



# The effect of low ascorbic acid content on tomato fruit ripening

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## Abstract

**Main conclusion** The oxidant/antioxidant balance affects the ripening time of tomato fruit.

**Abstract** Ripening of tomato fruit is associated with several modifications such as loss of cell wall firmness and transformation of chloroplasts to chromoplasts. Besides a peak in H<sub>2</sub>O<sub>2</sub>, reactive oxygen species (ROS) are observed at the transition stage. However, the role of different components of oxidative stress metabolism in fruit ripening has been scarcely addressed. Two GDP-L-galactose phosphorylase (GGP) *Solanum lycopersicum* L. cv Micro-Tom mutants which have fruit with low ascorbic acid content (30% of wild type) were used in this work to unravel the participation of ascorbic acid and H<sub>2</sub>O<sub>2</sub> in fruit maturation. Both GGP mutants show delayed fruit maturation with no peak of H<sub>2</sub>O<sub>2</sub>; treatment with ascorbic acid increases its own concentration and accelerates ripening only in mutants to become like wild type plants. Unexpectedly, the treatment with ascorbic acid increases H<sub>2</sub>O<sub>2</sub> synthesis in both mutants resembling what is observed in wild type fruit. Exogenous supplementation with H<sub>2</sub>O<sub>2</sub> decreases its own synthesis delaying fruit maturation in plants with low ascorbic acid content. The site of ROS production is localized in the chloroplasts of fruit of all genotypes as determined by confocal microscopy analysis. The results presented here demonstrate that both ascorbic acid and H<sub>2</sub>O<sub>2</sub> actively participate in tomato fruit ripening.

**Keywords** Antioxidants · *Solanum lycopersicum* · Chloroplast · Chromoplasts · Hydrogen peroxide · NADPH oxidase

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Charlotte Steelheart and Matías Leonel Alegre contributed equally to this work.

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## Introduction

Fruit ripening is a complex phenomenon that involves several physiological and metabolic changes with a strong impact on fruit nutritional and sensory qualities (Kumar et al. 2016). The distinctive plastid differentiation taking place during ripening is a highly coordinated process involving programmed molecular and hormonal components (Giovannoni et al. 2017). This is particularly evident in chloroplasts of fruit undergoing the transformation to chromoplasts, an event characterized by the synthesis and accumulation of natural carotenoids (Egea et al. 2011). These changes are accompanied by the green to red color shift in tomato fruit (Klee and Giovannoni 2011).

Increments in the levels of reactive oxygen species (ROS) have been observed and are associated with the transition from chloroplasts to chromoplasts during ripening of tomato fruit (Jiménez et al. 2002). This could involve cellular damage where lipids and proteins seem to be a target for oxidation as observed in pepper chromoplasts (Martí et al. 2009;

2011). Changes in the activities of superoxide dismutase (the enzyme catalyzing the dismutation of the superoxide radical to  $H_2O_2$ ) and in the components of the ascorbate–glutathione cycle scavenging  $H_2O_2$  have been observed during fruit ripening of different species (Jiménez et al. 2002; Martí et al. 2009; Palma et al. 2015; Huan et al. 2016). While ROS could contribute to ripening, associated processes that control oxidative stress by enhancing antioxidants might improve tomato fruit quality and shelf life (Cocaliadis et al. 2014). Genetic and physiological evidence suggests that  $H_2O_2$  acts as a signaling second messenger in plant cells, mediating the acquisition of tolerance to both biotic and abiotic stresses (Noctor et al. 2018). Bouvier et al. (1998) demonstrated that ROS act as a novel class of second messenger regulating the expression of chromoplast carotenoid biosynthesis genes.

As a direct scavenger of ROS, ascorbic acid protects carotenoids and regenerates tocopherols; while as the major redox buffer in plants it is present at high concentrations in most plant cell compartments, including the apoplast (Foyer and Noctor 2011). In addition, ascorbate serves as a co-factor for many enzymes such as violaxanthin de-epoxidase, which is required for the dissipation of excess excitation energy through the non-photochemical quenching of chlorophyll. It is also an electron donor in the reaction of the ascorbate–glutathione cycle where ascorbate peroxidase (APX) reduces  $H_2O_2$  (Asada 1999). Analysis of leaf transcripts in the *vtc1* mutant (vitamin C-deficient Arabidopsis) demonstrates that ascorbic acid is associated with proteins implicated in DNA-binding, cell cycle control, signaling and developmental processes, carbon, cell wall and lipid metabolism, and anthocyanin synthesis (Pastori et al. 2003). Moreover, ascorbic acid is also a regulator of cell division, cell elongation and growth (De Simone et al. 2017) and a signal transducing molecule; changes in ascorbic acid concentration can modulate PR gene expression and systemic acquired resistance (Pavet et al. 2005). Up to date, no study has been carried out regarding the consequence of ascorbic acid deficiency on the fruit ripening process, such as in tomato. Nevertheless, there is a close interaction between the metabolism of ascorbic acid and respiration and photosynthesis observed in leaves (Bartoli et al. 2006, 2009), cell wall synthesis in tomato fruit (Gilbert et al. 2009), hormones such as ethylene in rosette biomass accumulation in Arabidopsis (Caviglia et al. 2018) and tomato fruit yield (Alegre et al. 2020).

This work examines the hypothesis that ROS play an active role in the transition from green to red stages during the ripening of tomato fruit. With that aim, we used two independent lines of tomato mutants deficient in GDP-L-galactose phosphorylase (GDP), known to be the major controlling enzyme of the ascorbic acid biosynthesis pathway (Bulley and Laing 2016). As these GDP-deficient mutants display a strong decline in ascorbic acid content (30% of the wild type), their use could contribute to establish the role of

ascorbic acid and oxidative stress during the ripening phase, especially during the chloroplast to chromoplast transition in tomato fruit.

## Materials and methods

### Plant material and treatments

The experiments were performed with wild type (*Solanum lycopersicum* L. cv Micro-Tom) and two GDP-L-galactose phosphorylase-deficient mutant lines (*Slgdp1*, named GGP-5261 and GGP-49C12, respectively). GGP-5261 and GGP-49C12 lines were selected from the NBRP-Tomato population (Tsukuba-Japan) and TILLING-Tomato collection (Bordeaux-France), respectively (Baldet et al. 2013). The experiments were carried out in the greenhouses of the Institute of Plant Physiology, National University of La Plata, Argentina. Plants were grown in 3-L pots filled with fertilized soil under normal greenhouse irradiance (photosynthetic photon flux density of about  $700 \mu\text{mol m}^{-2} \text{s}^{-1}$  at midday) and temperatures of  $25 \pm 2$  and  $20 \pm 3$  °C, day and night, respectively. For the different treatments, fruits were sprayed three times a week before nightfall with 5 mL per plant of either 10 mM  $H_2O_2$  or 20 mM ascorbic acid at pH 5.7 for 20 days following a mature green (MG) fruit formation on trusses. Controls were sprayed with distilled water and 0.01% Tween 20 was used as surfactant in all treatments. The effect of ascorbic acid and  $H_2O_2$  treatments on “in vine” ripening was determined at MG, breaker (BR), orange (OR) and red ripe (RR) fruit collected 24 h after the last treatment. Ripening stages were selected according to the United States Department of Agriculture standard grades of fresh market tomatoes (Tu et al. 2000). The fruits were washed with distilled water after harvest. A batch of samples were frozen in liquid nitrogen and stored at  $-80$  °C until use.

Three independent experiments were performed using at least three plants for each treatment.

### Ripening time

The effect of ascorbic acid and  $H_2O_2$  treatments on ripening time was determined by counting the days from MG to RR stage by analyzing the color change of 20 fruit per genotype from different plants. For each treatment, the number of days for “in vine” ripening was taken when 50% plus one fruit reached the RR stage.

### Ascorbic acid concentration

The effect of ascorbic acid and  $H_2O_2$  treatments on total and reduced ascorbic acid content was measured in approximately 150 mg of frozen fruit pericarp powder.

The extraction of total ascorbic acid was carried out with a 3% (v/v) trifluoroacetic acid solution and centrifuged at  $16,000\times g$  for 10 min. The content was measured with a HPLC system (Shimadzu LC-10ATvp solvent delivery module) fitted with a Zorbax MicroSpher C-18 column (100 mm  $\times$  4.6 mm, Agilent Technologies) and detected at 265 nm (Shimadzu UV-Vis SPD-10Avp detector) as described in Bartoli et al. (2006). Dehydroascorbic acid (the oxidized form of ascorbate) was estimated as the difference between the contents of total and reduced forms (Bartoli et al. 2006). To calculate the redox state of the ascorbic acid, the following formula was used: % of dehydroascorbic acid =  $[(\text{dehydroascorbic acid content})/(\text{ascorbic acid content} + \text{dehydroascorbic acid content})^{-1}] \times 100$ .

### Glutathione concentration

The same homogenate used for ascorbic acid was used for glutathione measurements. Contents of total and oxidized glutathione were determined spectrophotometrically (spectrophotometer AV-160A Shimadzu, Japan) as described in Griffith (1980) in a reaction mixture containing 100 mM potassium phosphate (pH 7.5), 5 mM EDTA, 0.3 mM NADPH, 6 mM DTNB and approximately 50 units of glutathione reductase/mL. Oxidized glutathione was measured after incubating 100  $\mu$ L of each sample with 2  $\mu$ L of vinylpyridine. The redox state was calculated with the same formula used for ascorbate.

### Hydrogen peroxide production

The effect of ascorbic acid and  $\text{H}_2\text{O}_2$  treatments on fruit  $\text{H}_2\text{O}_2$  production was determined by immersing the entire fruit (fresh weight of about 3 g) immediately after harvest in 2 mL of a solution containing 10,000 U/L horseradish peroxidase, 10  $\mu$ mol/L Amplex Red (*N*-acetyl-3,7-dihydroxyphenoxazine) and 50 mmol/L Tris-HCl buffer, pH 7 (Amplex Red assay kit, Molecular Probes). The reaction was linear for several hours. The change in the fluorescence of the solution was recorded 100 min after beginning the incubation with a CLARIOstar® Plus Multi-mode Microplate Reader (Ortenberg, Germany) in fluorescence mode at 560 nm excitation wavelengths (Zhou et al. 1997).

### Subcellular ROS detection

Immediately after harvesting, fruits were washed and their pericarps cut in thin slices with a hand microtome for the subcellular location of ROS according to Ramos-Artuso et al. (2019). The segments were incubated in 50 mM Tris/HCl solution, pH 7.4, containing 0.025 mM 2',7'-dichlorofluorescein diacetate (DCFDA, Sigma-Aldrich®) for 30 min in the darkness. Then they were rinsed with the

same solution without the fluorescent probe. Tissues were observed using Confocal Spectral Laser Microscope (Leica TCS SP5, ex = 488 nm and em = 525 nm). Fruit slices incubated in the solution without DCFDA were used as a target to rule out the existence of autofluorescence.

### Malondialdehyde (MDA) concentration

Two hundred milligram of frozen pericarp fruit was ground in 1 mL of 50 mM potassium phosphate buffer, pH 7.0, and 100  $\mu$ L of a 0.2% (w/v) ethanol solution of butylated hydroxytoluene for the measurement of MDA. Homogenates were centrifuged at  $16,000\times g$  for 10 min. Supernatants were mixed with 1 M HClO (1:1), vigorously shaken and centrifuged again at  $16,000\times g$ . Then one volume of 0.8% (w/v) thiobarbituric acid was added to two volumes of the supernatant and incubated at 90 °C for 1 h. Samples were filtered and injected onto a HPLC system previously described and MDA detected at 590 nm. The HPLC conditions consisted in a running buffer of 70% 100 mM potassium phosphate buffer pH 7.0 and 30% methanol as described by Templar et al. (1999).

### Enzyme assays

The activity of different enzymes was measured by grinding approximately 500 mg of frozen fruit powder in 1 mL of 100 mM Bicine buffer pH 7.5 containing 10% (v/v) glycerol and protease inhibitors (1 nM of phenylmethylsulfonyl fluoride; 1  $\mu$ M E-64 and 1  $\mu$ M of EDTA, from Sigma-Aldrich®). The homogenates were centrifuged at  $16,000\times g$  for 10 min and the supernatant was used for the enzymatic assays performed with a UV-visible recording spectrophotometer (AV-160A Shimadzu, Japan). Catalase (CAT) activity was measured according to Bartoli et al. (1999), monitoring  $\text{H}_2\text{O}_2$  decomposition at 240 nm in a reaction medium consisting of 50 mM potassium phosphate buffer (pH 7.0), 15 mM  $\text{H}_2\text{O}_2$ , up to 100  $\mu$ L of homogenate (1 mg/mL protein) and 0.1% (v/v) Triton X-100. Ascorbate peroxidase (APX) activity was measured by a modification of the method described by Amako et al. (1994). Soluble and membrane fractions were extracted with 50 mM phosphate buffer, pH 7.0, 1 mM EDTA and 1 mM ascorbic acid. The reaction mixture contained 50 mM phosphate buffer pH 7.0, 0.5 mM ascorbate, and 0.1 mM  $\text{H}_2\text{O}_2$  and oxidation of ascorbic acid was followed by a decrease at 265 nm. The activity was calculated using an extinction coefficient of 2.8 m/mol cm for ascorbic acid. Peroxidase (PX) activity was measured spectrophotometrically at 430 nm according to Bartoli et al. (1995), in a reaction medium consisting of 50 mM potassium phosphate buffer (pH 6), 5 mM pyrogallol and 8 mM  $\text{H}_2\text{O}_2$ . The activity was calculated using an extinction coefficient of 2.47 m/mol cm. Superoxide dismutase (SOD) activity was determined due to its capacity to inhibit

the reduction on nitroblue tetrazolium generated by the xanthine–xanthine oxidase reaction (Beauchamp and Fridovich 1971). The reaction mixture contained 50 mM potassium phosphate (pH 7.8), 0.83  $\mu$ M xanthine and 0.015 mM EDTA. An aliquot of the crude extract including around 0.2–1 units of SOD was added. The reaction was started by the addition of an aliquot of the xanthine oxidase producing a change of 0.025 U of absorbance at 560 nm/min in each reaction. The activity was followed for 40 s at 560 nm. One unit of SOD is defined as the amount of enzyme that inhibits the reduction by 50%. NADPH oxidase (NOX) was measured by a modification of the method described by Li et al. (2004) by lucigenin-enhanced chemiluminescence in a reaction medium containing 50 mM potassium phosphate (pH 7.4), 5  $\mu$ M lucigenin and 40  $\mu$ M NADPH. Light emission was measured every 5 s for 40 s using the microplate reader in luminescence mode. Total soluble protein content was determined in the samples according to Bradford (1976) using bovine serum albumin (Sigma-Aldrich®) as standard.

### Ripening parameters

The respiration rate was determined with an infrared CO<sub>2</sub> sensor (Lutron Electronic Enterprise Co. L.T.D., Model GC-2028) at 23 °C. Four intact fruits (typically 15 g in total) were placed in a 1.74-L tight glass chamber and the atmosphere concentration of CO<sub>2</sub> was recorded every minute for at least 20 min. Results were expressed in mmol of CO<sub>2</sub> kg<sup>-1</sup> FW h<sup>-1</sup>. To determine the fruit firmness, a texture analyzer (TA. XT. PLUS, Micro Systems TM Goldalming, Surrey, UK) was used with a 2.5-mm-diameter flat probe. The deformation tests were set with the following parameters: distance 0.5 mm, speed test 0.25 mm/s and 0.058 N of trigger force. The maximum force of the test was recorded to analyze the fruit firmness. Results were expressed in N. Total soluble solids (TSS) content, total titratable acidity (TTA), pH and TSS/TTA ratio were measured according to Steelheart et al. (2019a). Ten fruits were analyzed for each treatment.

### Statistical analysis

Data represent an average of the results obtained from at least three independent experiments (spring/summer 2017–2019) with a minimum of three plants for each treatment. The results were analyzed by means of ANOVA and the means were compared by the Tukey test at a 0.05 significance level.

## Results

### Effects of ascorbic acid and H<sub>2</sub>O<sub>2</sub> treatments on ripening time

The two *Slggp* mutants displayed a delayed ripening time since they took about three more days than the wild type to reach the RR stage from the MG stage (Table 1). When they were treated with 20 mM ascorbic acid, the fruit ripening time was equivalent to wild type, while on the contrary, the treatment with H<sub>2</sub>O<sub>2</sub> extended the ripening time in both mutants but not in wild type (Table 1).

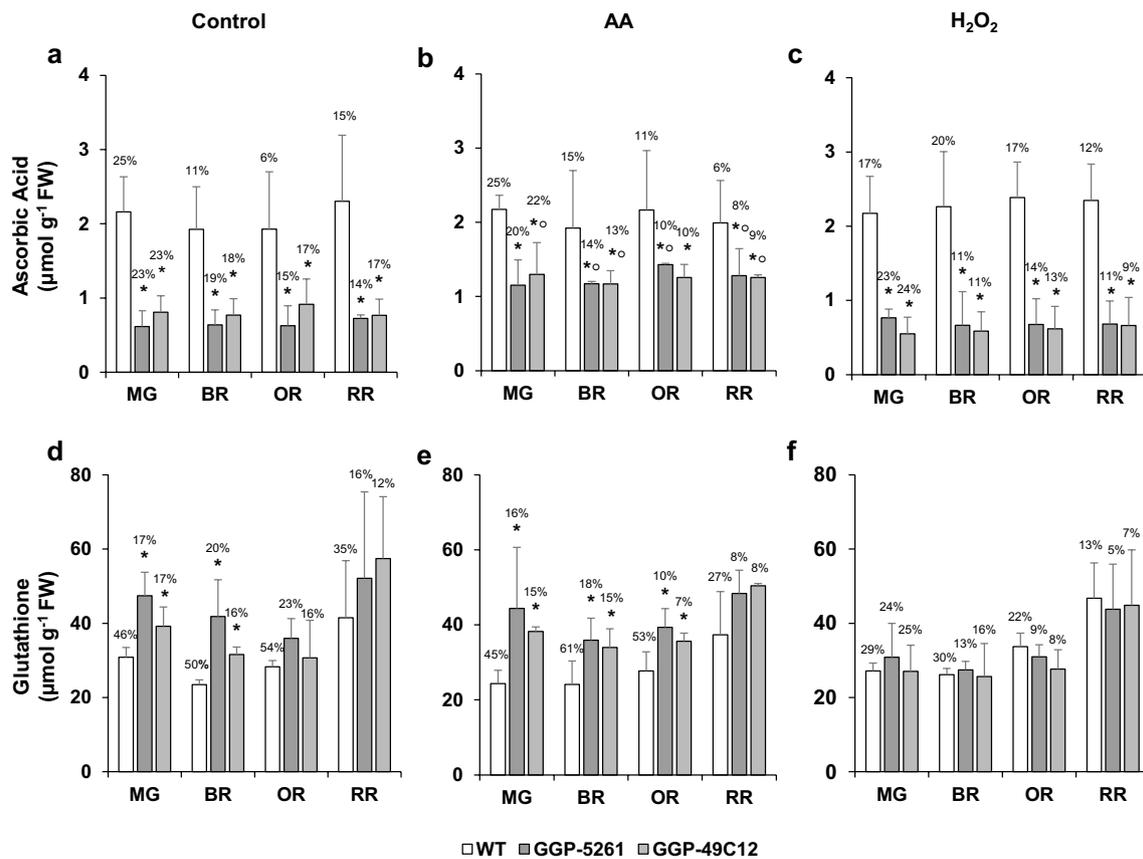
### Ascorbic acid and glutathione concentration and redox state during fruit ripening

Ascorbic acid concentrations remained constant during fruit ripening in wild type and both *Slggp* lines, though in the mutant fruits the ascorbate content is around 30% of the wild type (Fig. 1a). When ascorbate was exogenously supplied, AA fruit concentration increased by twofold in the two mutants whereas it remained unchanged and comparable to that of the untreated plants in the wild type fruit (Fig. 1b). H<sub>2</sub>O<sub>2</sub> treatment had no effect on ascorbic acid concentration in wild type and mutant fruits (Fig. 1c). The redox state of ascorbate decreased (i.e. the concentration of the oxidized form decreases), in wild type fruit during ripening whereas it remained constant in both mutant fruits (Fig. 1a). Ascorbic acid supplementation had no significant effect on the redox state in the wild type, while it decreased in *Slggp* plants at BR, OR and RR stages thus abolishing the differences between the genotypes (Fig. 1b). After H<sub>2</sub>O<sub>2</sub> treatment, ascorbic acid redox state increased at BR and OR stages in wild type fruit while it tended to decrease in mutant fruits, especially at the BR stage (Fig. 1c). The glutathione content remained constant during fruit ripening in all the genotypes but the mutants showed increased glutathione concentration at MG and BR stages (Fig. 1d). Ascorbic acid and H<sub>2</sub>O<sub>2</sub> treatments had no effect on glutathione content in

**Table 1** Number of days to observe 50% of red ripe fruits in wild type and *Slggp1* mutant plants in control, 20 mM ascorbic acid and 10 mM H<sub>2</sub>O<sub>2</sub> treatments

	Control	AA	H <sub>2</sub> O <sub>2</sub>
WT	14.0 ± 0.5 aAB	12.0 ± 0.5 aA	15.0 ± 1.0 aB
GGP-5261	17.0 ± 1.0 bB	13.0 ± 0.5 aA	19.0 ± 1.5 bC
GGP-49C12	17.0 ± 1.0 bB	13.0 ± 0.5 aA	19.0 ± 2.0 bC

Lower case letters denote statistical differences between genotypes for the same treatment and capital letters denote statistical differences between same genotypes for different treatments (ANOVA,  $p \leq 0.05$ ). Values are the mean of three-independent experiments



**Fig. 1** Ascorbic acid content and redox state (%) in control (a), 20 mM ascorbic acid (b) and 10 mM  $\text{H}_2\text{O}_2$  (c) treatments and glutathione content and redox state (%) in control (d), 20 mM ascorbic acid (e) and 10 mM  $\text{H}_2\text{O}_2$  (f) treatments. Values are the mean of three-independent experiments with two replicates each. Data are expressed based on fresh weight. Asterisk denotes statistical differ-

ences between different genotypes for the same ripening stage and treatment, open circle denotes statistical differences between the same genotype and ripening stage for different treatments and closed square denotes statistical differences compared to the MG stage for the same genotype (ANOVA,  $p \leq 0.05$ )

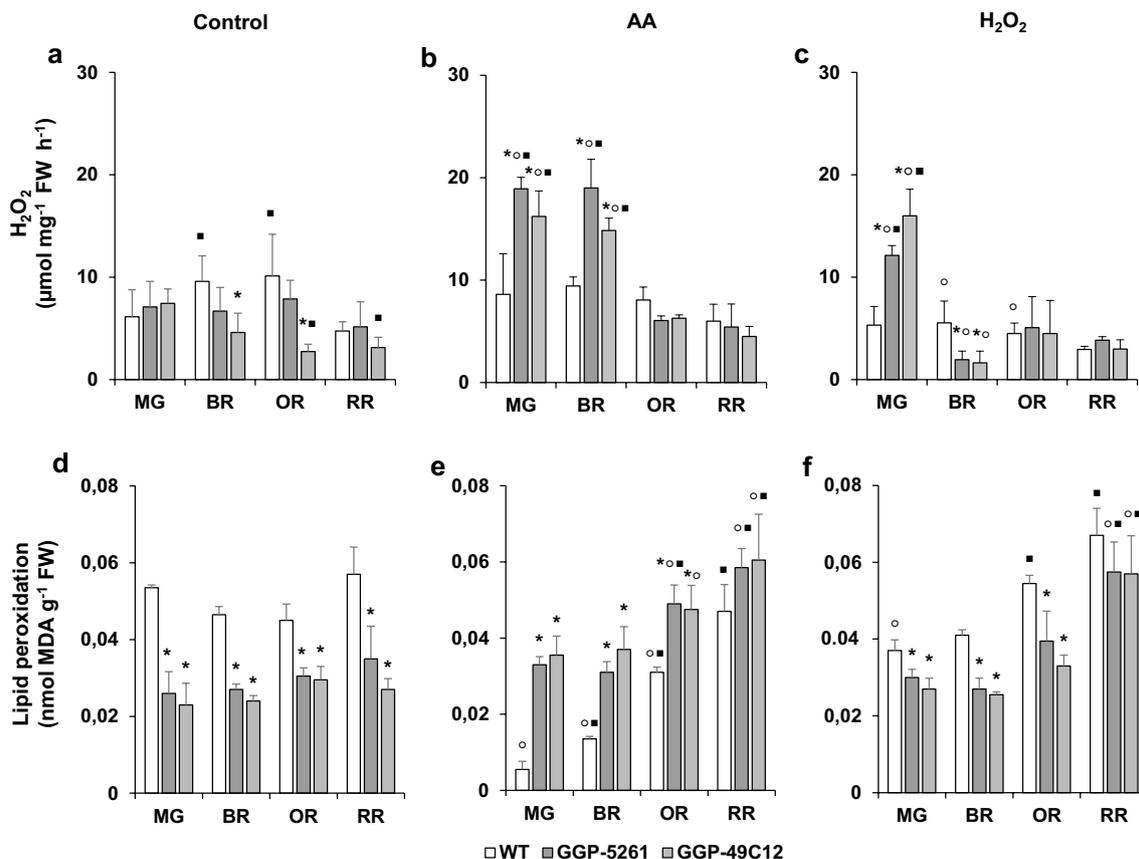
fruit of both mutants and wild type (Fig. 1e, f). In wild type, glutathione was more highly oxidized than in mutants fruit (Fig. 1d). Ascorbic acid treatment reduced the redox state at OR and RR in mutants but not in wild type fruit (Fig. 1e). The treatment with  $\text{H}_2\text{O}_2$  decreased the glutathione redox state in wild type fruits at all ripening stages and in mutants for OR and RR fruits (Fig. 1f).

### **$\text{H}_2\text{O}_2$ production and lipid peroxidation during fruit ripening**

The production of  $\text{H}_2\text{O}_2$  increased progressively in wild type fruit from MG up to OR stage and then declined at RR. Such a pattern was not observed in the mutants; on the contrary, in the GGP-49C12, fruits it tended to decrease during ripening (Fig. 2a). Ascorbic acid treatment had no effect on the wild type fruits, while unexpectedly it induced a significant increase in  $\text{H}_2\text{O}_2$  production

(three- to fourfold compared to control) at MG and BR stages in both *Slgpp* lines (Fig. 2b).  $\text{H}_2\text{O}_2$  treatment produced a small reduction in its synthesis in BR and OR wild type fruits; in mutant plants, it induced an increase at the MG stage followed by a strong decrease at the BR stage (Fig. 2c).

Under control conditions, the lipid peroxidation level remained constant at all ripening stages for all genotypes; however, the concentration of MDA was always higher in wild type compared to the mutants (Fig. 2d). Ascorbic acid treatment significantly reduced the lipid peroxidation level in wild type, mainly at MG and BR stages (ten- and fivefold, respectively). The reverse effect was observed in the mutant plants for which MDA content increased gradually by twofold up to the RR stage (Fig. 2e). The lipid peroxidation pattern was similar under  $\text{H}_2\text{O}_2$  treatment increasing in all genotypes during ripening (Fig. 2f).



**Fig. 2** H<sub>2</sub>O<sub>2</sub> production by tomato fruit in control (a), 20 mM ascorbic acid (b) and 10 mM H<sub>2</sub>O<sub>2</sub> (c) treatments and lipid peroxidation in control (d), 20 mM ascorbic acid (e) and 10 mM H<sub>2</sub>O<sub>2</sub> (f) treatments. Fruit were washed with distilled water and H<sub>2</sub>O<sub>2</sub> production measured for the following 100 min. Values are the mean of three-independent experiments with two replicates each. Asterisk denotes

statistical differences between different genotypes in the same ripening stage and treatment, open circle denotes statistical differences between same genotype and ripening stage for different treatments and closed square denotes statistical differences compared to the MG stage for the same genotype (ANOVA,  $p \leq 0.05$ )

## H<sub>2</sub>O<sub>2</sub> metabolism during fruit ripening.

To assay the capacity of H<sub>2</sub>O<sub>2</sub> removal, the APX, PX and CAT activities were measured (Fig. 3). CAT activity showed a peak at BR and OR stages only in the wild type tomatoes. In contrast, mutants presented similar activities throughout ripening with higher values than wild type at MG and RR (Fig. 3a). While ascorbic acid and H<sub>2</sub>O<sub>2</sub> treatments reduced CAT activity in mutants at the MG stage, H<sub>2</sub>O<sub>2</sub> increased CAT activity in the wild type (Fig. 3b, c).

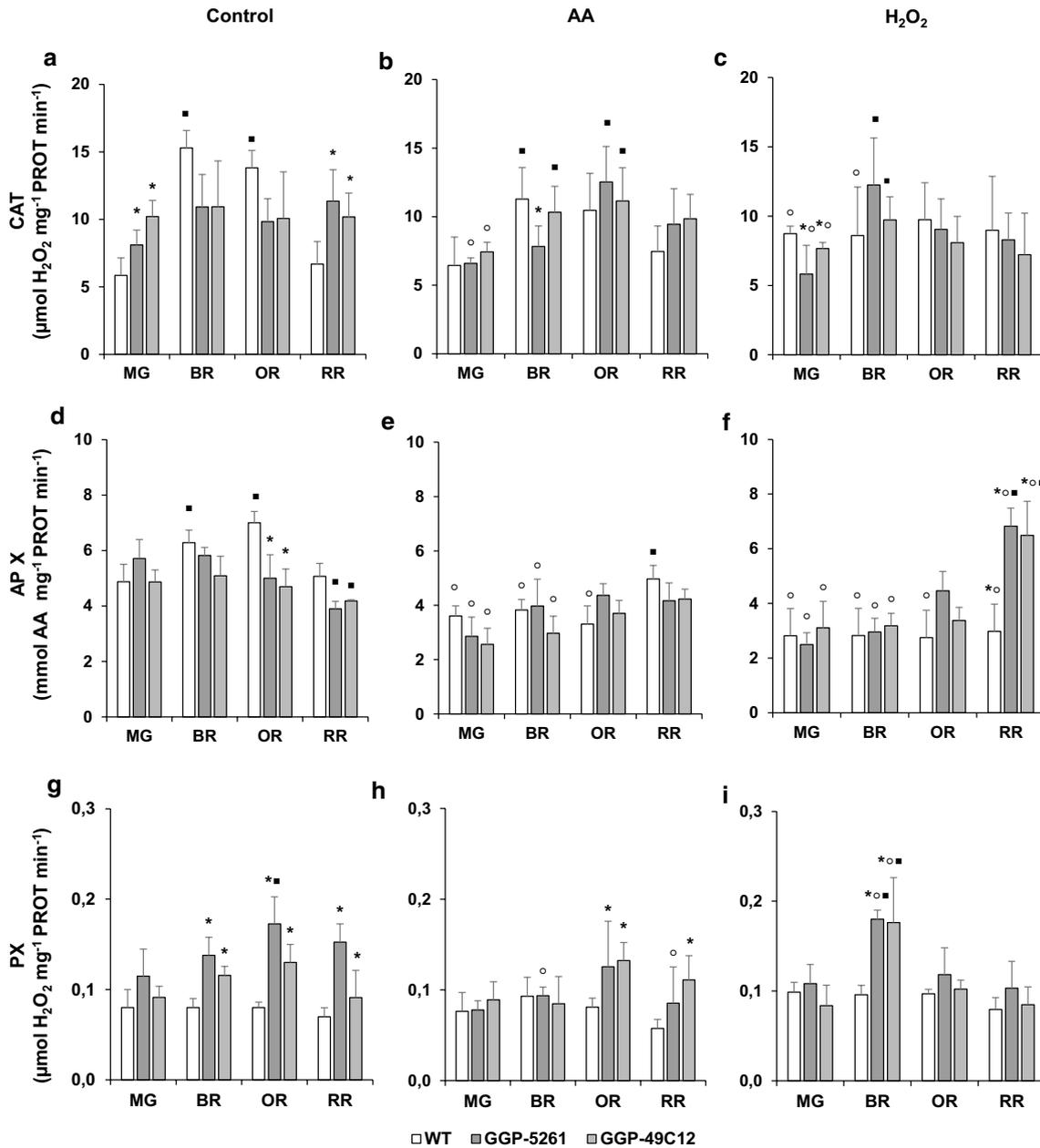
APX activity increased in wild type at BR and OR stages but not in mutants in untreated fruit (Fig. 3d). This trend was not observed in ascorbic acid and H<sub>2</sub>O<sub>2</sub> supplemented tomatoes and showed in general lower activity in all genotypes (Fig. 3e, f). In particular, H<sub>2</sub>O<sub>2</sub> treatment decreased the APX activity but activity increased in both mutants at the RR stage.

PX activity did not change during normal ripening in wild type but both mutants showed higher activities than wild

type from BR to RR stages and particularly a peak at the OR stage (Fig. 3g). Ascorbic acid application reduced PX activity avoiding this peak in one of the mutants (Fig. 3h). H<sub>2</sub>O<sub>2</sub> treatment produced an increase in PX activity in both mutants at the BR stage (Fig. 3i).

Besides the *in vivo* synthesis of H<sub>2</sub>O<sub>2</sub> analyzed with Amplex Red, ROS production was assayed by measuring the activities of NADPH oxidase and SOD.

NADPH oxidase activity showed a peak in fruit at BR stage in wild type with twofold higher activity than in the mutant tomatoes (Fig. 4a). Ascorbic acid application produced an increase in NADPH oxidase activity in wild type at MG stage, but no change was observed in *Slgpp* plants (Fig. 4b). H<sub>2</sub>O<sub>2</sub> treatments provoked increases in NADPH oxidase activity in both mutants at BR stage which were higher than in wild type (Fig. 4c). Similar to NADPH oxidase, SOD activity showed a peak in wild type only at BR stage (Fig. 4d). Ascorbic acid stimulated this enzyme activity in mutants reaching similar values to those observed in



**Fig. 3** Changes in catalase (CAT), ascorbate peroxidase (APX) and peroxidase (PX) activity in control, 20 mM ascorbic acid and 10 mM H<sub>2</sub>O<sub>2</sub> treatments. Values are the mean of three independent experiments with two replicates each. Asterisk denotes statistical differences between different genotypes for the same ripening stage and

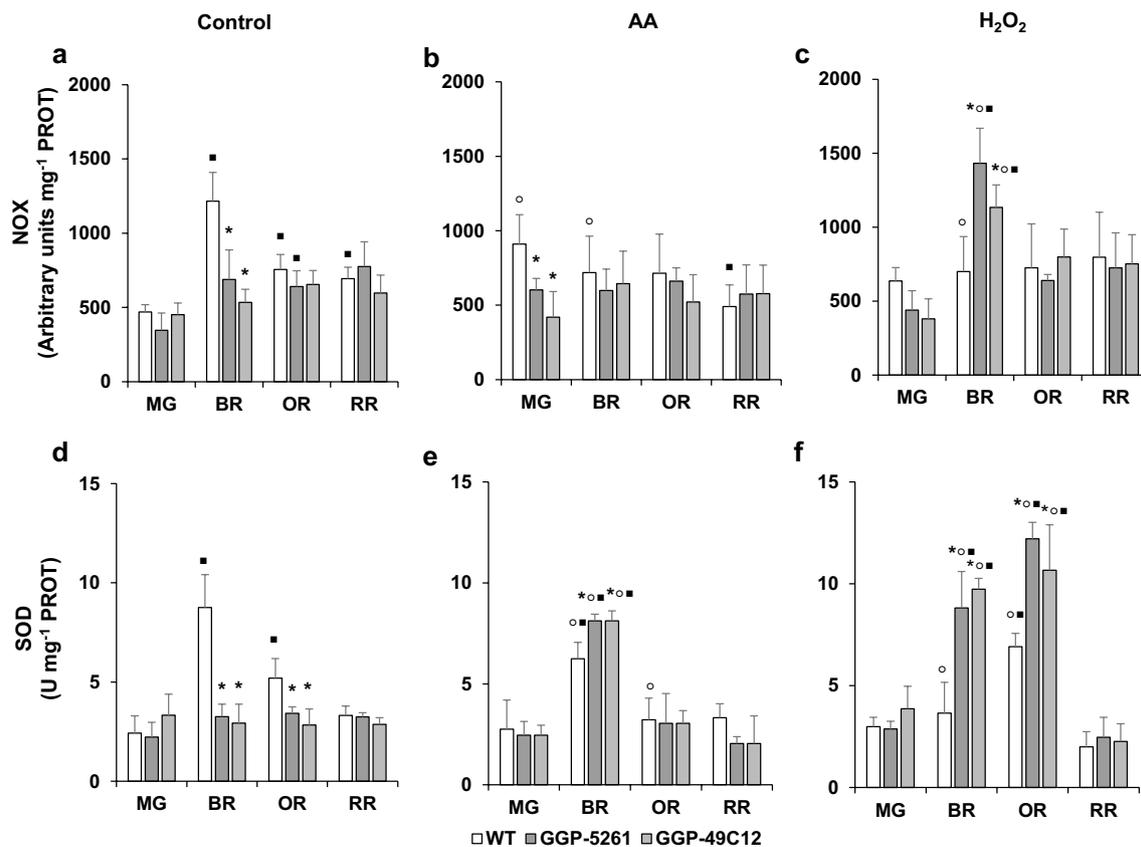
treatment, open circle denotes statistical differences between same genotype and ripening stage for different treatments and closed square denotes statistical differences compared to the MG stage for the same genotype (ANOVA,  $p \leq 0.05$ )

wild type at BR (Fig. 4e). H<sub>2</sub>O<sub>2</sub> treatment also produced an increase in SOD activity in both mutants at BR stage but in all genotypes at OR stage (Fig. 4f).

### Subcellular localization of H<sub>2</sub>O<sub>2</sub> production

Although DCFDA oxidation visualized by confocal microscopy cannot be used to quantify ROS production it

is useful to determine in which subcellular compartment H<sub>2</sub>O<sub>2</sub> is produced. Figure 5 shows DCFDA oxidation in all genotypes at BR stage. Chloroplasts were the only organelles where H<sub>2</sub>O<sub>2</sub> was detected in cells of the wild type and both mutant fruits. Similar effects were observed in ascorbic acid and H<sub>2</sub>O<sub>2</sub> treated fruit (Supplementary Fig. S1 and Supplementary Fig. S2, respectively).



**Fig. 4** Changes in superoxide dismutase (SOD) and NADPH oxidase (NOX) activity in control, 20 mM ascorbic acid and 10 mM H<sub>2</sub>O<sub>2</sub> treatments. Values are the mean of three-independent experiments with two replicates each. Asterisk denotes statistical differences between different genotypes for the same ripening stage and

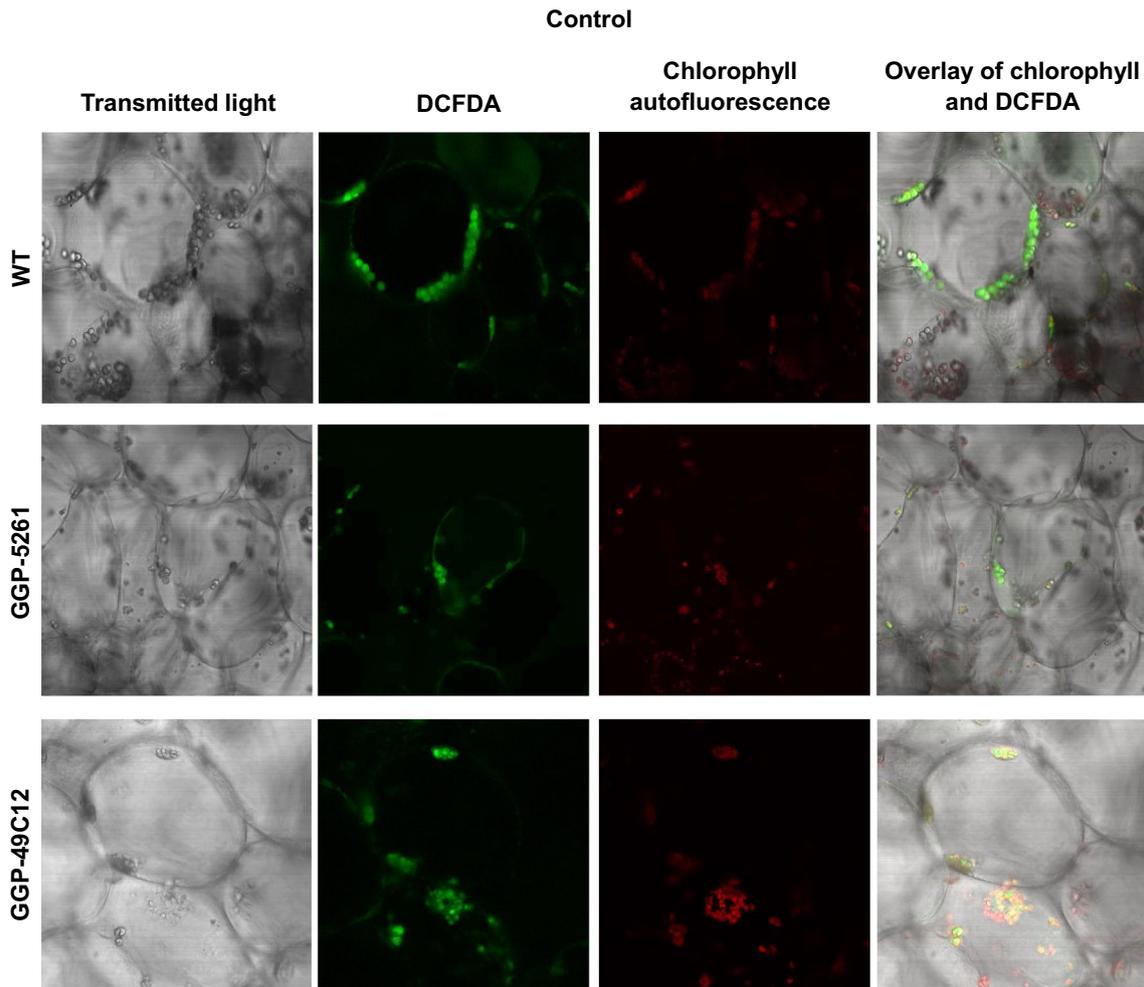
treatment, open circle denotes statistical differences between same genotype and ripening stage for different treatments and closed square denotes statistical differences compared to the MG stage for the same genotype (ANOVA,  $p \leq 0.05$ )

### Ascorbic acid and H<sub>2</sub>O<sub>2</sub> effects in the fruit quality parameters during ripening

Respiration decreased throughout the ripening of the fruit of all genotypes. A decrease in respiration in wild type fruit was observed between MG and BR stages but in both mutants between OR and RR (Fig. 6a–c). Firmness decreased with maturation, both mutants having higher values than wild type at RR stage in untreated fruit (Fig. 6d–f). None of the genotypes showed changes in the pH of the fruit (Suppl. Table S1) but the total soluble solids/total titratable acidity ratio that decreased with maturation remained higher in both mutants than in wild type at BR, OR and RR stages in untreated plants (Suppl. Table S1).

### Discussion

Changes in the production or concentration of ROS and antioxidants are associated with fruit ripening; however, their specific roles are still barely addressed. Tomato *Slggl* mutants, with low ascorbic acid content, and exogenous chemical treatments were used in this work to unravel the functions of this antioxidant as well that of H<sub>2</sub>O<sub>2</sub> during tomato fruit maturation.



**Fig. 5** Tomato pericarp tissues at BR stage analyzed by confocal microscopy. DCFDA probe was used for the detection of the sub-cellular  $H_2O_2$  localization. Bright field images, 0,025 mM DCFDA

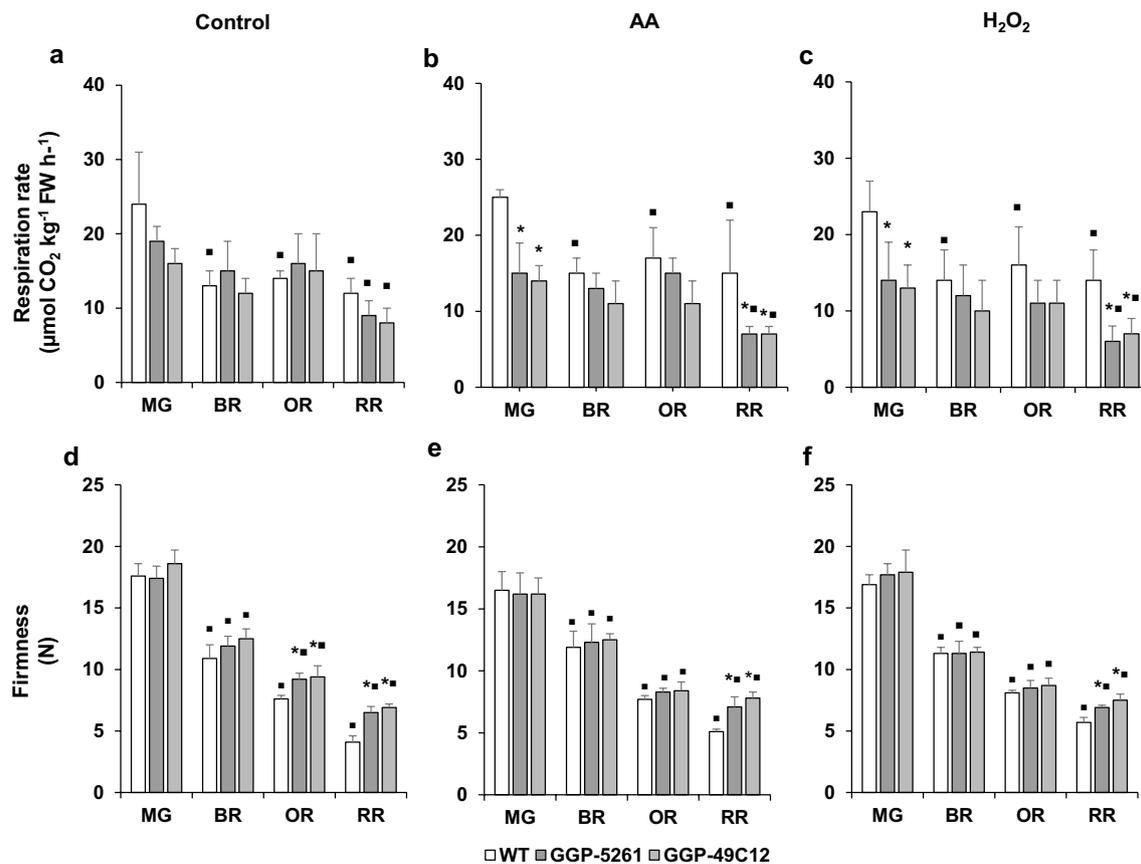
fluorescence, chlorophyll autofluorescence, for wild type, GGP-5261 and GGP-49C12 are shown. Colocalization of the DCFDA signal and chlorophyll autofluorescence was observed in all cases

### The effect of ascorbic acid concentration on fruit ripening

Many functions are established for ascorbic acid during the growth and development of plants. It is considered as the central water-soluble antioxidant, with specific roles mainly in photosynthesis and in cell expansion and division as well as acting as a cofactor for many enzymes (Foyer et al. 2020). Among these enzymes, 1-aminocyclopropane-1-carboxylic acid oxidase, which catalyzes ethylene formation, requires ascorbic acid (Dong et al. 1992); however, we have previously observed that fruits of both *Slgpp1* mutants show increased ethylene production (Alegre et al. 2020). Most studies dealing with ascorbic acid deficiency effects in plants have focused on leaves and a few in fruit. Several tomato plants have been generated with down- or up-regulation of gene expression for enzymes of ascorbate synthesis pathway and also those of the recycling pathways (Amaya et al.

2014; Gilbert et al. 2009; Bulley et al. 2012; Cronje et al. 2012; Cai et al. 2015; Alhagdow et al. 2007; Zhang et al. 2013). However, there is a lack of information regarding the consequences of ascorbic acid content modification on the timing of fruit ripening. To address this question, we used two *Slgpp1* mutant plants producing fruits whose ascorbic acid content is around 3 times lower than the wild type. This deficiency is associated with delayed ripening from green to red ripe stages of the fruit (Table 1). The return to wild type traits observed in both mutants after ascorbate treatment demonstrates the participation of this metabolite in fruit ripening.

During ripening, firmness of tomato fruits is characterized by a progressive decline, but firmness remains higher in both *Slgpp1* mutants at RR stage as previously observed (Alegre et al. 2020); however, this change in firmness was not reverted by ascorbic acid nor  $H_2O_2$  treatments (Fig. 6). These results suggest that the deficiency of *SIGGP1* activity



**Fig. 6** Respiration and firmness in control, 20 mM ascorbic acid and 10 mM  $\text{H}_2\text{O}_2$  treatments. Values are the mean of three-independent experiments with two replicates each. Respiration data are expressed based on fresh weight. Asterisk denotes statistical differences between different genotypes for the same ripening stage and treat-

ment, open circle denotes statistical differences between the same genotype and ripening stage for different treatments and closed square denotes statistical differences compared to the MG stage for the same genotype (ANOVA,  $p \leq 0.05$ )

results in changes of some fruit ripening processes that are not associated with ROS or antioxidant concentrations. This effect may be due to an accumulation of precursors destined for cell wall formation in *Slgpp1* mutant tissues. The low ascorbate content in GDP-D-mannose 3,5-epimerase (*SIGME*)-silenced plants (for both *SIGME1* and *SIGME2* genes) is linked with firmness reduction of the tomato fruit (Gilbert et al. 2009). Together these results suggest that silencing either GME or GGP, in addition to reducing ascorbic acid biosynthesis flux, also impairs processes related to fruit softening, and this may depend on the precise impairment which could occur before or after the delivery of sugars for cell wall formation. This conclusion must be confirmed by future experiments.

### $\text{H}_2\text{O}_2$ participation in fruit ripening

The increase in the ROS steady state at the beginning of tomato fruit ripening is well demonstrated by several studies. Jimenez et al. (2002) observed an increase in  $\text{H}_2\text{O}_2$  and

oxidative damage at the chloroplast-chromoplast transition stage. Similarly, Kumar et al. (2016) observed a peak of  $\text{H}_2\text{O}_2$  at BR stage during tomato ripening. Brennan and Frenkel (1977) demonstrated the participation of  $\text{H}_2\text{O}_2$  in the initiation and promotion of fruit ripening in pears. In our work, in vivo  $\text{H}_2\text{O}_2$  production increased during the transition from MG to OR stages only in wild type fruit (Fig. 2). Barsan et al. (2012) in a proteomic study observed an increase of stress responsive proteins associated with redox pathways such as components of the ascorbate–glutathione cycle or heat-shock proteins during the green to red transition in tomato. However, ROS production may be a secondary associated event, or in contrast, may be an active signal promoting ripening associated processes (Decros et al. 2019). Unexpectedly, the ascorbic acid supplementation provoked an increase in  $\text{H}_2\text{O}_2$  production. Although ascorbic acid has a central function as antioxidant, it may have also a prooxidant function. This ROS generating activity depends on its concentration and the presence of transition metals such as  $\text{Fe}^{3+}$  and  $\text{Cu}^{2+}$  (Buettner and Jurkiewicz

1996). Associated with this, increase in  $H_2O_2$  production the ripening time in both *Slggp1* mutants was shortened. In concordance with this observation, the treatment with  $H_2O_2$  provokes decreases in its own production at BR stage delaying fruit ripening in plants of both *Slggp1* lines. Taken together, these results highlight the participation of  $H_2O_2$  in the tomato chloroplast-chromoplast transition.

Increased oxidation may be a key component of ROS signaling; however, the absence of a link between MDA content and redox states of antioxidants (i.e. glutathione and ascorbate) with changes in ripening time suggests that oxidative damage is not a key determinant for fruit maturation and reinforces the evidence that  $H_2O_2$  itself represents a signal involved in fruit ripening (Steelheart et al. 2019b). The relative high stability of  $H_2O_2$  in cells is crucial for its accumulation and function in different plant cell compartments (Foyer and Noctor 2016). The high stability allows the diffusion of  $H_2O_2$  from different organelles to provide retrograde signaling by impacting gene expression (Galvez-Valdivieso and Mullineaux 2010).

Chloroplasts, mitochondria, peroxisomes and the apoplast represent the main subcellular sites of ROS production (Noctor et al. 2018). Photosynthetic and respiratory electron transport chains are considered as the main sources of ROS in chloroplast and mitochondria, respectively. In peroxisomes,  $H_2O_2$  is produced by enzymes associated with photorespiratory metabolism. In addition, the associated NADPH oxidase and SOD activities may also contribute to the accumulation of  $H_2O_2$  in the apoplast of plant cells. To determine the subcellular sites of  $H_2O_2$  production we performed confocal microscopy in fruit tissues. This analysis demonstrates that the production of ROS in control, ascorbic acid or  $H_2O_2$ -treated fruit matches with chlorophyll, indicating that the main  $H_2O_2$  production takes place in chloroplasts. The dismantling of thylakoid membranes observed during the chloroplast–chromoplast transition (Barsan et al. 2012) may cause an increase in ROS accumulation. In addition, the increase in the activities of NADPH oxidase and SOD in wild type fruit indicates they could participate in  $H_2O_2$  production at BR stage (Fig. 4). However, the absence of DCFDA oxidation in the apoplast suggests that the  $H_2O_2$  production in this compartment may be very low; furthermore, it does not mean is absent during fruit ripening. New experiments using more accurate methods must be carried out to determine the participation of ROS produced in the apoplast on fruit ripening.

The concentration of  $H_2O_2$  in plant tissues depends on the balance of its production and removal. Arabidopsis plants with low ascorbic acid show altered antioxidant activity (Colville and Smirnoff 2008). Similarly, in this work, tomato *Slggp1* plants present higher GSH content as well as greater catalase and peroxidase activities that may avoid the peak in  $H_2O_2$  accumulation observed in non-treated wild type fruit.

Ascorbic acid and  $H_2O_2$  treatments increased or decreased  $H_2O_2$  production, respectively, at the BR stage (Fig. 2). These modifications in ROS production cannot be explained by the change in the activity of a particular antioxidant but may result from the balance of them, such as CAT, APX or PX activities and glutathione content measured in this work.

Taken as a whole, the results presented here demonstrate that changes in the  $H_2O_2$  and ascorbic acid levels actively participate in the development of fruit ripening.

**Author contribution statement** CS, MLA, IG, GEGG, DJ, CB, YO and HE performed the experiments and made the statistical analysis of the data. PB, CR, CS and CGB planned different aspects of the research and wrote de manuscript. All authors read and approved the manuscript.

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