Journal of Experimental Botany, Vol. 69, No. 14 pp. 3449–3463, 2018
doi:10.1093/jxb/erx453 Advance Access publication 3 January 2018

REVIEW PAPER

Nitro-oxidative metabolism during fruit ripening

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Received 2 October 2017; Editorial decision 27 November 2017; Accepted 3 December 2017

Editor: Ismail Turkan, Ege University, Turkey

Abstract

Pepper (*Capsicum annuum* L.) and tomato (*Solanum lycopersicum* L.), which belong to the *Solanaceae* family, are among the most cultivated and consumed fleshy fruits worldwide and constitute excellent sources of many essential nutrients, such as vitamins A, C, and E, calcium, and carotenoids. While fruit ripening is a highly regulated and complex process, tomato and pepper have been classified as climacteric and non-climacteric fruits, respectively. These fruits differ greatly in shape, color composition, flavor, and several other features which undergo drastic changes during the ripening process. Such ripening-related metabolic and developmental changes require extensive alterations in many cellular and biochemical processes, which ultimately leads to fully ripe fruits with nutritional and organoleptic features that are attractive to both natural dispersers and human consumers. Recent data show that reactive oxygen and nitrogen species (ROS/RNS) are involved in fruit ripening, during which molecules, such as hydrogen peroxide (H_2O_2), NADPH, nitric oxide (NO), peroxynitrite ($ONOO^-$), and *S*-nitrosothiols (SNOs), interact to regulate protein functions through post-translational modifications. In light of these recent discoveries, this review provides an update on the nitro-oxidative metabolism during the ripening of two of the most economically important fruits, discusses the signaling roles played by ROS/RNS in controlling this complex physiological process, and highlights the potential biotechnological applications of these substances to promote further improvements in fruit ripening regulation and nutritional quality. In addition, we suggest that the term 'nitro-oxidative eustress' with regard to fruit ripening would be more appropriate than nitro-oxidative stress, which ultimately favors the consolidation of the plant species.

Keywords: Fruit ripening, nitric oxide, nitro-oxidative stress, reactive oxygen and nitrogen species, pepper, tomato.

Fruit ripening: pepper and tomato are agronomically important model plants

Fruit ripening is a highly regulated developmental process involving drastic internal transcriptional and biochemical modifications which coincide with seed maturation. Alterations in fruit coloration, texture, and palatability to animals make ripening a key evolutionary process which facilitates seed dispersal over great distances (Gapper *et al.*, 2013; Karlova *et al.*, 2014; Kumar *et al.*, 2014; Giovannoni *et al.*, 2017). Tomato and pepper are examples of the two main groups of climacteric and non-climacteric fleshy fruit species, respectively, according to their respiration profiles and dependence on the phytohormone ethylene during the

ripening process (Palma *et al.*, 2009; Cherian *et al.*, 2014; Liu *et al.*, 2015). With regard to the consumption of these fruits, tomatoes are almost exclusively consumed at the ripe stage (red, orange, or yellow in color), while peppers can be consumed at both the green (immature) and red/yellow/orange/purple stages, with the latter possessing greater economic market values. Though largely consumed when fresh, tomato and pepper are also used in the food, medical, and pharmaceutical industries.

Production and consumption of tomato and pepper, which constitute an important source of nutrients, such as vitamins A and C, in many countries, are increasing worldwide. According to data provided by the United Nations Food and Agriculture Organization (http://www.fao.org), between 1994 and 2014, world tomato production increased 2.1-fold from 83 Mt to 171 Mt. Likewise, pepper fruit production rose 2.4-fold from 13 Mt to 31 Mt in the same period.

Nitro-oxidative metabolism in higher plants

Plant nitro-oxidative metabolism has hitherto been regarded as a plant response to external and potentially harmful environmental conditions which can lead to cellular damage or even cell death (Pascual *et al.*, 2010; Airaki *et al.*, 2012; Begara-Morales *et al.*, 2013; Corpas and Barroso, 2013; Signorelli *et al.*, 2013; Simontacchi *et al.*, 2015; Corpas, 2017; Houmani *et al.*, 2017). However, it has now been demonstrated that some of the molecules—both reactive oxygen and nitrogen species (ROS and RNS, respectively)—involved in this plant response play a regulatory and signaling role in many plant physiological processes including seed germination, development, vegetative growth, and reproduction (Camejo *et al.*, 2010; Martí *et al.*, 2011; Airaki *et al.*, 2015; Huan *et al.*, 2016; Zafra *et al.*, 2016; Jiménez-Quesada *et al.*, 2017).

Hydrogen peroxide (H_2O_2) and nitric oxide ($^{\cdot}NO$) are among the most representative and studied molecules in the ROS and RNS families. However, given their mediation in post-translational modifications of macromolecules which affect their cellular functions, several H_2O_2- and NO-related substances, including superoxide radicals ($O_2^{\cdot-}$), peroxynitrite ($ONOO^-$), *S*-nitrosoglutathione (GSNO), and, more recently, nitro-fatty acids, are also of considerable physiological and biochemical importance (Huber and Hardin, 2004; Wang *et al.*, 2006; Lindermayr and Durner, 2009; Airaki *et al.*, 2011; Leterrier *et al.*, 2011; Astier *et al.*, 2012; Hu *et al.*, 2017; Mata-Pérez *et al.*, 2017). Table 1 summarizes the main components of these two families of inorganic and organic molecules in radical and non-radical species, among others. There is growing evidence to show that the increased cellular production of some of these molecules, such as $O_2^{\cdot-}$, H_2O_2, and $ONOO^-$, is associated with cellular damage to biomolecules, with lipid oxidation/nitration and protein oxidation of sulfur-containing methionine and nitration of tyrosines being used as potential cellular markers of nitro-oxidative processes (Corpas *et al.*, 2009; Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011; Jacques *et al.*, 2013; Farmer and Mueller, 2013). However, these molecules can also play a regulatory role through the direct or indirect modification of proteins such as *S*-nitrosylation (formerly *S*-nitrosation), which are then endowed with a signaling function (Corpas *et al.*, 2015). Thus, some proteins involved in the plant immune responses have been studied in depth. For example, the TGA1 transcription factor and the non-expresser of pathogenesis-related gene1 (NPR1) are two proteins involved in the systemic acquired resistance in plants which can be *S*-nitrosylated. Accordingly, in the presence of NPR1, the *S*-nitrosylation at Cys260 and Cys266 of TGA1 enhances the DNA binding activity (Lindermayr *et al.*, 2010). Another example is the *Arabidopsis thaliana* respiratory burst oxidase homolog D (AtRBOHD also known as NADPH oxidase) which can be *S*-nitrosylated at Cys890, provoking a decrease in ROS production and consequently limiting the hypersensitive response (Yun *et al.*, 2011).

Table 1. *Main ROS and RNS including inorganic and organic molecules*

Non-radicals	Radicals
Inorganic molecules	
Hydrogen peroxide (H_2O_2)	Superoxide radical ($O_2^{\cdot-}$)
Singlet oxygen ($^1O_2$)	Hydroxyl radical ($^{\cdot}OH$)
Ozone (O_3)	Alkoxyl radicals ($RO^{\cdot}$)
Hypochlorous acid (HClO)	Peroxyl rdicals ($ROO^{\cdot}$)
Nitroxyl anion (NO^-)	Nitric oxide ($^{\cdot}NO$)
Nitrosonium cation (NO^+)	Nitrogen dioxide ($^{\cdot}NO_2$)
Nitrous acid (HNO_2)	
Dinitrogen trioxide (N_2O_3)	
Dinitrogen tetroxide (N_2O_4)	
Peroxynitrite ($ONOO^-$)	
Peroxynitrous acid (ONOOH)	
Organic molecules	**Organic molecules**
Nitrotyrosine (Tyr-NO_2)	Lipid peroxyl radicals ($LOO^{\cdot}$)
Nitrosoglutathione (GSNO)	
Nitrosothiols (SNOs)	
Nitro-γ-tocopherol	
Nitro-fatty acids (NO_2-FA)	

Pepper fruits: ripening-associated changes in ROS and RNS metabolism

The genus *Capsicum*, a member of the *Solanaceae* family, includes ~25 species. Of the five domesticated species of *Capsicum* (*C. annuum*, *C. baccatum*, *C. frutescens*, *C. pubescens*, and *C. chinense*), the most agronomically important is *C. annuum*, which is extensively cultivated and consumed around the world. This species has many varieties, whose fruits differ in size, shape, color, and pungency, which makes it possible to distinguish between hot and sweet peppers. Depending on their shape, sweet peppers can be further classified into California, Lamuyo, and Dulce Italiano categories. California sweet peppers have similarly sized transverse and longitudinal axes (Fig. 1A), while Lamuyo and Dulce Italiano

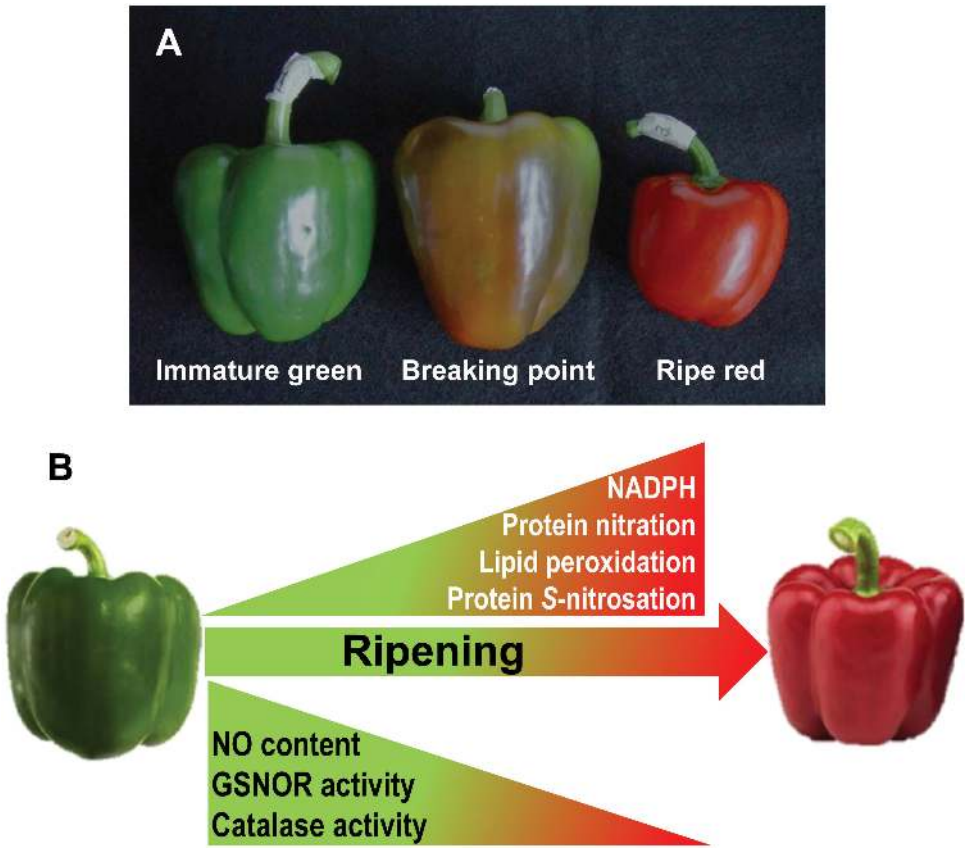

Fig. 1. Temporal changes in nitro-oxidative metabolism during pepper fruit ripening. (A) Representative picture of sweet pepper (*Capsicum annuum* L. California phenotype) fruits at different ripening stages which can be found in the same plant at the same time. (B) Schematic representation of the temporal changes in ROS/RNS metabolism during pepper fruit ripening. GSNOR, nitrosoglutathione reductase.

peppers have longer longitudinal axes. It is important to note that, unlike tomato, the genetic engineering of *Capsicum* has been very limited due to the lack of effective *Agrobacterium*-mediated genetic transformation protocols for this plant genus (Heidmann and Boutilier, 2015). Thus, the important improvements in different agronomic pepper varieties have, up to now, been obtained through conventional breeding.

Pepper fruits contain metabolites, such as ascorbic acid (vitamin C), carotenoids (provitamin A), flavonoids, and capsaicinoids (unique to hot peppers), which, given their antioxidant capacities, contain potential health-promoting properties. However, the content of these components depends on both internal genotypic and developmental factors as well as external environmental growth conditions (Wahyuni *et al.*, 2011, 2013). For example, the red color of ripe pepper fruit is due to the high level of accumulated total carotenoids, with the expression of genes encoding key carotenoid biosynthetic enzymes, such as phytoene synthase (PSY), phytoene desaturase (PDS), and capsanthin-capsorubin synthase (CCS), being relatively higher in red fruits (Ha *et al.*, 2007; Li, 2013; Lado *et al.*, 2016).

To the best of our knowledge, most studies of ROS and RNS metabolism in peppers have been carried out on sweet peppers (*C. annuum*), particularly on the California phenotype (Mateos *et al.*, 2003, 2013; Palma *et al.*, 2015; Chaki *et al.*, 2015; Rodríguez-Ruiz *et al.*, 2017*a*, *b*). Recent data reveal that NO and RNS profiles can be used as an index of ripening progression in pepper fruit, which makes this species an excellent model to study the metabolism of antioxidants, ROS, and RNS during non-climacteric fruit ripening.

Ascorbate and other antioxidant systems during pepper fruit ripening

Ascorbate: a central player in the ascorbate–glutathione cycle

Pepper, which contains the highest level of ascorbic acid among fleshy fruits (>100 mg 100 g^{-1} FW), constitutes an important source of vitamin C in the human diet. From a plant perspective, this high vitamin C content plays a key physiological role due to its powerful antioxidative capacity (Palma *et al.*, 2011). In this respect, increasing data demonstrate that sweet pepper ripening involves an active ROS metabolism in the different subcellular compartments including peroxisomes (Mateos *et al.*, 2003), mitochondria (Jiménez *et al.*, 2002*a*), and chloroplasts/chromoplasts (Martí *et al.*, 2009), where ascorbate may alleviate potential ripening-related damage given its ubiquitous presence in cell compartments (Palma *et al.*, 2015).

Among the different possible pathways of ascorbate biosynthesis in plants, the most consensual route is the L-galactose pathway (Valpuesta and Botella, 2004), with the final step requiring L-galactono-1,4-lactone (GalL) oxidation to

ascorbic acid in a reaction catalyzed by the mitochondrial membrane-bound L-galactono-1,4-lactone dehydrogenase (GalLDH). This enzyme, whose activity, protein content, and gene expression remain virtually unchanged during ripening, has been characterized in sweet pepper fruit (Rodríguez-Ruiz *et al.*, 2017*a*). Ascorbate has also been shown to remain at relatively stable levels during pepper ripening (Rodríguez-Ruiz *et al.*, 2017*a*). In contrast, the pepper ripening process is characterized by an active nitro-oxidative metabolism and an increase in both lipid peroxidation and protein nitration (Chaki *et al.*, 2015; Rodríguez-Ruiz, 2017). Consequently, it has been proposed that, by maintaining high and constant ascorbate levels through ripening, pepper fruits minimize undesirable collateral cellular damage which would otherwise be caused by a ripening-associated oxidative burst (Palma *et al.*, 2015). In this respect, it is important to note that *in vitro* enzymatic assays of GalLDH in the presence of peroxynitrite, a strong oxidant and nitrating molecule, show that the activity was unaffected (Rodríguez-Ruiz *et al.*, 2017*a*), which further points to the great physiological importance of an adequate ascorbate supply during the ripening process.

Ascorbate is also part of the ascorbate–glutathione cycle, a key antioxidant mechanism in plants. By oxidizing ascorbate, ascorbate peroxidase (APX) activity contributes to the regulation of H_2O_2 content. A significant increase in APX activity (Table 2) has been reported during pepper ripening (Mateos *et al.*, 2013). An increase in the activity of other enzymatic components in the ascorbate–glutathione cycle, such as monodehydroascorbate reductase (MDAR) and glutathione reductase (GR), was also detected during pepper ripening (Rodríguez-Ruiz, 2017). All enzymatic components in the ascorbate–glutathione cycle have diverse cell localizations, such as the cytosol, plastids, mitochondria, and peroxisomes. Pepper fruits therefore need to maintain an adequate and co-ordinated balance between these components, as some of these organelles, particularly chloroplasts, undergo notable structural and metabolic changes during ripening (Palma *et al.*, 2015). The RNS-mediated post-translational regulation of APX is of particular importance, as this enzyme can be inactivated by irreversible nitration and activated by reversible *S*-nitrosylation (Begara-Morales *et al.*, 2014*a*), with APX highlighting the connection between the metabolism of ROS and RNS. Though not yet fully characterized, evidence indicates that APX is also subject to finely tuned modulation by RNS in pepper fruits (Rodríguez-Ruiz, 2017).

Reduced glutathione (GSH), which is present in relatively high concentrations (millimolar), plays a key role in plant cell antioxidant defenses. The thiol group present in GSH can be readily oxidized, leading to the formation of oxidized glutathione (GSSG), which can be recycled by GR activity at the expense of NADPH oxidation. During pepper fruit ripening, NADPH supply undergoes a 2-fold increase (Table 2) due to the activity of a group of NADP-dehydrogenases (NADP-DHs), which are also necessary to support the cellular antioxidant system (Mateos *et al.*, 2009). Thus, analysis of the main NADP-DH groups, including NADP-isocitrate dehydrogenase (NADP-ICDH), NADP-malic enzymes (MEs), as well as glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), which belong to the pentose phosphate pathway, showed that all activities, with the exception of G6PDH, increased by between 54% and 100% in ripe pepper fruits (Mateos *et al.*, 2009).

Lipid peroxidation, a recognized marker of oxidative stress, increases by ~50% during pepper ripening, indicating active production of ROS, particularly superoxide radicals and hydrogen peroxide, at this stage of fruit development (Rodríguez-Ruiz, 2017). Superoxide dismutase (SOD) activity, which regulates superoxide radical levels, shows differential responses during pepper ripening. Pepper fruits contain at least four SOD isozymes: one MnSOD, one FeSOD, and two CuZnSODs (I and II), with only CuZnSOD II activity showing a significant increase during ripening (Rodríguez-Ruiz, 2017). This increase in at least one of the CuZnSOD isozymes resembles that observed in plants exposed to specific environmental stresses (Manai *et al.*, 2014;

Table 2. *Summary of the specific changes in ROS and RNS metabolism reported in ripening sweet pepper (Capsicum annuum L, California phenotype)*

Biochemical parameters	Green fruit	Red fruit	Reference
ROS metabolism			
Lipid peroxidation (nmol MDA mg^{-1} protein)	9	36	Rodríguez-Ruiz (2017)
H_2O_2 (μmol H_2O_2 g^{-1} FW)	96	88	Camejo *et al.* (2015)
Ascorbate (mg ascorbate 100 g^{-1} FW)	141	153	Rodríguez-Ruiz *et al.* (2017*a*)
Catalase (μmol H_2O_2 min^{-1} mg^{-1} protein)	59	14	Chaki *et al.* (2015)
Ascorbate peroxidase (nmol ascorbate min^{-1} mg^{-1} protein)	700	2100	Mateos *et al.* (2013); Rodríguez-Ruíz (2017)
SOD isozymes[a]		Increased CuZnSOD II activity[a]	Rodríguez-Ruiz (2017)
NADPH (pmol g^{-1} FW)	6.1	10.8	Mateos *et al.* (2009)
RNS metabolism			
NO content		Decrease	Chaki *et al.* (2015)
GSNOR activity (nmol NADH min^{-1} mg^{-1} protein)	66.0	37.5	Rodríguez-Ruiz *et al.* (2017*b*)
Nitrated and *S*-nitrosylated proteins		Increase[a]	Chaki *et al.* (2015); Rodríguez-Ruiz *et al.* (2017*b*)
Total SNOs		Increase[a]	Chaki *et al.* (2015); Rodríguez-Ruiz *et al.* (2017*b*)

[a] Evaluated by specific electrophoretic techniques (i.e. isozymatic pattern activity, 2D immunoblot analysis, DAF gels).

Houmani *et al.*, 2016), suggesting that particular CuZnSOD isozymes are more closely associated with fruit responses to ripening-associated increases in oxidative stress.

Catalase, a key peroxisomal antioxidant enzyme, is affected by nitration

Catalase (CAT), another important antioxidant enzyme, is exclusively located in peroxisomes, which can be positively or negatively modulated depending on the plant species, organ, and environmental conditions (Corpas *et al.*, 1999; Vandenabeele *et al.*, 2004; Mhamdi *et al.*, 2012). During pepper ripening, CAT activity is down-regulated (Chaki *et al.*, 2015). Nitro-proteomic analysis of pepper fruits using an antibody against nitro-tyrosine identified CAT as a principal target of nitration, which inhibits its activity (Chaki *et al.*, 2015). This is in line with studies of other plant species which indicate that CAT activity is modulated by NO- and ONOO-dependent post-translational modifications (Clark *et al.*, 2000; Corpas and Barroso, 2017). This inhibition of CAT activity by NO-related molecules may reduce H_2O_2-removing capacity and consequently increase ripening-associated nitro-oxidative burst in pepper peroxisomes (Corpas *et al.*, 2017).

RNS metabolism is altered during ripening of pepper fruit

Data regarding RNS metabolism in pepper fruits are relatively new and focus on the ripening of sweet pepper (*C. annunm*, California phenotype) from the immature green to ripe red stages. The endogenous NO content has been reported to be down-regulated during pepper ripening, which is accompanied by a concomitant increase in *S*-nitrosothiols (SNOs), natural reservoirs of NO (Chaki *et al.*, 2015). This process is supported by a ripening-associated reduction in *S*-nitrosoglutathione reductase (GSNOR) activity, which catalyzes the NADH-dependent reduction of GSNO to GSSG and ammonium (Leterrier *et al.*, 2011) and consequently regulates cellular SNO levels. Recently, a comprehensive analysis of SNO pools showed that *S*-nitrosylated proteins also increase in ripe pepper fruits (Rodríguez-Ruiz *et al.*, 2017*b*), suggesting that endogenous circulating NO is accumulated as SNO in the ripening tissues. This enables an important protein pool to be regulated via trans-nitrosylation and nitration, as observed in the case of pepper CAT, which further highlights the recently reported interplay between ROS and RNS metabolism (Begara-Morales *et al.*, 2016).

Table 2 summarizes the principal changes observed in ROS and RNS metabolism in ripening sweet pepper. Taken together, these data confirm the biochemical co-ordination among the different pathways involved in ROS and RNS metabolism in order to support the physiological and biochemical changes associated with progressive and visible changes in color during fruit ripening. Figure 1B shows the proposed model of ripening-associated temporal fluctuations in ROS and RNS metabolism in this plant material.

In an ongoing study, a differential transcriptomic analysis, using RNA sequencing (RNA-Seq; Illumina) during pepper ripening, of five replicates from immature green and ripe red fruits harvested from the same pepper plant was carried out. This revealed that ~2200 genes are up-regulated during ripening, many of which are involved in responses to different stresses such as salinity, cold, heat, oxidative stress, wounding, and high light intensity (Fig. 2; SGG, unpublished results). These results point to a highly active modulation of stress-related genes in this physiological process, in which molecules, such as H_2O_2, GSNO, and nitro-fatty acids, may be involved (Neill *et al.*, 2002; Quan *et al.*, 2008; Begara-Morales *et al.*, 2014*b*; Mata-Pérez *et al.*, 2016).

Tomato fruits: ROS and NO metabolism

ROS metabolism during tomato fruit development and ripening

Numerous reports focused on tomato nitro-oxidative metabolism can be found in the literature; however, relatively few provide comprehensive data concerning the ROS and RNS production, scavenging, and signaling through tomato fruit development and ripening (Jiménez *et al.*, 2002*b*; Mondal *et al.*, 2004; Murshed *et al.*, 2014). This contrasts with the high commercial value of this fruit crop and its worldwide adoption as a model species for understanding fleshy fruit development and climacteric ripening.

A more complete understanding of the mechanisms behind the nitro-oxidative balance in this fruit crop species may have important agronomic and economic implications since many injuries in tomato fruits are attributed to disturbances in oxidative metabolism, including the sunscald injury caused by photo-oxidative stress and fruit tissue damage due to the oxidative burst events after chilling or hypoxia treatments (Stevens *et al.*, 2008; Torres *et al.*, 2006). The existence of extensive mutant, transgenic, and introgression lines collections, as well as TILLING platforms (Gur *et al.*, 2004; Carvalho *et al.*, 2011; Okabe *et al.*, 2011; Saito *et al.*, 2011), also makes the tomato an attractive model species for genetic and molecular characterization of nitro-antioxidant defenses as well as ROS and RNS interaction and signaling in climacteric fruits.

Ripening-associated changes in tomato fruit oxidative metabolism

The ripening-associated changes in oxidative metabolism have been assessed by monitoring the content of H_2O_2, protein/lipid oxidation, as well as the transcript abundance and activities of antioxidant enzymes in fruits of wild-type and ripening-impaired tomato mutants (Jiménez *et al.*, 2002*b*; Mondal *et al.*, 2004; Murshed *et al.*, 2014). Some studies revealed high activities of antioxidant enzymes such as CAT, SOD, GR, MDAR, and dehydroascorbate reductase (DHAR) in immature tomato fruits, presumably reflecting the intensified antioxidant defenses against ROS production from photosynthesis-associated processes at this fruit developmental stage (Jiménez *et al.*, 2002*b*). However, the activities

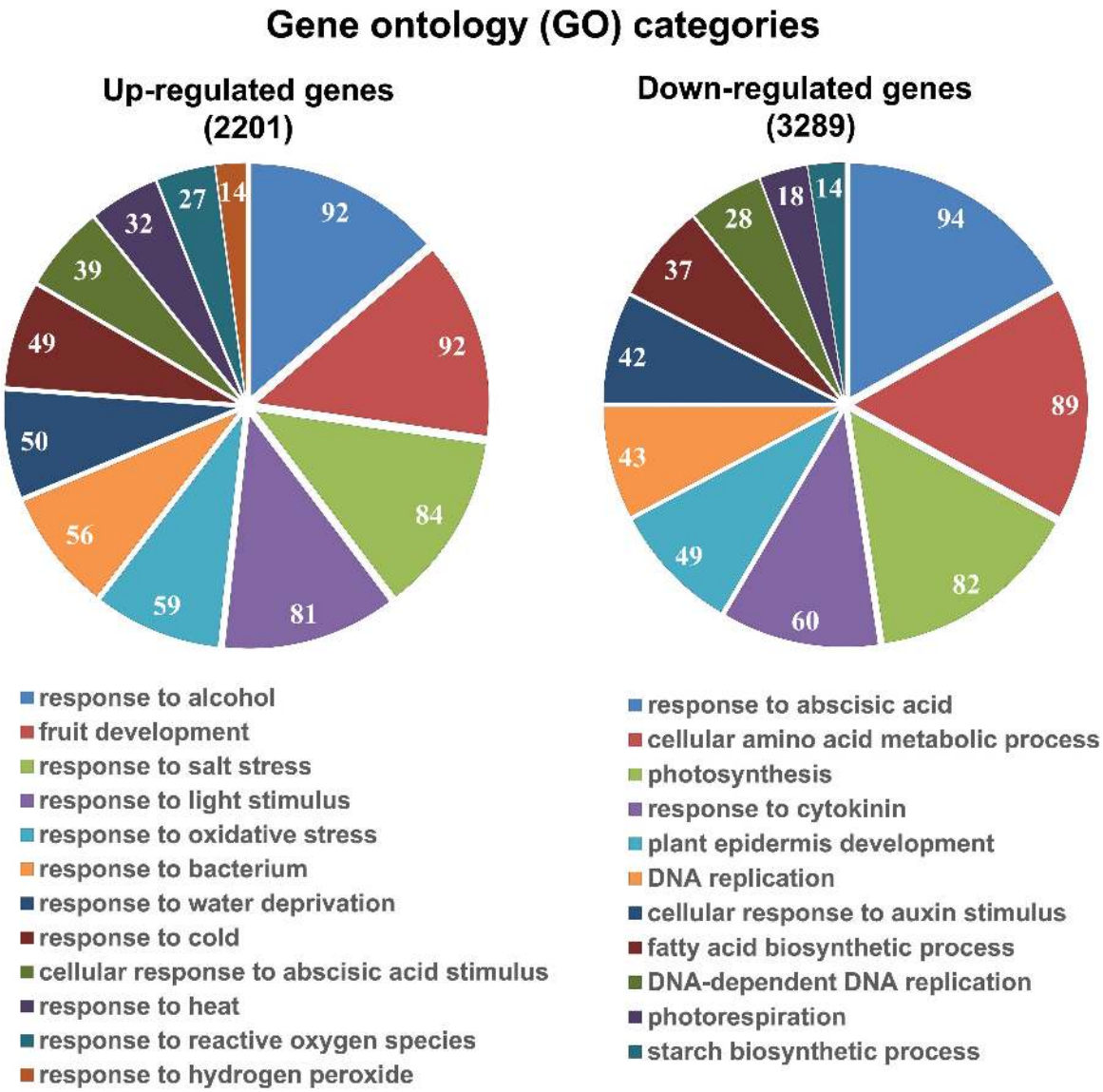

Fig. 2. Differential transcriptomic analysis by RNA-Seq between green and red pepper (*Capsicum annuum*) fruits. The charts show selected categories significantly enriched ($P \leq 0.01$). Functional classification of up-/down-regulated genes was achieved by Gene Ontology (GO) according to biological processes. Five biological replicates from five different plants each were included in the analysis for the two ripening stages. Total RNA was isolated using a two-step method based on Trizol reagent and the RNAeasy Plant Mini Kit (Qiagen), following the manufacter's instructions. Sequencing was performed to generate 2 × 75 bp paired-end reads for transcriptome sequencing on an Illumina NextSeq550 platform. Reads were pre-processed to remove low-quality sequences, specific features [such as poly(A) or poly(T) tails, terminal transferase tails, and adaptors], contaminant sequences, and trimming the undesired segments, using SeqTrim-Next. Clean reads were assembled using Brujin algorithms (Oases, SOAPdenovo-Trans, and RAY). Transcriptomes were analyzed by principal component analysis (PCA) using FactoMineR to find which assembly was closer to the *Populus trichocarpa* and *Arabidopsis thaliana* complete transcriptomes. Our RNA-Seq analysis pipeline uses Bowtie2 to align the reads to the reference transcriptome and Samtools for the quantification of known transcripts (count reads per transcript). The transcriptome reference was annotated against *A. thaliana* using FullLengther-Next. Differential expression analyses were performed using DEgenes-Hunter. Finally, a functional enrichment analysis was conducted using PlantRegMap (GO enrichment tool) using default parameters.

of these same enzymes are found to be particularly reduced before the ripening phase by other authors (Murshed *et al.*, 2014, 2013), suggesting substantial differences within the tomato cultivars and the experimental conditions analyzed.

As tomato fruit ripens, chloroplasts develop into chromoplasts, and the photosynthetic apparatus is progressively disassembled, presumably leading to a gradual reduction in fruit photosynthesis-dependent ROS production. There are ample grounds to consider that only a very limited fraction of ROS generation in photosynthetic tissues is derived from the mitochondrial respiration (Maxwell *et al.*, 1999), which suggests that oxidants in unripe fruits are mainly derived from the photosynthetic activity. However, it seems reasonable to consider that the relative contribution of mitochondrial respiration to ROS production may progressively increase as chloroplasts develop into chromoplasts (Fanciullino *et al.*, 2014). As most of the chromoplast differentiation and the associated accumulation of liposoluble antioxidant compounds, such as lycopene and β-carotene, takes place during the climacteric phase, this particular step of the ripening process may involve drastic

changes in the fruit cell antioxidant scenario (Considine, 2006). Coincidently, a transitory rise in H_2O_2 levels and lipid/protein oxidation and reduction in the activities of SOD, CAT, and most enzymes associated with the ascorbate–glutathione cycle have been described during the breaker stage (Rabinowitch *et al.*, 1982; Jiménez *et al.*, 2002*b*; Malacrida *et al.*, 2006; Kumar *et al.*, 2016). Moreover, analysis of a large RNA-Seq data set available for multiple tomato cultivars revealed marked changes in transcript abundance of numerous tomato genes encoding SOD, CAT, APX, MDAR, DHAR, and GR during tomato fruit ripening (Fig. 3), further suggesting extensive alterations in oxidative metabolism during climacteric ripening.

Because H_2O_2 content and the activity of antioxidant enzymes are co-ordinately changed at the breaker stage (Fig. 4), the peak in ROS levels during the tomato fruit climacteric phase is currently interpreted as a highly co-ordinated process rather than only the by-product of an increase in respiration (Jiménez *et al.*, 2002*b*). Moreover, as the peak in ROS during the breaker stage was significantly reduced in fruits of tomato ripening-impaired mutants, the rise in oxidant levels has been suggested as a signal to fruits to ripen (Kumar *et al.*, 2016).

While suggesting oxidative stress as an integrative factor for triggering tomato fruit ripening has a lot of appeal, one may still wonder whether the oxidative burst at the beginning of the climacteric phase is a cause or a consequence of the ripening process. A definite answer to this question is still missing, but accumulating evidence supports the hypothesis that the rise in oxidants promotes tomato ripening initiation

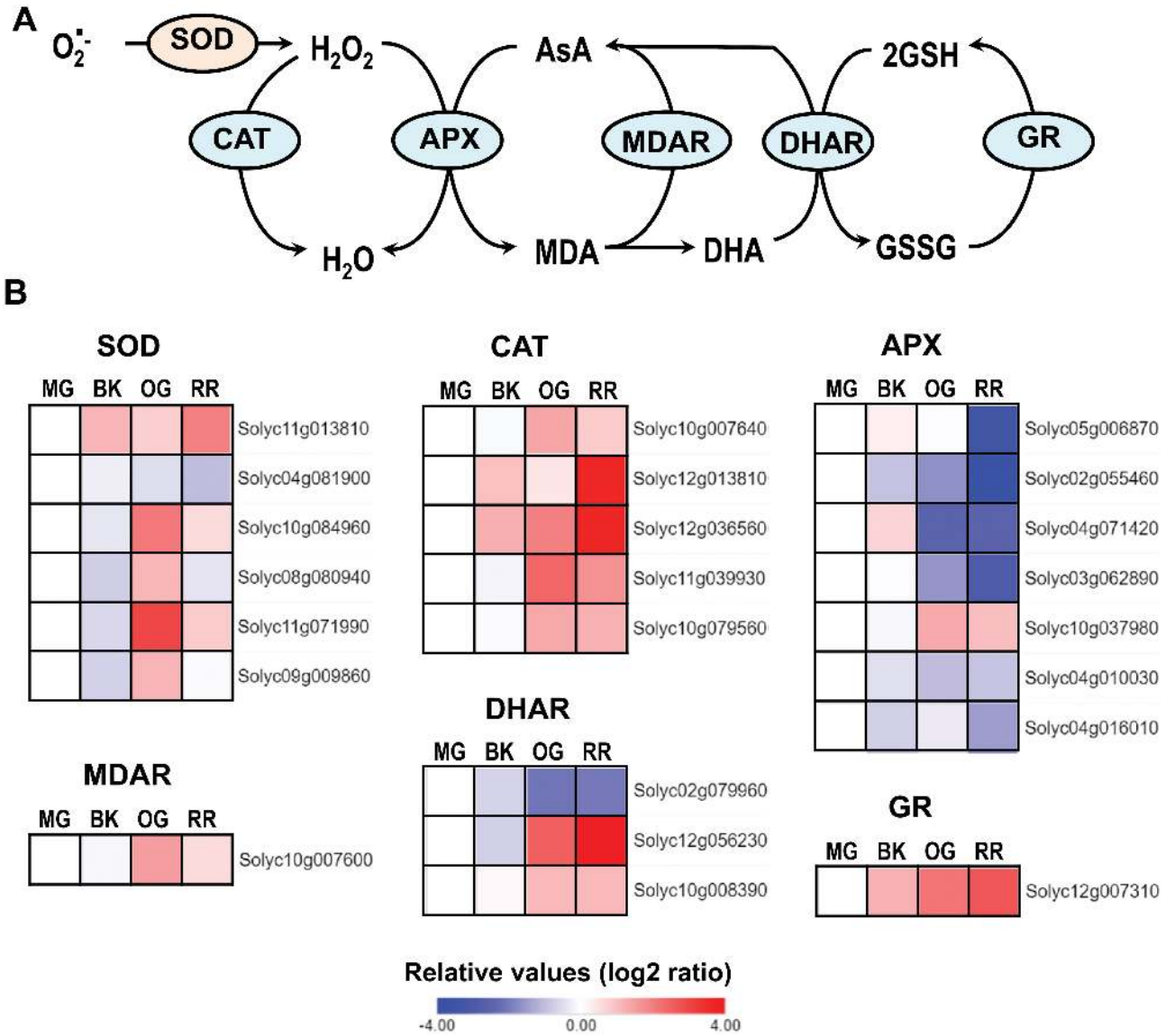

Fig. 3. Transcriptional profile of genes encoding antioxidant enzymes during tomato ripening. (A) The ascorbate–glutathione cycle. (B) Heat map representation of the expression pattern of tomato genes encoding antioxidant enzymes during tomato ripening. Blue and red correspond to low and high relative expression values, respectively. For a given gene and ripening stage, the expression value corresponds to the mean of normalized expression available in the TomExpress platform (including all available RNA-Seq data sets). Only genes whose transcript abundance at BK (Breaker), OG (Orange), and RR (Red ripe) stages exceeded a 2-fold variation compared with the MG (mature green) stage are presented. AsA, ascorbate; APX, ascorbate peroxidase; CAT, catalase; GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase, DHA(R), dehydroascorbate (reductase); MDA(R), monodehydroascorbate (reductase); SOD, superoxide dismutase.

and progression (Mehta *et al.*, 2002; Mondal *et al.*, 2004; Zhang *et al.*, 2013). For example, stage-based comparisons performed in two tomato cultivars with distinct ripening patterns revealed increased oxidative stress and reduced radical scavenging activity in the cultivar with a shorter shelf life (Mondal *et al.*, 2004), implying that disturbances in ROS production or scavenging may be associated with the contrasting ripening patterns observed between these two genotypes. These data suggest that the tomato fruit ripening process may be accelerated when the reduced ROS scavenging activity results in excess free radicals. Consistent with this, fruit shelf life can be significantly altered in tomato transgenic lines engineered for higher antioxidant levels, as revealed by the delayed senescence and reduced ROS levels detected in anthocyanin-enriched tomato fruits (Zhang *et al.*, 2013) and the extended fruit vine life and increased lycopene content observed in lines engineered for increased fruit content of spermidine and spermine, two polyamines with important antioxidant properties (Mehta *et al.*, 2002).

In wild-type tomato fruits, both ascorbate and glutathione levels progressively increase during ripening, reinforcing the antioxidant defenses as fruit ripening progresses (Fig. 4B; Jiménez *et al.*, 2002*b*; Mondal *et al.*, 2004; Murshed *et al.*, 2014). Moreover, the glutathione redox state (i.e. reduced glutathione/total glutathione) and GR activity also gradually increase throughout ripening (Jiménez *et al.*, 2002*b*; Andrews *et al.*, 2004). Therefore, maximum levels of antioxidants such as carotenoids, flavonoids, tocopherols, ascorbate, and reduced glutathione are usually detected in fully ripe tomato fruits (Fig. 4B), potentially acting as a mechanism to attenuate the intensive oxidative stress observed at the final ripening stages. Melatonin, another important antioxidant in biological systems, also progressively accumulates during tomato fruit ripening (Okazaki and Ezura, 2009; Huang and Mazza,

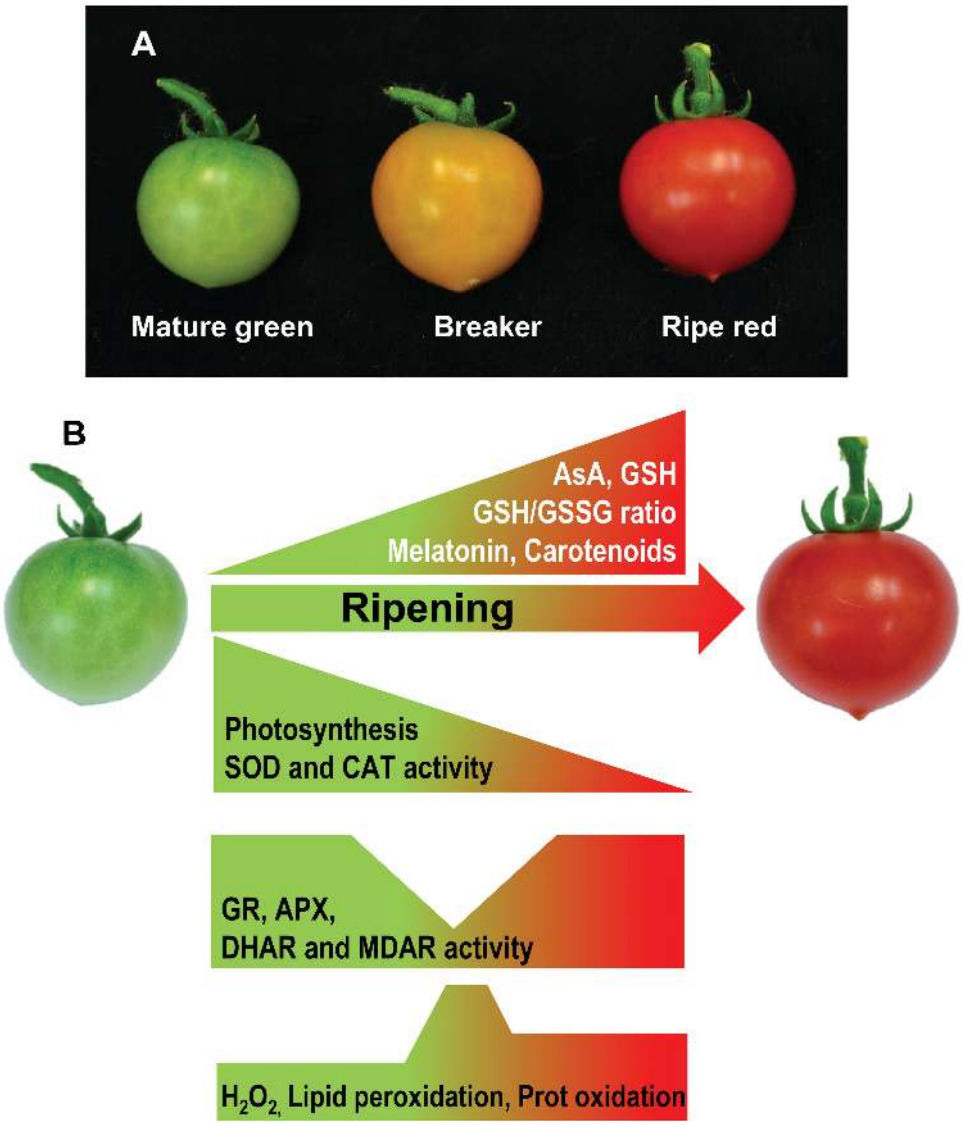

Fig. 4. Temporal changes in oxidative metabolism during tomato fruit ripening. (A) Representative picture of tomato (*Solanum lycopersicum* L., cultivar Micro-Tom) fruits at different ripening stages. (B) Schematic representation of the temporal changes in ROS metabolism during tomato fruit ripening. AsA, ascorbate; APX, ascorbate peroxidase; CAT, catalase; GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase, DHAR, dehydroascorbate reductase; MDAR monodehydroascorbate reductase; Prot, protein; SOD, superoxide dismutase.

2011; Arnao and Hernández-Ruiz, 2014). Interestingly, recent pharmacological evidence indicates that melatonin promotes ripening in tomato (Sun *et al.*, 2015); however, whether the promotive influence of this molecule on tomato ripening is related to its action as a free radical scavenger or via some direct signaling mechanism remains to be determined.

Despite the progressive increases in antioxidant molecules, oxidative stress normally intensifies during the last stages of tomato ripening (Jiménez *et al.*, 2002*b*; Mondal *et al.*, 2004), possibly due to a gradual loss in the capacity of antioxidant enzymes to scavenge the excessive free radical production in over-ripe fruits. This phenomenon has been proposed as an integral part of the tomato ripening program since ROS may facilitate the induction of metabolic processes typically observed at the final ripening stages (Jiménez *et al.*, 2002*b*; Dumville and Fry, 2003). Evidence suggests that the ascorbate-dependent generation of ROS in the apoplast facilitates the non-enzymatic solubilization of plant cell wall polysaccharides, particularly pectins, thus promoting tomato fruit softening (Dumville and Fry, 2003). Moreover, data also indicate that the cellular redox state may either directly or indirectly regulate the synthesis of compounds typically accumulated in ripening tomato fruits such as carotenoids and phenylpropanoids (Fanciullino *et al.*, 2014). According to the current theory, the oxidative stress caused by the ripening-associated oxidative burst may activate fruit redox-sensitive systems to regulate carotenoid biosynthesis-related enzymes transcriptionally and post-translationally, thus promoting the accumulation of these compounds in ripe fruits. Ripening-associated oxidative stress may also stimulate the conversion of fruit chloroplasts into chromoplasts, which in turn also facilitates carotenoid synthesis and accumulation within tomato fruit tissues (Fanciullino *et al.*, 2014).

The equilibrium between ROS production and scavenging in plant vegetative tissues can be disturbed by stress stimuli (Sharma *et al.*, 2012). Similarly, tomato fruit oxidant metabolism is influenced by environmental factors such as light, salt, temperature, and oxygen availability (Murshed *et al.*, 2014, 2013). Salt and drought stress have been shown to stimulate a co-ordinated increment in antioxidant levels and antioxidant metabolism, and the intensity of the salt- or drought-induced changes in the fruit antioxidant machinery seems to depend on the fruit development stage as well as the treatment duration and severity (Murshed *et al.*, 2014). Significant differences in the fruit antioxidant responses to salinity were also observed within distinct tomato cultivars (Gautier *et al.*, 2010).

As in other photosynthetically active tissues, the absorption of solar energy by the chloroplast-rich pericarp cells of immature green fruits may surpass the photosynthetic apparatus capacity, leading to photo-oxidative stress (Foyer *et al.*, 1994). As tomato fruits are virtually devoid of functional stomata, it is believed that light-triggered oxidative stress may also be linked to increments in photosynthetically produced O_2 within the fruit tissues (Cocaliadis *et al.*, 2014). The excessive ROS production under these circumstances may lead to fruit plastid bleaching and other agronomical disorders such as fruit cracking and heterogeneous ripening. The incidence of these agronomical disorders is particularly high in tomato cultivars possessing the functional *Golden 2-like 2* (*GLK2*) transcription factor gene, which promotes fruit chloroplast development (Powell *et al.*, 2012) and consequently increases the incidence of oxidative stress under high light conditions (Cocaliadis *et al.*, 2014). The increased susceptibility of tomato fruits at mature green and breaker stages to sunscald is believed to be associated with the increased superoxide production when the photosynthetic apparatus begins to be dismantled, giving rise to chromoplasts (Rabinowitch *et al.*, 1982). Light irradiation has been shown to regulate tomato fruit ascorbate and carotenoid accumulation (Alba *et al.*, 2000; Gautier *et al.*, 2008, 2009; Bianchetti *et al.*, 2017), but the direct influence of light on other components of tomato fruit antioxidant metabolism remains to be investigated.

In vegetative tissues, temperature stress usually promotes ROS production and stimulates ROS detoxification systems (Suzuki and Mittler, 2006). As high temperatures accelerate fruit metabolism and ripening, low temperatures are frequently employed to extend fruit shelf life before human consumption. However, tomato fruit is especially sensitive to chilling injury due to oxidative stress and other physiological disturbances when storage is below 10 °C (Malacrida *et al.*, 2006; Biswas *et al.*, 2016). Studies revealed a positive correlation between MDAR activity and ascorbate levels in tomato, particularly during chilling stress, designating this enzyme as a priority target for genetically engineering increased post-harvest chilling resistance (Stevens *et al.*, 2008). Accordingly, fruit shelf life under freezing stress was significantly improved in tomato introgression lines exhibiting increased fruit MDAR activity (Stevens *et al.*, 2008) whereas fruit tolerance to chilling was slightly reduced in tomato *MDAR*-down-regulated lines (El Airaj *et al.*, 2013). Besides ascorbate, polyamines are among the antioxidant compounds typically accumulated in cold-treated tomato fruits (Goyal *et al.*, 2016). In line with this, the chilling injury symptoms on tomato fruits can be ameliorated either by treating wild-type fruits with polyamines or by engineering tomato fruits for the overaccumulation of these compounds (Javanmardi *et al.*, 2013; Goyal *et al.*, 2016). Numerous studies also indicate a pivotal role for polyamines in regulating chilling responses during tomato vegetative growth (Song *et al.*, 2014; Diao *et al.*, 2017).

Physiological disorders related to oxidative stress can also be observed following fruit storage under controlled atmosphere conditions. In mature green tomato, post-anoxia stress has been demonstrated to trigger a rapid and co-ordinated up-regulation of all ascorbate-related biosynthetic and recycling genes, resulting in a transitory increase in ascorbate levels (Ioannidi *et al.*, 2009). This agrees with the key role played by ascorbate in attenuating the burst in ROS production usually observed within the first hours after aerobic conditions are re-established (Ioannidi *et al.*, 2009).

Ripening-associated changes in tomato fruit nitrosative metabolism

While the metabolism and interaction of NO and other RNS in tomato vegetative development and stress responses are

increasingly well described (Manai *et al.*, 2014; Melo *et al.*, 2016; Wen *et al.*, 2016), the nitrosative metabolism and signaling during tomato fruit development and ripening are far from being well understood. Most of the current knowledge on the relationship between NO and tomato fruit ripening is based on pharmacological approaches (Eum *et al.*, 2009; Lai *et al.*, 2011). NO supplementation via either fumigation or treatments with NO donors [e.g. sodium nitroprusside (SNP)] were demonstrated to delay tomato fruit ripening (Eum *et al.*, 2009; Lai *et al.*, 2011). An antagonistic interaction between NO and ethylene metabolism seems to be responsible for the delayed-ripening phenotype of NO-treated tomato fruits, as indicated by the reduced ethylene emission detected in both intact and fresh-cut tomato fruits exposed to NO treatment (Eum *et al.*, 2009; Aboul-Soud, 2010; Lai *et al.*, 2011). The exact mechanism behind NO-triggered negative impacts on climacteric ethylene production in tomato is far from being completely elucidated; however, it seems to involve changes in the transcript abundances of specific *ACS* and *ACO* tomato genes (Eum *et al.*, 2009; Aboul-Soud, 2010; Lai *et al.*, 2011). *In vitro* studies suggest that NO may also post-translationally regulate ACS and ACO activities (Tierney *et al.*, 2005; Zhu *et al.*, 2006); however, the actual implications of such regulatory mechanisms during tomato fruit ripening remain unexplored. The antagonistic relationship between ethylene and NO has also been described during tomato vegetative growth (Melo *et al.*, 2016), suggesting some level of conservation of this signaling interaction across vegetative and reproductive tissues.

Corroborating the pharmacological evidence, recent data revealed a delayed-ripening phenotype in fruits of the NO-overaccumulating tomato mutant *short root* (*shr*) (Bodanapu *et al.*, 2016). Fruit size and metabolite levels in the *shr* mutants significantly differ from that in their wild-type counterparts; however, as the *shr* impacts NO levels throughout plant vegetative and reproductive growth (Negi *et al.*, 2010) and its molecular nature still remains elusive (Bodanapu *et al.*, 2016), further genetic evidence is still required to explain mechanistically the relationship between endogenous NO levels and tomato fruit ripening initiation and progression.

Very limited information is currently available on the biosynthesis, conjugation, and removal of NO and other RNS in tomato fruits. However, accumulating data reveal that NO production during tomato vegetative growth may involve either nitrate reductase (NR) (Graziano and Lamattina 2007; Shi *et al.*, 2015; Melo *et al.*, 2016) or nitric oxide synthase (NOS)-like activities (Negi *et al.*, 2010; Yang *et al.*, 2016). In tomato fruit tissues, the application of L-NAME (N^G-nitro-L-arginine methyl ester), an inhibitor of animal NOS enzymes, reduced NO and ethylene levels, resulting in delayed ripening (Yang *et al.*, 2016). Moreover, some of the phenotypical differences of the *shr* mutant have been successfully rescued via the application of L-NAME, but remained unchanged upon the inhibition of NR activity (Negi *et al.*, 2010). However, since L-NAME is an arginine analog, negative collateral impacts in global amino acid metabolism and protein synthesis cannot be ruled out in these pharmacological approaches.

The mechanisms and relevance of RNS and ROS interaction during tomato fruit development and ripening also remain elusive. However, data obtained in tomato vegetative tissues indicate that NO treatments can significantly alter both ROS production and antioxidant metabolism (Manai *et al.*, 2014). SNP application has been shown to reduce H_2O_2 contents and increase SOD, APX, and GR activities in tomato leaf tissues (Manai *et al.*, 2014). Conversely, Laxalt *et al.* (2007) showed that NO was crucial for phosphatidic acid production which, in turn, caused an increase in ROS production in tomato cells. Moreover, Piterkova *et al.* (2013) found that NO and ROS interact synergistically to promote the accumulation of a heat-shock protein in tomato leaves in response to wounding or heat stress. A complex interaction involving NO, ROS, ethylene, and salicylic acid has been demonstrated in tomato cells (Gémes *et al.*, 2011; Poór *et al.*, 2013, 2015). Whether similar ROS, RNS, and hormonal interactions also take place during tomato fruit development and ripening remains to be determined.

Biotechnological applications

It is increasingly evident that both climacteric and non-climacteric ripening are accompanied by marked changes in nitro-oxidative metabolism, which, in turn, may regulate ripening-associated processes ranging from modifications in texture to the accumulation of health-promoting compounds (Dumville and Fry, 2003; Fanciullino *et al.*, 2014). Although the judicious manipulation of key components of fruit nitro-oxidative metabolism has enormous potential to adjust agronomically important traits of fruit crops, very few attempts to genetically engineer ROS or RNS metabolism in fruit tissues have been carried out (Stevens *et al.*, 2008; Javanmardi *et al.*, 2013; Goyal *et al.*, 2016).

Table 3 summarizes some beneficial effects of exogenous applications of NO to both climacteric and non-climacteric fruits. In general, NO supplementation has been observed to have a beneficial impact, especially during post-harvest handling given its promotion of fruit quality preservation and consequent crop loss reduction. The finely tuned regulatory mechanism, through which NO influences fruit ripening, has not been fully characterized. Nevertheless, it has been observed that NO is capable of repressing ethylene metabolism and signaling, while, at the same time, inducing antioxidative enzymes which, in turn, prevent oxidative damage. Given the increasingly reported effectiveness of NO supplementation in extending fruit shelf life and quality (Manjunatha *et al.*, 2010), other gas transmitters with regulatory properties similar to those observed for NO, such as hydrogen sulfide (H_2S), are now being explored for this purpose (Hu *et al.*, 2012; Zhu *et al.*, 2014).

In the specific case of *C. annuum*, it has recently been reported that the application of NO gas (5 ppm for 1 h) to sweet pepper fruits at the breaking point stage delays ripening, as previously described for tomato and other climacteric fruits. However, the most important finding of this study is the concomitant increase (24%) in ascorbate content, as a consequence of a simultaneous increment in GalLDH activity and gene expression (Rodríguez-Ruiz *et al.*, 2017*a*). These data highlight the dual beneficial effects of NO. These involve, first,

Table 3. *Examples of the beneficial effects of NO supplementation during the ripening of climacteric and non-climacteric fruits*

Fruit	NO donor	Effects	Reference
Climacteric			
Apple (*Malus domestica* L.)	Diethylenetriamine-nitric oxide (DETANO)	Inhibition of browning in apple slices	Pristijono *et al.* (2008)
Mango (*Mangifera indica* L.)	NO	Alleviates chilling injury and delays fruit color development, softening, and ripening	Zaharah and Sing (2011)
		Disease resistance	Hu *et al.* (2014)
		UV-B irradiation induces NO content, alleviating cold damages	Ruan *et al.* (2015)
Banana (*Musa* spp.)	NO	Enhances chilling tolerance	Wang *et al.* (2015)
Japanese plum fruit (*Prunus salicina*)	NO	Delays ripening and alleviates chilling injury symptoms during cold storage	Singh *et al.* (2009)
Peach (*Prunus persica* L.)	NO	Up-regulates the abundance of SOD and HSP70, promotes the production of the complex ACO–NO–ACC, which affects ethylene biosynthesis.	Kang *et al.* (2016)
Tomato (*Solanum lycopersicum* L.)[a]	NO	Suppresses fruit growth and ripening	Bodanapu *et al.* (2016)
	Sodium nitroprusside (SNP)	Delays tomato fruit ripening	Eum *et al.* (2009); Lai *et al.* (2011)
Kiwi (*Actinidia chinensis* L)	NO	Extends post-harvest life and enhances antioxidant activity	Zhu *et al.* (2010)
Non-climacteric			
Sweet pepper (*Capsicum annuum*)	NO gas	Delays fruit ripening	Chaki *et al.* (2015)
		Increases vitamin C content	Rodríguez-Ruiz *et al.* (2017*a*)
Strawberry (*Fragaria anannasa*)	NO	Extends post-harvest life	Leshem and Pinchasov (2000); Wills *et al.* (2000, 2007)
Cucumber (*Cucumis sativus*)	NO	Extends post-harvest life	Leshem *et al.* (1998)

[a] *short root* (*shr*) tomato mutant, whose phenotype results from hyperaccumulation of NO.

post-harvest regulation of pepper fruit by delaying the ripening process and, secondly, increased antioxidant capacity and consequent greater health value for consumers due to higher vitamin C content (not yet described with regard to other fruits).

Conclusions and future perspectives

Up to now, the reported data indicate that ROS and RNS metabolism are prominently involved in pepper and tomato fruit ripening, which, according to stress parameters such as lipid oxidation and protein nitration, could be classified as nitro-oxidative stress. The ripening-associated changes in fruit nitro-oxidative metabolism are an integral part of the biochemical and genetic reprogramming required for the progression of both climacteric and non-climacteric ripening. This physiological phenomenon can therefore be categorized as 'nitro-oxidative eustress', a term already used in animal biology to describe a stress situation linked to beneficial effects in living systems (Sies, 2017).

Despite the considerable recent advances made in characterizing ROS and RNS metabolism in climacteric and non-climacteric fruits, significant gaps in information remain. For example, the discovery of genes and gene networks associated with the regulation of ROS and RNS metabolism through analysis of transcriptome, proteome, and/or metabolome data could facilitate the identification of candidate genes for reverse genetics and biotechnological applications in both climacteric and non-climacteric crop fruits. The application of such technologies to fruit ripening under NO-enriched conditions could provide important information to improve fruit nutritional quality, minimize economic losses due to precocious fruit deterioration during production and transportation, and also provide the safety features required by present-day consumers. Tomato and pepper, two representative crop species of the *Solanaceae* family with contrasting ripening behaviors, appear to be particularly promising model species to decipher further the key components and regulatory mechanisms associated with ROS and RNS homeostasis and signaling during both climacteric and non-climacteric fruit ripening.

Acknowledgements

The work of FJC and JMP is supported by an ERDF-co-financed grant from the Ministry of Economy and Competitiveness (AGL2015-65104-P) and Junta de Andalucía (group BIO192), Spain. The authors also acknowledge the computer resources and the technical support provided by the Plataforma Andaluza de Bioinformática of the University of Malaga for the differential transcriptomic analysis of pepper fruits. The work of LF is supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant nos #2013/18056-2 and #2016/01128-99).

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