductance (**a**), leaf respiration (**b**), assimilation (**c**) and Fv/Fm ratio (**d**) during a sunny (*filled circles*) or a cloudy day (*open squares*) in young fully developed leaves of *S. lycopersicum* 'WVa 106' (see "Materials and methods"). Values are means of five replicates and *different letters* indicate significant differences (LSD test, $P < 0.05$). The *bar* below each graph indicates dark and light periods: sunrise took place at 0415 and sunset at 1900 hours

it could be seen that during step 7 (GGP, Fig. 1), *VTC2* gene responded to shade, and not *VTC5*, suggesting that these two genes may not play the same role in regulating AsA synthesis. Finally, and contrary to the findings of Li et al. (2009) in apple leaves, the transcription of the last two steps of the AsA synthesis pathway (*GalDH* and *GLDH* genes) was not significantly down-regulated during long-term shading of tomato leaves. Previous studies have indeed shown that the reduction in the expression of the gene coding for GalDH (Gatzek et al. 2002) or GLDH (Alhagdow et al. 2007) did not affect AsA synthesis in leaves indicating that these steps were not limiting for AsA synthesis.

Furthermore, we demonstrated that T-AsA levels in leaves showed large fluctuations during the day. Moreover, almost all transcripts in the AsA biosynthesis pathway varied during the day. Previous studies have also reported diurnal fluctuations in leaf T-AsA content in *Arabidopsis* (Tamaoki et al. 2003; Dowdle et al. 2007; Maruta et al. 2008), tobacco (Chen and Gallie 2004) or potato (Tedone

et al. 2004). However, these periodic oscillations in T-AsA content were only studied with respect to *PMI1* and *PMI2* (Maruta et al. 2008) or *VTC2* and *VTC5* gene expression (Dowdle et al. 2007), the latter displaying a fivefold increase in *Arabidopsis* during a light cycle. As found in our study, Dowdle et al. (2007) reported that diurnal fluctuations of T-AsA content were delayed with diurnal fluctuations of *VTC2* and *VTC5* expression, as *VTC2* and *VTC5* transcripts peaked in the first few hours of the light cycle, while T-AsA content and GPP activity increased toward the end of the light period (delay of 6 h). In our study, most of the genes peaked early at 0400 hours, but T-AsA content increase up to 1200 hours (delay of 8 h). This delay between gene expression and maximum T-AsA content could be explained by the time needed to synthesize enzymes involved in AsA synthesis, but also by light regulation at a post-transcriptional level or enzyme activity level as suggested by the results of Dowdle et al. (2007). Moreover, as T-AsA pool size is also controlled by recycling, T-AsA content variations could also be due to light regulation of AsA recycling as reported by Kuzniak (2004) and Chen and Gallie (2004). However, in terms of *VTC2* transcript variations (the gene that mostly varied during the day), we observed a good correlation between *VTC2* transcript level and T-AsA content 8 h later in leaves ($R^2 = 0.72$, Supplemental Fig. S5). Contrary to *VTC2*, *VTC5* peaked at the end of the day (1600 hours) suggesting complementary expression of these two genes. Finally, our study over the course of a day enabled us to emphasize the important role of *GME1* whose transcript level was also well related to T-AsA content 8 h later ($R^2 = 0.55$, Supplemental Fig. S5), alongside *VTC2*, in the diurnal fluctuations of the AsA biosynthetic pathway in tomato. Indeed, the two steps GME and GGP (steps 6 and 7) were previously reported as being determinant regulatory points of AsA synthesis in tobacco leaves (Dowdle et al. 2007; Linster and Clarke 2008).

The comparison between a sunny and a cloudy day at sunrise underlined that the differences in light intensity (-50% on a cloudy day versus a sunny day between 0400 and 0500 hours), caused a significant down-regulation of practically all genes in the biosynthetic pathway including the last two, *GalDH* and *GLDH*. It therefore appears that even a weak light signal, at sunrise, is sufficient to cause a rapid response, which is suggestive of a signal-type regulation (Bartoli et al. 2009). These results underlined the tight regulation of the AsA biosynthetic pathway at diurnal level and according to radiation. The pattern of T-AsA daily fluctuations reported in the present study might be explained either by the response to changes in daily irradiance or by an intrinsic rhythm. However, this last point looks more likely considering, on one hand, the present pattern of diurnal gene expression (high level of transcripts
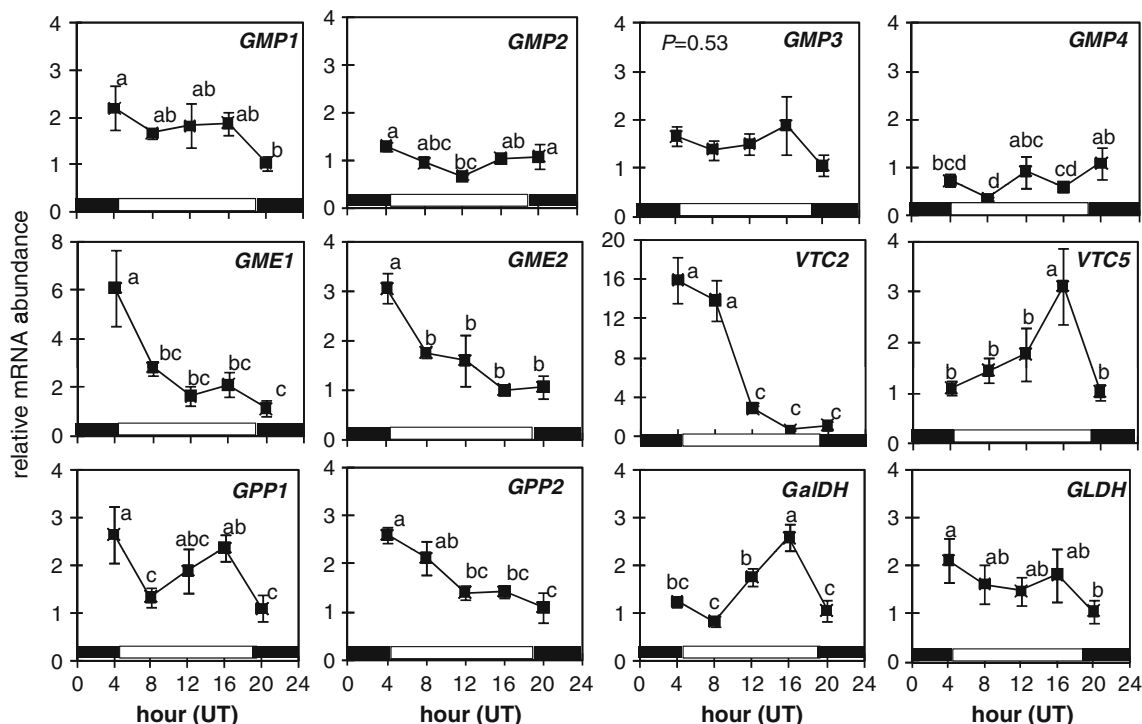
Fig. 6 Diurnal variations of transcript levels of enzymes of the AsA biosynthetic pathway in *S. lycopersicum* 'WVa 106' leaves during a sunny day. Data were obtained by quantitative real-time PCR normalized against Actin1 and expressed relative to level at 20 h. Data are means of four replicates (±SE) and *different letters* indicate significant differences (LSD test, $P < 0.05$). The *bar* below the graph indicates dark and light periods: sunrise took place at 0415 and sunset at 1900 hours
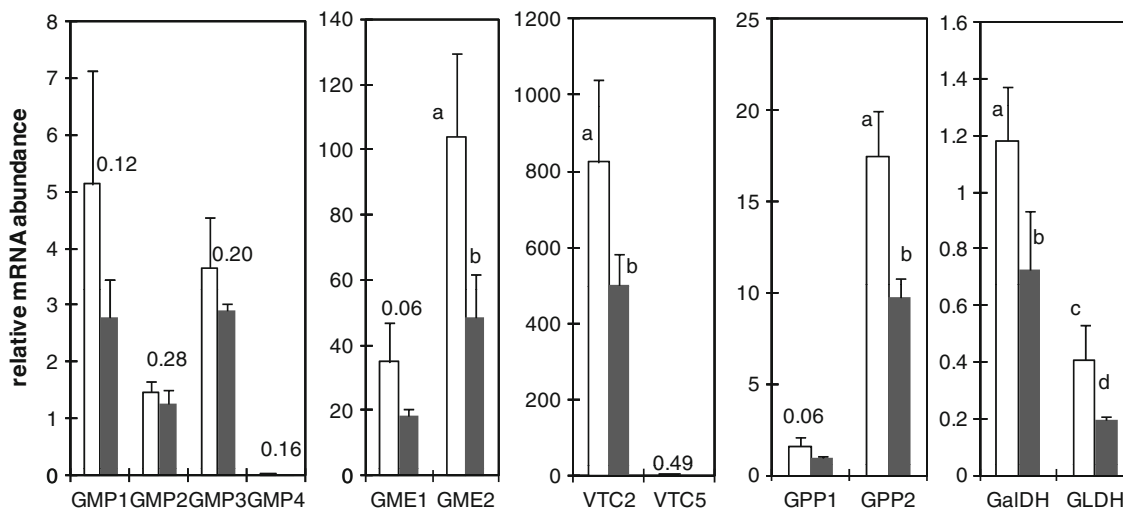
Fig. 7 Relative abundances of AsA-related mRNAs in *S. lycopersicum* 'WVa 106' leaves during a sunny (*open bars*) or a cloudy (*gray bars*) day at sunrise. Data were obtained by quantitative real-time PCR normalized against Actin1. Data are means of four replicates (±SD), *different letters* indicate values that are significantly different between harvest days else numbers above bars indicate *P* values (Tukey test, $P < 0.05$)

at sunrise for key genes coding for GME and GGP) and, on the other hand, data from Dowdle et al. (2007) that reported an intrinsic rhythm for *VTC2*.

Diurnal variations in T-AsA levels followed variations in stomatal conductance, respiration, chlorophyll fluorescence and carbon assimilation, which is consistent with the role of T-AsA in these different processes. Indeed, AsA, through its role as a redox buffer, is involved in the functioning of leaves as a regulator of stomatal opening (Chen and Gallie 2004). It also plays a key role in photosynthesis; firstly, to

protect the photosynthetic apparatus from damage caused by the synthesis of reactive oxygen species (Smirnoff 2000), and secondly in the mechanisms that dissipate excess light energy, such as the water–water cycle (Asada 1999) and the xanthophyll cycle (Demmig-Adams and Adams 1996). Finally, because the last enzyme in the biosynthetic pathway is situated in the electron transport chain in mitochondria, AsA is closely linked to respiration (Bartoli et al. 2000) andalso to photosynthetic electron transport (Yabuta et al. 2008). Our study over the course of a day thus confirmed the tight regulation of the AsA content, which may be linked to its important role in these different processes.

Transcripts in leaves and fruits

We found transcripts of all genes of the AsA biosynthetic pathway in red fruits as reported earlier by Ioannidi et al. (2009; steps 5–10, Fig. 1). These results confirm that tomato fruits are able to synthesize their own AsA, as demonstrated in apple (Razavi et al. 2005) or blackcurrant (Hancock et al. 2007). No other studies reporting AsA-related gene expression variations in both fruit and leaf are available, although these two organs accumulated ascorbate differently. Indeed, T-AsA levels are highly different between these two organs (twice more T-AsA in leaves than in fruits), and one could suppose that its synthesis and regulation also differ (Badejo et al. 2008; Li et al. 2009). We observed that transcripts of the *GMP2* gene were abundant in red fruits. Gilbert et al. (2009) and Badejo et al. (2008) also found more *GMP* transcripts in fruits than in leaves in tomato or acerola, with a strong correlation between *GMP* expression and T-AsA contents in acerola leaves and fruits. The GMP enzyme produces GDP-D-mannose, a precursor of AsA, and also of cell wall polysaccharides, which is essential for N- and O-glycosylation of proteins (Conklin et al. 1999). This protein glycosylation is an important step in the ripening process of tomato (Handa et al. 1985). Indeed, inhibition of glycosylation can delay ripening in tomato. Consequently, the abundance of *GMP2* transcripts that we observed in fruits may be linked to the use of GDP-D-mannose for protein glycosylation during ripening.

Light regulation of vitamin C-related gene expression in fruits

Even though the effects of light on T-AsA levels in fruits are important and have been known since 1945 (Hamner et al. 1945; Mc Collum 1946; Madamba et al. 1974; El-Gizawi et al. 1993), the mechanisms governing the light regulation of AsA biosynthetic pathway have been the subject of few studies in fruits (only one study published in apple, Li et al. 2009). The present study showed that, in

comparison to leaves, T-AsA levels in tomato fruits tend to decline under shade ($P = 0.06$), which could be linked to a reduction in the expression of two genes, *GPP1* and *VTC2*. This latter gene is also well known as a major factor in regulation of the L-galactose pathway (Dowdle et al. 2007; Linster and Clarke 2008). This is the first time that light regulation of the expression of these two genes in fruits has been observed. Indeed, Li et al. (2009) recently demonstrated a reduction in the quantity of *GLDH* transcripts in apple that was proportional to the intensity of shade. However, gene transcripts coding for GGP and GPP were not included in that study. Furthermore, these authors did not observe any significant difference in gene expression in fruit flesh, but only in skin. This could be linked to the fact that organs or tissues exposed to radiation, such as fruit skin, respond more markedly to variations in light intensity than flesh (Li et al. 2009, 2010). In agreement with this hypothesis, studies have shown that AsA levels decreased from the outside to the inside of the fruit (Li et al. 2010). Another explanation for the small number of genes responding to shade in fruits may be linked to the developmental stage of the fruits in our study. Indeed, several authors have demonstrated that expression of *GMP*, *GME*, *GalDH* and *GLDH* genes diminishes during ripening, while that of *GGP* and *GPP* genes increases (Alhagdow et al. 2007; Ioannidi et al. 2009). During our study, genes that were weakly expressed at the red ripe stage, *GMP*, *GME*, *GalDH* and *GLDH*, did not appear to respond to shade, while *GGP* and *GPP*, which were strongly expressed at the red ripe stage, did respond. These results suggested that these two steps, GGP and GPP, could be key points of AsA regulation during ripening. It could thus be seen that even in nearly ripe fruits, the radiation conditions affecting the plant might affect the AsA biosynthetic pathway in fruits and consequently their final content at harvest. However, fruits were more buffered, which may be linked to their volume and their light interception capacity. By contrast, the epicarp of fruit might respond much better to light, as suggested by the findings of Li et al. (2009) in apple.

Our results demonstrate the importance of light regulation of AsA synthesis-related gene expression in both short and longer terms in leaves and fruits. These light-regulated genes could explain part of the light induced changes in T-AsA content, but other regulatory levels by light might also exist at the post-transcriptional stage and in addition light may also directly affect enzyme activity as was reported for GLDH (Tamaoki et al. 2003). Thus, complementary experiments in which AsA-related synthesis proteins will be quantified may help in the understanding of light regulation of the AsA pathway. Moreover, after its synthesis, AsA content also depends on its recycling (Chen and Gallie 2004; Zhou et al. 2009) and its degradation (Pallanca and Smirnoff 2000). Future work to determine

the importance of these processes is needed to help in the understanding of how T-AsA level and its redox state contribute to the adaptation of plants to their light environment, particularly in fruit where light regulation is poorly studied.

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