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High light stress induces H_2O_2 production and accelerates fruit ripening in tomato

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ABSTRACT

Increased synthesis of H_2O_2 is observed during the initiation of fruit ripening. However, its association with plant cell processes triggering the maturation of fruit has not yet been demonstrated. The aim of this work is to investigate whether H_2O_2 participates in the tomato ripening process and particularly through its association with the ethylene signaling pathway. The experiments were carried out with two ethyl methanesulfonate mutant lines of Micro-Tom tomato deficient in GDP-L-galactose phosphorylase activity and displaying lower ascorbic acid content than the corresponding parental genotype (i.e. wild type). Plants were subjected to a high irradiance (HI) treatment to stimulate H_2O_2 synthesis. HI treatment enhanced H_2O_2 production and reduced the timing of fruit ripening in both mutants and wild-type fruits. These results could be linked to an increase of the expression of H_2O_2-related genes and changes in the expression of ethylene-related genes. The fruit H_2O_2 production increased or decreased after applying the treatments that induced ethylene synthesis or blocked its action, respectively. The results presented in this work give an evidence of the association of redox and hormonal components during fruit ripening in which H_2O_2 participates downstream in the events regulated by ethylene.

Key words: antioxidants, ascorbate, ethylene, fruits, glutathione, hydrogen peroxide, ripening, *Solanum lycopersicum* L. cv Micro-Tom

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1. Introduction

The ripening phase in tomato fruit is characterized by the transformation of chloroplasts in chromoplasts a process that involves the dismantling of chloroplast structures, degradation of chlorophyll accompanied by a concomitant synthesis and accumulation of lycopene [1]. The resultant chromoplast is an active plastid where takes place the synthesis of carotenoids, aminoacids, vitamins and lipids [2]. As a climacteric species, tomato requires the participation of ethylene for the initiation of ripening. This hormone is essential to the chromoplast formation but also to the softening of the fruit and accumulation of several metabolites such as reducing sugars and volatile compounds [3]. Fruits of mutants with deficiency of ethylene synthesis or ethylene sensitivity do not ripen and consequently stay at the green stage of their development [4]. The regulation of the ripening process has been extensively studied in the climacteric fruits in which ethylene is responsible for initiating and/or coordinating ripening-associated modifications, including changes in fruit colour, firmness, respiratory crisis and ethylene burst [3,5,6]. In tomato, ethylene is produced through the non-autocatalytic ethylene system 1 until the mature green stage while the ripening-associated ethylene peak is autocatalytic (system 2) [5]. Different isoforms of 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO), the two enzymes that catalyze the committed steps of ethylene biosynthesis, are involved in system 1, system 2 and stress-induced ethylene production. The ethylene signaling and response pathway includes ethylene receptors, one of which is mutated in the tomato Never ripe mutant [6], and Ethylene Response Factors (ERFs) that mediate the diversity of responses to ethylene and its interactions with other hormones and redox signaling [7,8].

During the transition from immature to mature stages, several studies show an increase of oxidative stress in fruit of different species [9,10]. In bell pepper disks treated with menadione, tert-butylhydroperoxide, paraquat, diamide or buthionine sulfoximine in order to

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induce oxidative stress the expression of genes encoding enzymes of lycopene synthesis is enhanced [11]. Our previous work shows the association of a peak of H_2O_2 production with the initiation of tomato fruit ripening [12]. Additionally, the fruits of tomato mutants displaying lower lipid peroxidation and higher levels of glutathione, all associated with a reduced H_2O_2 production show a delayed ripening phase [12]. These results suggest that components of the redox metabolism, like H_2O_2, participate in the process of tomato maturation. During the last two decades, several works have described the role of reactive oxygen species (ROS) and antioxidant molecules in numerous processes throughout plant development [13]. However, the role of the redox metabolism as a component of the events participating in fruit ripening, and particularly H_2O_2 - ethylene interaction, has not been established yet. Here, the experiments were carried out to confirm the hypothesis that H_2O_2 is integrated to the physiological mechanisms triggering fruit ripening. In this sense, we explored the possible interaction of this ROS with the ethylene signaling pathway. To achieve this task we used tomato plants with altered oxidative metabolism and performed treatments stimulating H_2O_2 synthesis or modifying ethylene signaling.

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2. Material and methods

2.1. Plant material and treatments

The experiments were carried out in the greenhouses of the Institute of Plant Physiology, National University of La Plata, Argentina. Plants of tomato wild type (*Solanum lycopersicum* L. cv Micro-Tom) and GDP-L-galactose phosphorylase-deficient ethyl methanesulfonate (EMS) mutant lines (*Slggp1*) were isolated using TILLING approach from the NBRP-Tomato population (Tsukuba-Japan) and TILLING-Tomato collection (Bordeaux-France) and named *ggp-5261* and *ggp-49C12*, respectively [14]. Furthermore, to reduce the number of mutations distributed across the genome, the original mutant lines were back-crossed with the wild-type Micro-tom parent several times. In the present study, the plants corresponded to BC6F2 seeds. The EMS mutant GGP-5261 was identified as previously described [15] using Endo1 endonuclease coupled with a LI-COR DNA analyzer, and was further confirmed by sequencing. This mutation corresponds to a nucleotide change in the exon 8 of the *SlGGP1* gene (SoLyc06g073320) from G to T resulting in the change of GAG encoding glutamic acid to TAG encoding a stop codon as described in [14]. This point mutation corresponds to a nonsense mutant, leading to the truncation of the GGP protein. The plants were grown in 3 L pots filled with fertilized soil at about 700 µmol m^{-2} s^{-1} (Photosynthetic photon flux density at midday), and temperatures of 25 ± 2 and 20 ± 3 °C, day and night, respectively. The diary light/dark periods were about 14 and 10 h, respectively.

A high irradiance treatment (HI, about 2000-2500 µmol m^{-2} s^{-1} PPFD at midday) was used to induce an increase of H_2O_2 production during ripening. Plants with fruits at mature green (MG) stage on trusses were placed outside of the greenhouse at full sunlight for 20 d while control plants were still cultured in the greenhouse conditions (GI). Besides, for the

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ethylene-modulated ripening analysis treatments with 1-aminocyclopropane-1-carboxylic acid (ACC, an ethylene precursor) and with 1-methyl cyclopropene (1-MCP, an ethylene inhibitor) were applied to plants growing in the greenhouse for 20 d. Since ACC treatments were shown to increase the ethylene production of *chrysanthemum* petals during senescence [16], plants harboring MG fruits were sprayed three times a week before nightfall with 5 ml of 0.5 mM ACC (Sigma-Aldrich®), using distilled water as control and Tween 20 at 0.01 % (v/v) as surfactant. In addition, since the effect on the ethylene synthesis can be seen in the combined treatment of 1-MCP and S-nitrosoglutathione during postharvest [17], related with previous results with 1-MCP alone in several tomato cultivars and ripening stages [18], 1-MCP treatments were carried out three times a week before nightfall, on plants harboring MG fruits placed into a 40L sealed chamber with 8 ppm of 1-MCP (EthylBloc™), or a chamber without ethylene inhibitor as the control treatment for 12 h.

The effect of the treatments on "*in vine*" ripening was determined at MG, breaker (BR), orange (OR) and red ripe (RR) fruit. These developmental stages were selected as indicated by the United States Department of Agriculture standard grades of fresh market tomatoes [19]. The pericarps taken from a batch of samples were cut and frozen in liquid nitrogen and stored at -80 °C until use.

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2.2. Fruit temperature determination

The temperature of the fruits was recorded at 12 and 14 h UT with an infrared thermometer (Raynger® ST6™).

2.3. Ripening time

The effect of HI and ethylene on ripening time was determined as described by Steelheart et al. [12], *i.e.* by counting the days needed for the color change from MG to RR

stage using 20 fruits per genotype from different plants. For each treatment, the number of days during "*in vine*" ripening was taken when 50% plus one fruit reached the RR stage.

2.4. H_2O_2 production and lipid peroxidation

The H_2O_2 production was determined as previously reported in Steelheart et al. [12] by immersing the entire fruit (fresh weight of about 3 g), immediately after harvest in a 50 mL conical bottom tube with 2 mL of Amplex Red™ solution, containing 10,000 U/L horseradish peroxidase, 10 μmol/L Amplex Red (N-acetyl-3,7-dihydroxyphenoxazine) and 50 mmol/L Tris–HCl buffer, pH 7 (ThermoFisher Scientific). One hundred min after beginning the incubation, fluorescence change of the solution was measured using a CLARIOstarPlus Multi-mode Microplate Reader (Ortenberg, Germany), in fluorescence mode at 560 nm excitation wavelengths [20]. The H_2O_2 production was estimated comparing the fluorescence changes with a standard curve of H_2O_2 (Amplex Red™). Lipid peroxidation was estimated by measuring the malondialdehyde content (MDA); homogenates were prepared according to Steelheart et al. [8], with frozen pericarp fruit grounded in 1 mL of 50 mM potassium phosphate buffer, pH 7.0 and 100 μL of a 0.2% (w/v) ethanol solution of butylated hydroxytoluene and centrifuged at 16,000×g for 10 min. Supernatants were mixed with 1 M HClO (1:1), and 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. MDA was detected at 590 nm with HPLC conditions according to Templar et al. [21]. The MDA used for the standard curve were prepared with 1,1,3,3-tetramethoxypropane (Sigma-Aldrich®).

2.5. Photosynthesis measurements

Photosynthesis measurements were carried out on fruits at MG and BR stages "*in vine"* during HI and GI treatments. Nevertheless, photosynthesis was not measured during the

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stages undergoing senescence-associated chloroplast dismantling, as it takes place at OR and RR stages, because the chlorophyll fluorescence analysis cannot be properly assayed. The electron transport rate (ETR) was determined with a chlorophyll modulated fluorescence system (FMS-2, Hansatech Instruments Ltd., Norfolk, UK) and calculated according to Genty et al. [22] at the irradiance received *in situ* at the fruit surface. The absorptivity was considered as 0.85 since this is a highly conserved value in non-senescent leaves of various species [23]. The PSII efficiency (Fv/Fm) was measured using the saturating pulse method [23]. Adapted fixed clamps were used to darken the fruit for 30 min. Measurements were carried out after acclimation to darkness and relaxing the non-photochemical dissipation mechanisms. Non-photochemical quenching (NPQ) was calculated according to Baker [24] from chlorophyll fluorescence parameters.

2.6. Ascorbic acid and glutathione concentration

The ascorbic acid and glutathione contents were assayed using approximately 150 mg of frozen fruit pericarp powder. The extraction was carried out as described in Bartoli et al. [25], with a 3% (v/v) trifluoroacetic acid solution and centrifuged at 16,000×g for 10 min and the homogenate was used to assay both ascorbic acid (reduced form) and dehydroascorbic acid (oxidized form) using a HPLC system (Shimadzu LC-10ATvp solvent delivery module) and L-ascorbic acid with a purity of 99.9% (Sigma-Aldrich®), as standard. The redox state of ascorbate was calculated according to Steelheart et al. [12], using the following formula: % of dehydroascorbic acid = [(dehydroascorbic acid content)/(ascorbic acid content + dehydroascorbic acid content)] × 100. The same homogenates were also used to measure glutathione content with an enzymatic assay using a UV-visible recording spectrophotometer (AV-160A Shimadzu, Japan) as described by Griffith [26], in a reaction mixture containing 100 mM potassium phosphate (pH 7.5), 5 mM EDTA, 0.3 mM NADPH, 6 mM DTNB and

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approximately 50 units of glutathione reductase/mL, using L-Glutathione ≥98% (Sigma-Aldrich®), as standard. Oxidized glutathione was measured after incubating 100 µL of each sample with 2 µL of vinylpyridine. The redox state of glutathione was calculated with the same formula used for ascorbate.

2.7. Enzyme assays

The homogenates were prepared and processed as previously described [12] to assay the enzymatic activities with a UV-visible recording spectrophotometer (AV-160A Shimadzu, Japan), 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 µM E-64 and 1 µM of EDTA, from Sigma-Aldrich®). The homogenates were centrifuged at 16,000×g for 10 min, and the supernatant was used for the enzymatic assays.

Catalase (CAT) activity was assayed according to Bartoli et al. [27], monitoring H_2O_2 decomposition at 240 nm, in a reaction medium consisting of 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2, up to 100 µL of homogenate (1 mg/mL protein) and 0.1% (v/v) Triton X-100.

Ascorbate peroxidase (APX) activity was measured according to Amako et al. [28] with modifications as previously described [8] following ascorbic acid disappearance at 265 nm. 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 H_2O_2. The activity was calculated using an extinction coefficient of 2.8 mM^{-1} cm^{-1} for ascorbic acid.

Total soluble protein concentration was determined as described in Bradford [29] and using bovine serum albumin (Sigma- Aldrich®) as a standard.

2.8. Quantitative RT-PCR analysis

Approximately 200 mg of frozen fruit powder were used to extract total RNA using the TRIzol™-Reagent (Sigma Aldrich®), following the manufacturer's instructions with modifications; the resulting RNA pellet was resuspended in 100 µl of milli Q water treated with DEPC and 33 µl of 8 M lithium chloride (1/3 ratio) and precipitated overnight at 4°C. Subsequently, it was centrifuged for 10 min at 10,000 x g at 4°C, and the resulting pellet was washed with 500 µl of cold ethanol 70% (v/v) and centrifuged for 10 min at 12000 x g and 4°C. Then, RNA pellet was resuspended in 88 µl of milli Q water treated with DEPC and next mixed with 10 µl of 10X RQ (RNA Qualified) buffer and 2 µl of RNase free-DNase RQ (1U / µl) (Promega®). The mixture was incubated at 37°C in a water bath for 1 h, then mixed by inversion with 200 µl of pure isopropanol and was finally centrifuged for 10 min at 10,000 x *g* and 4 °C. The supernatant was discarded, and the pellet was washed with 500 µl of cold ethanol 70% (v/v) followed by a centrifugation for 10 min at 10,000 x *g* and 4°C.

Finally, the pellet was resuspended in 20 µl of milli Q water treated with DEPC. RNA concentration and purity was determined using a CLARIOstarPlus microplate reader (BMG Labtech). RNA integrity was analyzed by TAE-agarose 1% gel electrophoresis.

Purified RNA (2 µg) was used to prepare cDNA using M-MLV reverse transcriptase (Promega®) with random primers according to the manufacturer's instructions. Quantitative Real-time PCR was performed with a StepOne Plus™ Real-Time PCR System (Applied Biosystems) using the Master Mix qPCR 2.0 (Sybr/Rox) (PB-L Productos Bio-Lógicos), and PCR conditions were set according to the manufacturer's instructions.

The PrimerQuest Tool from Integrated DNA technologies was used for the primers design. The chosen parameters were: 85 - 150 base pairs (bp) of the amplicon, 18 - 25 bp of

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the primer, 59 - 63 ^{0}C melting temperature and a guanine/cytosine ratio of approximately 40-60%.

For the relative quantification, Ct values of gene expression were quantified according to ΔΔCT method. Relative fold changes were calculated by averaging the data of three biological replicates of ripening fruits of the three genotypes (wild type ΔCT). Elongation factor α 1 (SlEfα1) was used as a constitutively expressed gene to normalize the expression of the target genes according to Badejo et al. [30]. Accession numbers and primer sequences of genes analyzed are listed in the Supplementary material Table S1.

2.9. Statistical analysis

Data represent the mean ± SEM of the results obtained from at least three independent experiments (spring/summer 2017-2019) with a minimum of five 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.

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3. Results

3.1. The effect of high irradiance on fruit ripening

The relationship between the redox elements and the fruit ripening process was studied in two tomato *slggp1* mutants displaying an altered redox phenotype and compared with the wild type Micro-Tom under an abiotic stress condition, in the present case a high irradiance treatment. Hence, the plants transferred from inside the greenhouse (GI) to outside *i.e.* under full sun (HI) underwent three times more irradiance, and this resulted in a decrease of the duration of the fruit ripening in all genotypes (Table 1). This drastic irradiance variation was also accompanied by a change of the fruit temperature from 27°C to 37°C (Supplementary material Fig. S1). The effect of other environmental factors such as the UV light present outside the greenhouse may also affect the ripening of HI treated fruits.

In GI condition, the H_2O_2 production displayed an increase from MG up to OR stage and then declines in wild type fruits whereas it remained constant at all stages and equal to the MG value in the mutants (Fig. 1A). HI condition induces a significant increase of H_2O_2 production in fruits of all genotypes only at BR stage (Fig. 1B). In contrast, the quantification of oxidative damage assayed as MDA concentration increased by a factor of five under HI conditions in all samples but it did not show a peak as observed for H_2O_2 during transition stages (Fig. 1C, D). More interestingly, *slggp1* mutant fruits displayed lower oxidative damage under both control and stress treatments possibly due to a higher ROS scavenging capacity.

Since we previously observed that during fruit ripening chloroplasts were a main source of ROS [12], fruit photosynthesis was measured. Under GI, fruit ETR was merely similar between genotypes at MG stage but it slightly declined in both mutants at BR stage. In HI condition, fruit ETR increased in all genotypes by a factor 7 to 10 at the two stages (Table 2).

Fv/Fm slightly declined in both mutants at BR under GI but only one mutant line showed difference under HI. NPQ was very low in all genotypes at GI but largely increased in wild type and *slggp1* mutant fruits at MG and BR stages under HI (Table 2).

Ascorbic acid content, 50% lower in mutant than WT, remained unchanged during fruit ripening in all genotypes under GI condition, but it increased under HI treatment more markedly in wild type than in *slggp1* mutants (Fig. 2A, B). The oxidized form only increased at MG stage in all lines under stress. Similarly, glutathione content did not change whatever the genotype during all the ripening phases under GI and HI treatments. Unlike ascorbate, glutathione content was significantly higher under GI than HI conditions, as well it was higher in the two mutants than WT under GI condition (Fig. 2C, D). However, under HI conditions all genotypes present similar concentrations of glutathione. The oxidized form only increased in both mutants at MG stage under HI. Under GI condition, catalase and APX activities increased up to the middle of the ripening phase and then declined in the WT but remained high and constant in the *slggp1* mutant fruits. With exception of the significant increase of the catalase activity at MG and BR stages in the WT as well as the decrease of the APX at BR stage observed under HI condition, these two enzyme activities displayed no change between GI and HI (Fig. 2E, H).

3.2. Gene expression associated with oxidative stress and fruit ripening

To investigate the association of oxidative stress with the initiation of fruit ripening, specific genes related to H_2O_2 and ethylene signalling were selected and their expression was analysed. The expression of H_2O_2 related genes such as *JUNGBRUNNEN1* (*JUB1)*, a (H_2O_2)-induced NAC transcription factor [31], gradually increased up to the OR stage before declining at RR under GI (Fig. 3A), and showed a higher induction under HI stress conditions in the WT and both ascorbic acid deficient mutants (Fig. 3B). *DEHYDRATION-*

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RESPONSIVE ELEMENT BINDING PROTEIN 1 (*DREB1*), is another H_2O_2 related gene, belonging to the ethylene-responsive element binding protein (*EREBP*) families of TFs and can therefore regulate the expression of many stress-inducible genes in plants [32]. Its expression that was reported to be regulated by *JUB1*, increased later to reach a maximum at RR stage in the WT whereas it drastically declined in the mutants at RR stage. This increase of the *DREB1* expression is even higher under HI (Fig. 3C, D).

Since the overexpression of a constitutively active form of *DREB* (*35S:DREB2A CA*) resulted in the upregulation of an important number of both abiotic and heat shock-related genes [33], besides certain *SMALL HEAT-SHOCK PROTEIN* (*sHSP*) genes, like *SlHSP17.6*, *SlHSP20.0* and *SlHSP20.1*, in tomato were demonstrated to be transcriptionally regulated by ethylene during MG fruit transition into ripening [34], the expression of these *sHSP* was analysed. In a general manner, in the WT under GI condition, the expression of the three selected *sHSP* increased gradually to reach a peak at OR stage to decline at RR, whereas in the mutants, their expression was always lower (Fig. 3E, G, I). In contrast, HI induced a larger increase in *slggp1* than in fruits of wild type plants what is particularly evident at transition stages (Fig. 3F, H, J).

In another hand, considering that H_2O_2 and ethylene are both involved in the modulation of stress responses in plants, although their interrelation is not well understood [35], and since ripening and *sHSP* have been demonstrated to be regulated by ethylene [34] the expression of *ACS6*, *ACO4* and *NEVER-RIPE - ETHYLENE RECEPTOR 3 (ETR3)*, three ethylene-related genes, was determined. *ACS6* gene showed the highest expression at MG stage and then declined during ripening of all genotypes under GI and HI (Fig. 3K, L). *ACO4* gene was induced at OR stage with higher expression in mutants than in wild type fruit under GI (Fig. 3M) while under HI its expression was increased at MG and BR but strongly reduced subsequently in fruits of all lines (Fig. 3N). Under GI condition, *ETR3* gene expression

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showed a peak at OR stage in wild type and this expression was even higher in *slggp1* fruits at OR, but under HI, this expression peak totally disappeared (Fig. 3O, P).

3.3. Ethylene effect in ripening and oxidative stress

To demonstrate the interaction of the oxidative metabolism with hormonal signalling during tomato ripening, fruits were treated with ACC or 1-MCP, compounds that stimulate the synthesis of the hormone or inhibit its action, respectively. As expected, while exogenous ACC application triggered an acceleration of fruit ripening in wild type and in both *slggp1* mutants, the 1-MCP treatment induced the opposite effect (Table 3). Variations observed between control samples might be due to the different manipulations associated to ACC or 1-MCP treatments.

The trend of H_2O_2 production changed after the treatments manipulating ethylene synthesis or signalling comparing the genotypes during fruit maturation (Fig. 4A-D). ACC treatment increased H_2O_2 content by about 2 times in the wild type whereas it increased by 3 to 4 times in the mutants at MG stage and then declined in all lines (Fig. 4A, B). In contrast, 1-MCP drastically reduced the oxidant production in wild type and mutants along all ripening fruit stages (Fig. 4C, D). Oxidative damage was not altered by the ACC treatment, it only decreased in wild type fruits after 1-MCP treatment (Fig. 4E-H).

The concentration of ascorbic acid was merely similar in ACC-treated or untreated fruits, but 1-MCP treatment resulted in the decrease of the ascorbate content in all genotypes at MG and BR stages only (Fig. 5A-D). The redox state of ascorbic acid was not modified in ACC or 1-MCP treated samples (Fig. 5A-D). Glutathione content was increased by ACC treatment in wild type fruits at BR and RR stages but was decreased in both mutants at all ripening stages (Fig. 5E-H). However, 1-MCP also decreased glutathione content in both *slggp1* mutants from BR to RR stages, but no change was observed in the wild type. While

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ACC did not change its redox state, 1-MCP produced a decrease in wild type and an increase in both mutant fruits.

4. Discussion

ROS are continuously produced in subcellular compartments of plant tissues [13]. There is a growing evidence of their participation in many aspects of plant growth and development [36]. An important support to this function is the interaction of the redox components with hormone signalling pathways that has now been clearly demonstrated for many plant processes [37-39]. Associated with fruit ripening, a peak in H_2O_2 is induced during the chloroplast to chromoplast transition in wild type tomato varieties [10,40] (Fig. 1). The time to ripen is delayed in fruits of *slggp1* mutants which display a decreased oxidative stress as suggested by their lower H_2O_2 production and lipid peroxidation level and higher glutathione content compared to the wild type (Fig. 1, 2). Additionally, the treatments that induce an increment in the production of this oxidant speed up tomato maturation (Table 1). This is consistent with the lower activities of CAT and APX in both mutants than in wild type at transition stages under HI (Fig. 2). Remarkably, we consistently observed similar effects in the two *slggp1* mutants, regardless of the fact that they were obtained from two different EMS populations and that they carry different mutations (splice junction and nonsense mutations) located in different S*lGGP1* exons [14].

This promoting effect of H_2O_2 on fruit ripening has been observed in tomato but also in other species such as pears or grape [9,41,42]. However, the participation of endogenous H_2O_2 as a constituent of cell processes involved in fruit development is far from being established. Since we previously observed that chloroplasts are a main source of ROS [12], a HI treatment was applied to stimulate H_2O_2 synthesis derived from photosynthesis; however, other cell processes may also contribute to the generation of H_2O_2 under HI conditions such

as photorespiration, respiration or NADPH oxidase activity. This treatment produces a large increase in photosynthesis without inducing photo-damage (Table 2) and increases H_2O_2 production in all lines at BR stage (Fig. 1). In addition, HI treatment reduces the timing of ripening in all lines (table 1) further showing an association of H_2O_2 changes with fruit development. Although the analysis here is focused on the induction of oxidative metabolism by HI, higher photosynthesis may lead to changes in several metabolites (i.e. sugars) that may also have an impact in fruit ripening. Furthermore, the interaction of ethylene and auxin with light signaling is involved in the synthesis of carotenoids during tomato maturation [43].

The analysis of the expression of genes specifically induced by this oxidant may add a proof of its participation on fruit ripening triggered by stressful conditions. The expression of *JUB1*, a negative regulator of leaf senescence, is induced by H_2O_2 treatment and abiotic stress [44]. In fruits, this gene is induced at BR stage only in wild type but under oxidative stress conditions, that is here under HI, its expression is observed in all lines (Fig 3). In addition, *JUB1* activates the expression of transcription factors of the genes of the *DREB* family which are possibly involved in controlling the cellular levels of ROS by increasing the antioxidant activity [44,45]. In this work *DREB1* is also highly expressed at OR stage under stress conditions in all lines.

Small heat shock proteins protect chloroplasts and mitochondria from oxidative stress in leaf and root cells [46,47]. In tomato fruits, these proteins accumulate at BR stage having a proved role in the promotion of carotenoid synthesis [48]. Here we observed a higher expression of *sHSPs* during chloroplast-chromoplast transformation in all genotypes. These results might suggest a linkage of oxidative metabolism with a physiological process specifically associated with fruit ripening. In addition, these *sHSPs* are negatively regulated by ethylene [34] in agreement with their lower expression in *slggp1* mutants which have a

higher production of this hormone at fruit transition stages previously observed under greenhouse conditions [49].

Ethylene constitutes a key signalling element controlling tomato fruit maturation [50,6]. The expression data for *ACO4*, *ACS6* and *ETR3* genes suggests that the synthesis and signalling of ethylene are altered by HI stress conditions (Fig. 3). A peak of ethylene production is associated with a higher *ACO* expression [51] which is higher in both ascorbic acid deficient mutants than in the wild type at OR transition stage under GI condition. However, HI treatment increases the expressions of *ACO4* and *ACS6* at MG and BR suggesting a higher and anticipated ethylene production under stress (Fig. 3). The *ETR* family of genes encodes receptor proteins that negatively regulate ethylene action [52] and degradation of these proteins stimulates fruit ripening [53]. The lower expression of *ETR3* suggests a higher sensitivity to ethylene of fruits under HI conditions. To test the linkage between oxidative metabolism with ethylene signalling, fruits were treated with ACC or 1-MCP, precursor of the synthesis of the hormone or inhibitor of its action, respectively. The treatment with ACC produces a large increase in H_2O_2 production at MG and BR stages while 1-MCP drastically reduces it (Fig. 4). These results suggest that the H_2O_2 peak is linked to ripening-associated modifications induced by ethylene; however, how ethylene regulates H_2O_2 production is unknown.

Increase of the oxidative damage is observed during the maturation of fruits such as tomato, peach or pepper [10,54-56]. Carbonylation of mitochondria proteins has been determined in fruit tissues that may be involved in organelle dysfunction and senescence of the organ [57]. However, the results presented here show that alteration of H_2O_2 synthesis induced by ACC or 1-MCP treatments does not make a significant change in the pattern of oxidative damage in tomato fruits (Fig. 4). Antioxidants like ascorbate or glutathione are important redox components regulating metabolic changes during ripening of fruit [58, 59].

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Nevertheless, the increment of glutathione concentration after ACC application without change in ascorbic acid and the decrease in the concentration of both antioxidants after 1-MCP application (Fig. 5) may indicate that the effect of ethylene on the H_2O_2 steady state is associated with an increase of its synthesis, and not with a decrease of the scavenging capacity. Although many other antioxidants were not measured in the present work, these results further suggest that H_2O_2 is a natural player in fruit ripening of climacteric species, such as tomato, and that other components of oxidative metabolism (i.e. ascorbate or glutathione) may not be directly involved.

5. Conclusions

The specific induction of H_2O_2 associated genes (like *JUB1*) at transition stages suggests its participation during tomato fruit maturation. In addition, the results presented here show the linkage of oxidative metabolism and hormone signalling where H_2O_2 is integrated downstream in the events triggered by ethylene.

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Appendix A. Supplementary data

Supplementary material Fig. S1. Fruit temperature in plants grown inside or outside the greenhouse during tomato ripening.

Supplementary material Table S1. Primer sequences used to study gene expression during tomato ripening.

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Author contributions

MCS, MA, GEGG, PB, DJ, OY, EH, CB, IG carried out the experiments working at the greenhouses and/or labs and performed the statistical analysis of the results. PB, CR and CGB designed the trials and wrote de manuscript.

Declaration of Competing Interest

The authors declare that they have no known conflicts of interest that could have appeared to influence the work reported here.

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Table 1. Duration of fruit maturation according to the irradiance conditions of the culture.

	GI	HI
WT	14.0 ± 0.5 aA	10.0 ± 1.0 aB
GGP-5261	17.0 ± 1.0 bA	13.0 ± 1.0 bB
GGP-49C12	18.0 ± 1.0 bA	15.0 ± 0.5 cB

The duration of fruit maturation is determined as the number of days to get 51% of red ripe tomatoes in WT and *Slggp1* mutant plants cultured under control (Greenhouse irradiance, GI) and high irradiance (HI) treatments. Lower-case letters denote statistical differences between genotypes on the same treatment and capital letters denote statistical differences between treatments for the same genotype (ANOVA, $P \leq 0.05$). Values are the mean ± SEM of three-independent experiments.

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Table 2. Photosynthesis in fruits from plants cultured under control (Greenhouse irradiance, GI) and high irradiance (HI) treatments.

	ETR (µmol electrons m^{-2} s^{-1})				Fv / Fm				NPQ			
	GI		HI		GI		HI		GI		HI	
	MG	BR	MG	BR	MG	BR	MG	BR	MG	BR	MG	BR
WT	37.2 ± 2.2aA	37.4 ± 5.2bA	268.0 ± 10.8bB	224.4 ± 35.6aB	0.87 ± 0.01aA	0.85 ± 0.04bA	0.84 ± 0.03aA	0.83 ± 0.02bA	0.11 ± 0.04aA	0.15 ± 0.04bA	1.33 ± 0.05aB	2.21 ± 0.40aB
GGP-5261	41.5 ± 2.9aA	28.0 ± 3.5aA	227.2 ± 18.1aB	309.4 ± 30.3bB	0.86 ± 0.03aB	0.74 ± 0.00aA	0.80 ± 0.02aA	0.78 ± 0.01aB	0.12 ± 0.09aA	0.05 ± 0.04aA	2.01 ± 0.35bB	1.72 ± 0.30aB
GGP-49C12	39.8 ± 1.5aA	25.6 ± 4.7aA	245.6 ± 23.8aB	299.3 ± 27.6bB	0.87 ± 0.02aB	0.78 ± 0.01aA	0.79 ± 0.03aA	0.78 ± 0.02aA	0.14 ± 0.10aA	0.06 ± 0.04aA	1.97 ± 0.20bB	1.43 ± 0.23aB

Fruit photosynthesis was measured as photosynthetic electron transport rate (ETR), PSII maximum efficiency (Fv/Fm) and non-photochemical quenching (NPQ) in *Slggp1*mutants and wild type plants. Lower-case letters denote statistical differences between genotypes on the same treatment and capital letters denote statistical differences between treatments for the same genotype (ANOVA, P ≤ 0.05). Values are the mean ± SEM of three-independent experiments.

Table 3. Days to get 51% red mature tomatoes in WT and *Slggp1* mutant plants in control, 0.5 mM ACC and 8 ppm 1-MCP treatments.

	Control	ACC 0.5mM	Control	1-MCP 8ppm
WT	14.0 ± 0.5 aB	10.0 ± 1.0aA	12.0 ± 0.5 aA	17.0 ± 0.5 aB
GGP-5261	16.0 ± 0.5 bB	13.0 ± 0.5bA	15.0 ± 0.5 bA	17.0 ± 0.5 aB
GGP-49C12	17.0 ± 1.0 bB	15.0 ± 0.5 bA	14.0 ± 1.0 bA	17.0 ± 0.5aB

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

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Legends to figures

Fig. 1. Effects of control and high irradiance treatments on fruit oxidative metabolism. H_2O_2 production (A and B) and lipid peroxidation (C and D) were analyzed in *Slggp1* mutants and wild type tomato plants under GI and HI conditions. Values are the mean of three independent experiments with two biological replicates. Asterisk denotes statistical differences between different genotypes in the same ripening stage and treatment, open circle denotes statistical differences between the two treatments in the same genotype and ripening stageand closed square denotes statistical differences between BR, OR and RR stages compared to the MG stage for the same genotype (ANOVA, $p \leq 0.05$). Values are the mean ± SEM of three-independent experiments.

Fig. 2. Effects of control and high irradiance treatments on fruit redox metabolism. Fruit ascorbic acid content and redox state (expressed as percentage) (A and B), glutathione content and redox state (expressed as percentage) (C and D), catalase activity (CAT, E and F) and ascorbate peroxidase activity (APX, G and H) were measured in *Slggp1* mutants and wild type tomato plants under GI and HI conditions. Values are the mean of three-independent experiments with two biological replicates. Data are expressed based on fresh weight. Asterisk denotes statistical differences between different genotypes for the same ripening stage and treatment, open circle denotes statistical differences between the two treatments in the same genotype and ripening stage and closed square denotes statistical differences between BR, OR and RR stages compared to the MG stage for the same genotype (ANOVA, $p \leq 0.05$). Values are the mean ± SEM of three-independent experiments.

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Fig. 3. Effects of control and high irradiance treatments on gene expression. Analysis by quantitative RT-PCR of the expression of *JBU1* (A and B), *DREB1* (C and D), *class-I SlHSP* (17.6, 20.0 and 20.1, E-J), and ethylene-related (*ACS6*, *ACO4* and *ETR3*, K-P) genes in fruits from *ggp1* mutants and wild type tomato plants cultured under GI and HI conditions. Values are the mean of three-independent experiments with two biological replicates. Data are expressed based on fresh weight. Asterisk denotes statistical differences between different genotypes for the same ripening stage and treatment, open circle denotes statistical differences between the two treatments in the same genotype and ripening stage and closed square denotes statistical differences between BR, OR and RR stages compared to the MG stage for the same genotype (ANOVA, $p \leq 0.05$). Values are the mean ± SEM of three-independent experiments.

Fig. 4. H_2O_2 production and lipid peroxidation in fruits related to ethylene signaling. *Slggp1* mutant and wild type plants were treated with 0.5 mM ACC and 8 ppm 1-MCP and the fruit H_2O_2 production (A-D) and lipid peroxidation (E-H) were measured. Values are the mean of three independent experiments with two biological replicates. Asterisk denotes statistical differences between different genotypes in the same ripening stage and treatment, open circle denotes statistical differences between the two treatments in the same genotype and ripening stage and closed square denotes statistical differences between BR, OR and RR stages compared to the MG stage for the same genotype (ANOVA, $p \leq 0.05$). Values are the mean ± SEM of three-independent experiments.

Fig. 5. Redox metabolism in fruits related to ethylene signaling. *Slggp1* mutant and wild type plants were treated with 0.5 mM ACC and 8 ppm 1-MCP and the fruit ascorbic acid content and redox state (expressed as percentage) (A-D) and glutathione content and redox state

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(expressed as percentage) (E-H) were measured. 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 the two treatments in the same genotype and ripening stage and closed square denotes statistical differences between BR, OR and RR stages compared to the MG stage for the same genotype (ANOVA, $p \leq 0.05$). Values are the mean ± SEM of three-independent experiments.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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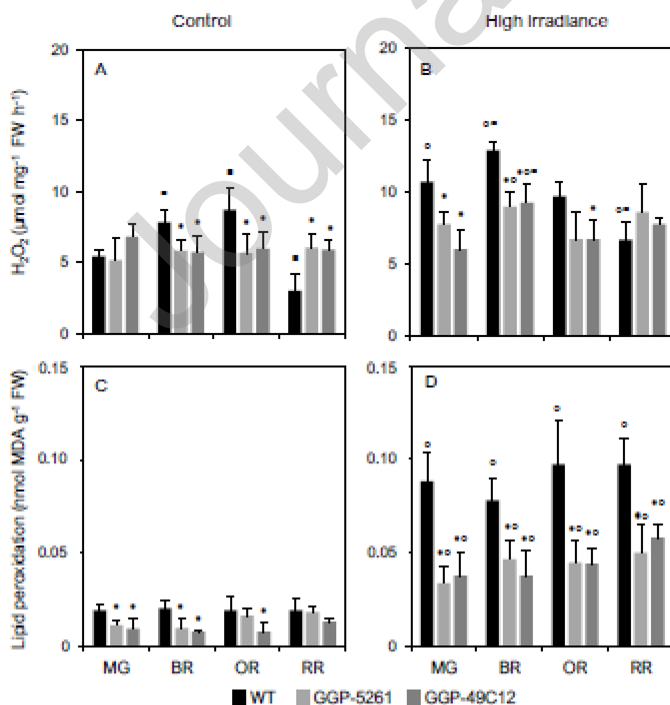

Fig 1

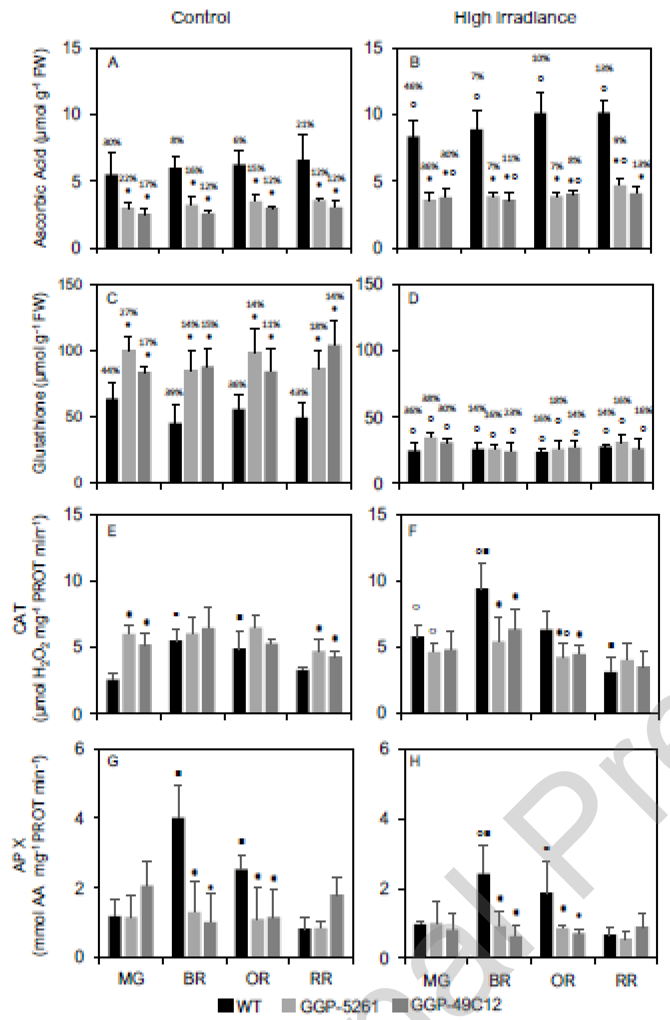

Fig 2

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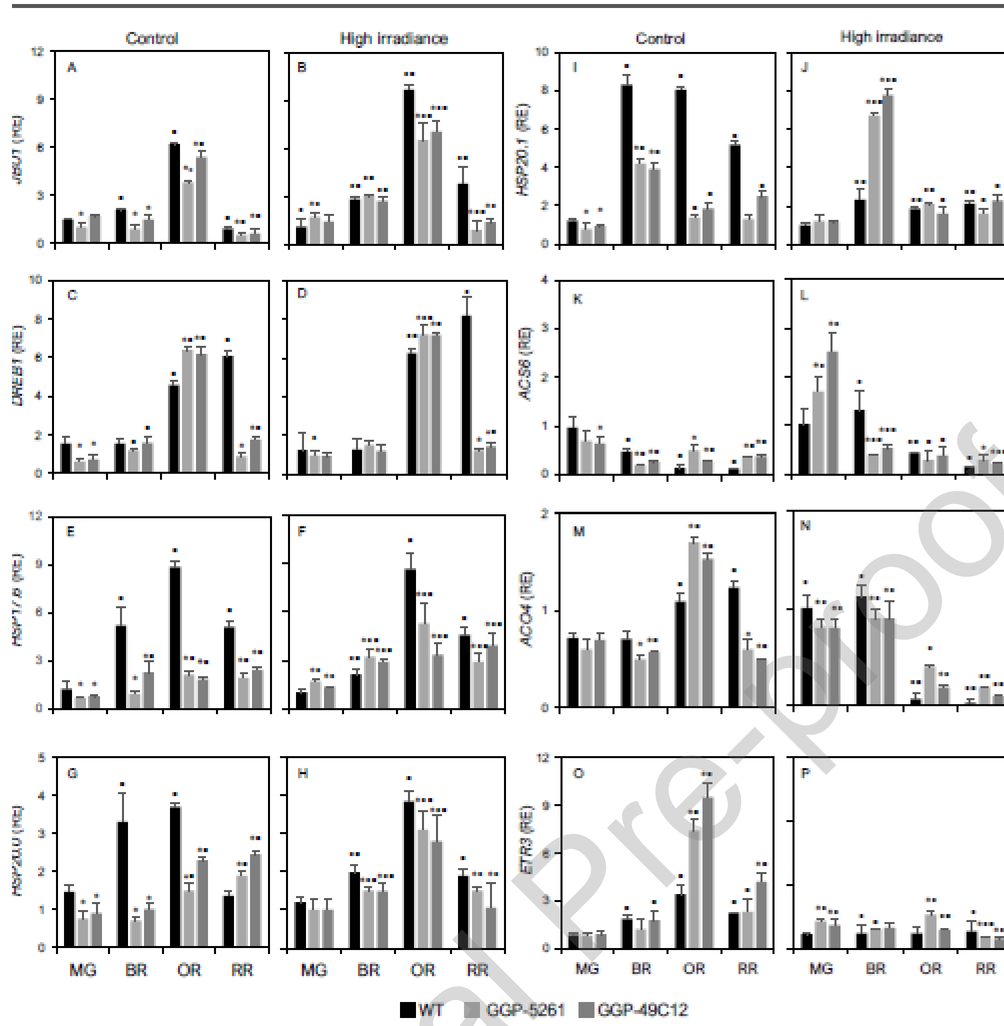

Fig 3

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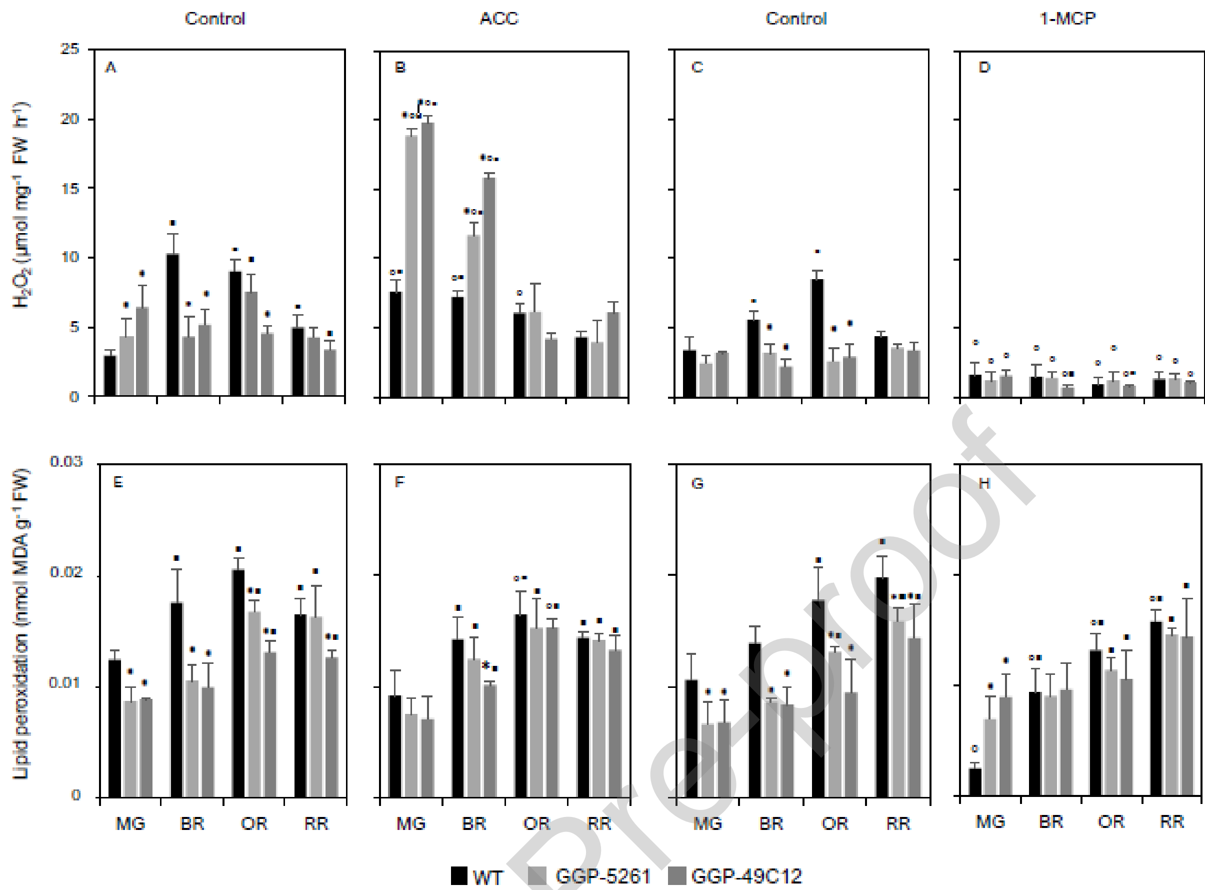

Fig 4

Highlights

- High irradiance (HI) treatment speeds up *in vine* tomato fruit ripening
- The reduction of time to ripen is linked to a higher H_2O_2 production at breaker stage
- HI treatment increases the expression of H_2O_2 associated genes
- Ethylene signaling controls H_2O_2 generation during *in vine* tomato fruit ripening

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