



RESEARCH PAPER

Nitric oxide-dependent regulation of sweet pepper fruit ripening

Salvador González-Gordo¹, Rocío Bautista², M. Gonzalo Claros², Amanda Cañas¹, José M Palma¹, and Francisco J. Corpas^{1,*} 

¹ Antioxidant, Free Radical and Nitric Oxide in Biotechnology, Food and Agriculture Group, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Granada, Spain

² Plataforma Andaluza de Bioinformática and Department of Molecular Biology and Biochemistry, Universidad de Málaga, Málaga, Spain.

* Correspondence: javier.corpas@eez.csic.es

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Abstract

Ripening is a complex physiological process that involves changes in reactive nitrogen and oxygen species that govern the shelf-life and quality of fruits. Nitric oxide (NO)-dependent changes in the sweet pepper fruit transcriptome were determined by treating fruits at the initial breaking point stage with NO gas. Fruits were also harvested at the immature (green) and ripe (red) stages. Fruit ripening in the absence of NO resulted in changes in the abundance of 8805 transcripts whose function could be identified. Among these, functional clusters associated with reactive oxygen/nitrogen species and lipid metabolism were significantly modified. NO treatment resulted in the differential expression of 498 genes framed within these functional categories. Biochemical analysis revealed that NO treatment resulted in changes in fatty acid profiling, glutathione and proline contents, and the extent of lipid peroxidation, as well as increases in the activity of ascorbate peroxidase and lipoxygenase. These data provide supporting evidence for the crucial role of NO in the ripening of pepper fruit.

Keywords: Ascorbate peroxidase, fatty acids, fruit ripening, lipid peroxidation, nitric oxide, pepper, proline, RNA-Seq, transcriptome.

Introduction

Nitric oxide (NO) is generated endogenously by plant cells and regulates the functions of many biomolecules, including proteins, nucleic acids, and lipids, either directly or through a family of derived molecules called reactive nitrogen species (RNS) (Corpas, 2016; Mata-Pérez *et al.*, 2016). Although most studies have focused on the analysis of NO-induced protein post-translational modifications, such as S-nitrosation, tyrosine nitration, and metal nitrosylation (Corpas *et al.*, 2008, 2019; Astier and Lindermayr, 2012; Holzmeister *et al.*, 2015; Begara-Morales *et al.*, 2016), accumulating data indicate that NO is also involved

in gene regulation (Grün *et al.*, 2006; Tossi *et al.*, 2011). The development of next-generation sequencing technologies has enabled large-scale analyses of NO-responsive genes to be carried out in different plant systems. Among these technologies, RNA sequencing (RNA-Seq) has become a very useful tool for performing comparative plant transcriptome analyses under physiological and stress conditions (Begara-Morales *et al.*, 2014; Ocaña *et al.*, 2015; Mata-Pérez *et al.*, 2016; Mizzotti *et al.*, 2018).

Pepper (*Capsicum annuum* L.) plants belong to the Solanaceae family, which includes species such as tomatoes,

whose fruits are of notable agro-economic importance worldwide. Among the five domesticated species of *Capsicum*, the most agronomically important is *C. annuum*, which has many varieties differentiated by external features such as color (green, red, orange, yellow, white, or purple), size, or shape. At the biochemical level, the capacity for biosynthesis of capsaicinoids also allows distinction between ‘hot’ and ‘sweet’ peppers. Both types of peppers have nutritional value, owing to their content of ascorbic acid (vitamin C) or carotenoids (provitamin A). The majority of studies have focused on hot peppers because capsaicinoids are bioactive molecules with biomedical and industrial applications (Egan *et al.*, 2019; Naves *et al.*, 2019); the number of scientific studies in sweet peppers is significantly lower. However, sweet peppers have a high agro-economical relevance in Europe, especially in south-east Spain because of its optimal climate conditions (temperature and sunlight hours per day) for growth. Over the past 10 years, biochemical data have indicated that, owing to significant modulations in key reactive oxygen species (ROS) and RNS components during ripening from the green to red stage, sweet pepper fruits develop a dynamic nitro-oxidative metabolism (Mateos *et al.*, 2009, 2013; Chaki *et al.*, 2015; Palma *et al.*, 2015; Rodríguez-Ruiz *et al.*, 2017a, b; Muñoz-Vargas *et al.*, 2018; Chu-Puga *et al.*, 2019). However, the molecular information in sweet pepper, especially during the transition from green to red fruits during ripening, is very scarce.

Based on pioneering work by Leshem’s group on NO and fruits (Leshem *et al.*, 1998; Leshem and Pinchasov, 2000), exogenous NO application started to be used in research, with the main goal of extending the post-harvest stage of both climacteric and non-climacteric fruit ripening. Initially, Leshem and colleagues found that in climacteric fruits, NO and ethylene displayed opposite profiles, thus indicating their antagonism. An increasing number of studies showed that the fumigation of fruits with NO could extend their post-harvest life and also preserve their qualities (i.e. turgor, aroma, or flavor) to a different degree depending of the type of fruits (Zhu *et al.*, 2010; Corpas and Palma, 2018). Accordingly, it has been reported that the application of NO during the post-harvest stage can delay softening of papaya (Guo *et al.*, 2014) or provide disease resistance to some types of fungus in orange (Zhou *et al.*, 2016) and peach (Li *et al.*, 2017). Recently, other potential modulators such as hydrogen sulfide (H₂S), melatonin, and abscisic acid (ABA) have begun to be used. Many studies have focused on the interaction of NO, H₂S, ABA, and ethylene in climacteric fruits (Wu *et al.*, 2018; Cao *et al.*, 2018; Mukherjee, 2019). By contrast, in non-climacteric fruits, research into the role and interactions of specific phytohormones as driving forces in the ripening process is still at an early stage.

The present study has a different aim because it was designed to understand how NO gas could modulate the physiological transition of ripening (from the green to the red stage) of sweet pepper fruit, with the goal of identifying potential signal transduction networks that govern this transition. Accordingly, we provide the first *de novo* assembly of the sweet pepper fruit transcriptome, which allowed a comparative time-course analysis of its modulation during the transition from green to red

fruits in the absence of NO and after exposure to NO gas. Data indicate that NO governs fruit ripening by affecting the transcriptome and fatty acid (FA) metabolism; this suggests that FA-derived molecules could participate in this process, since FAs are key membrane components that play diverse roles during plant growth, development, and pathogen defense, and as aroma volatile compound precursors in fruits (Ties and Barringer, 2012; Li *et al.*, 2016; Lim *et al.*, 2017). The analysis of other specific biochemical parameters, such as the content of proline and glutathione (both reduced and oxidized), lipid peroxidation (an oxidative stress marker), and the activity of ascorbate peroxidase (an antioxidant enzyme), also suggests that NO exerts an anti-maturation effect.

Materials and methods

Plant material

California-type sweet pepper (*C. annuum* L.) fruits were collected from plants grown in plastic-covered greenhouse (Syngenta Seeds, Ltd, El Ejido, Almería, Spain). Fruits from five different plants were harvested at three different developmental stages: green immature, breaking point (BP), and ripe red. The selected fruits had the same visual characteristics (size, shape, and color) for each stage.

NO treatment at the BP stage was carried out according to Palma *et al.* (2018). Basically, BP pepper fruits were placed in an NO-enriched atmosphere (5 ppm) in a hermetic box for 1 h, and then stored at room temperature for 3 days. Then, samples were frozen in liquid nitrogen and kept at -80 °C until processing.

RNA extraction, library preparation, and sequencing

Total RNA was isolated from pepper fruits using a two-step method based on Trizol® Reagent (Gibco BRL) and the RNAeasy Plant Mini Kit (Qiagen), following the manufacturer’s instructions. The RNA was quantified spectrophotometrically (NanoDrop, mySPEC, VWR®). Absorbance ratios at 260/280 nm and 260/230 nm were used in order to determine sample quality. RNA integrity was checked by agarose gel electrophoresis and visualized using the ChemiDoc-It®^{TS3} Imaging Systems (DBA Analytik Jena, USA). Total RNA samples were sequenced by the High-Throughput Sequencing Unit of the University of Malaga, Spain, in the NextSeq550 platform using 2×75 bp reads, and 40–50 million reads were generated for each replicate.

Sequence pre-processing, assembling and annotation

Raw reads were pre-processed, assembled, and evaluated using TransFlow (Seoane *et al.*, 2018), which produces up to 190 different assemblies (including primary and reconciled transcripts) that are then evaluated to find which assembly is closer to the *Arabidopsis thaliana* and *Populus trichocarpa* complete transcriptomes. Briefly, reads were pre-processed to remove low-quality, vector, adaptor, low-complexity and contaminant sequences, and organelle DNA, and the undesired segments were trimmed using SeqTrimNext (Falgueras *et al.*, 2010). The resulting useful reads were assembled using different assemblers based on Bruijn algorithms: Oases (Schulz *et al.*, 2012), SOAPdenovo-Trans (Luo *et al.*, 2012), and Ray (Boisvert *et al.*, 2010), executing *k*-mers 25 and 35. The resulting contigs were clustered with CD-HIT (Fu *et al.*, 2012) and then reconciled with CAP3 or Minimus (Sommer *et al.*, 2007). Evaluation of each set of tentative transcripts (TTs) was performed by Principal Component Analysis (PCA) using FactoMineR (Lê *et al.*, 2008). Finally, the set of TTs from the best transcriptome assembly (the one closest to *A. thaliana* and *P. trichocarpa* transcriptomes in the PCA) was annotated against the plant division of UniProtKB using Full-LengtherNext (Seoane *et al.*, 2018) with default parameters (minimal identity of ≥45% and minimal TT coverage of ≥25%). This software also allowed annotation with orthologs

from the proteomes of *A. thaliana* (TAIR 10 database, 27 416 proteins), *Solanum lycopersicum* (ITAG 2.4, 34 725 proteins), *Solanum tuberosum* (PGSC_DM_v3.4, 52 925 proteins), and *C. annuum* (bell pepper, UP000222542, 35 548 proteins).

Mapping and expression analysis

Useful reads were mapped on to the reconstructed transcriptome using Bowtie2 (Langmead and Salzberg, 2012). Transcript counts were obtained using Sam2count.py. Data normalization and differential expression analysis were carried out using DEgenes-Hunter (Gayte *et al.*, 2017) with default parameters. The differential expression analysis examined the relative change in expression between the different samples using different algorithms (EdgeR, DESeq2, Limma, and NOISeq), with the requirement that any differentially expressed gene (DEG) must appear in at least three of the four algorithms. A time-course analysis of gene expression was performed using the RNA-Seq ready maSigPro package (Nueda *et al.*, 2014), where different ripening stages were considered as their corresponding time points. Gene datasets were used for gene ontology (GO) and KEGG pathway enrichment analyses using different web tools [AgriGO v2.0 (Tian *et al.*, 2017), PlantRegMap (Jin *et al.*, 2017), and KOBAS 3.0 (Xie *et al.*, 2011)]. Significantly enriched GO terms were submitted to REVIGO (Supek *et al.*, 2011) to remove redundant categories. Supplementary Fig. S1 at JXB online schematically illustrates the process of the experimental procedure used in the sequence pre-processing, assembly, and annotation of the pepper fruit transcriptome.

Preparation of samples for biochemical analyses

Samples were ground in liquid N₂ using a mortar and pestle, and the resulting powder was suspended in 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM EDTA, 2 mM DTT, 0.1% (v/v) Triton X-100, 1 mM MgCl₂, and 10% (v/v) glycerol, to a final plant material/buffer ratio of 1:1 (w/v). Homogenates were filtered through two layers of Miracloth and centrifuged at 27 000 × *g* at 4 °C for 30 min. The supernatants were used for the enzymatic assays.

Ascorbate peroxidase (APX) activity was determined by monitoring the initial ascorbate oxidation by H₂O₂ at 290 nm (Hossain *et al.*, 1984). In this case, 2 mM ascorbate was added to the homogenizing medium to preserve this activity (Miyake and Asada, 1996).

Lipoxygenase (LOX) activity was determined by an in-gel assay according to Heinisch *et al.* (1996) with minor modifications. Samples (6 µg proteins were loaded per lane) were separated using discontinuous gel electrophoresis under non-denaturing conditions. After electrophoresis, the gel was briefly rinsed in distilled water and then incubated at 4 °C for 1 h under continuous shaking with a solution containing 0.2 M glycine-NaOH buffer (pH 9.0) and 50 µl linolenic acid (Sigma) prepared in 50 µl ethanol. The gel was then rinsed with distilled water and incubated with 20 ml staining solution containing 0.2 g *N,N*-dimethyl-*p*-phenylenediamine, 1.8 ml methanol, and 0.2 ml acetic acid. Gels were shaken at room temperature until the appearance of pink bands representing enzyme activity. Bands were quantified by using ImageJ software.

Determination of lipid peroxidation and proline and glutathione content

Lipid peroxidation was assessed by measuring the concentration of malondialdehyde (MDA) through the formation of thiobarbituric acid reactive substances (Buege and Aust, 1978). Proline was measured spectrophotometrically at 520 nm using the ninhydrin assay (Bates *et al.*, 1973). Reduced glutathione (GSH) and oxidized glutathione (GSSG) were quantified by liquid chromatography electrospray mass spectrometry (LC-ES/MS) according to Airaki *et al.* (2011). Protein concentration was determined with the Bio-Rad Protein Assay (Hercules, CA, USA) using bovine serum albumin as a standard. Pairwise analysis of variance was used to detect differences between samples with the aid of the Statgraphics Centurion program.

Gas chromatography-mass spectrometry assay of fatty acids

Total lipids were extracted in triplicate based on previous methods (Bligh and Dyer, 1959; Saini and Keum, 2016) with minor modifications. For extraction, only the edible parts of the pepper fruit (pericarp) were used. Samples were ground in liquid N₂ using a mortar and pestle, and 2.5 g of the resulting powder was suspended in 25 ml of a solution of chloroform and methanol (2:1, v:v) with continuous shaking for 1 h. Samples were then centrifuged at 5000 × *g* for 10 min at 25 °C, and the supernatants were recovered. This process was repeated at least twice until the supernatant became colorless. The supernatants obtained during the whole process were transferred to decanting ampoules, 15 ml 0.85% (w/v) NaCl was added, and the mixture was gently shaken. After decanting, the lower organic phase was collected into a pre-weighed glass tube, evaporated to dryness under a flow of N₂, and the total lipid content was then determined gravimetrically. Then, the extracted material was derivatized with 1 ml boron trifluoride (BF₃)-MeOH for 15 min at 60 °C to obtain the fatty acid methyl esters (FAMES). After cooling to room temperature, 700 µl hexane and 700 µl Milli-Q water were added and mixed vigorously for 5 min. Approximately 500 µl of the organic phase was transferred to a chromatography vial for loading on to the GC-MS system. A Varian 450 GC 240 MS ion trap mass spectrometer was used with the following conditions: 1 µl injected at 300 °C and split 20:1 with 1 ml min⁻¹ of He as carrier gas. A DB-5MS UI (30 m×0.25 mm×0.25 µm) capillary column (Agilent) was used, with the following temperature program: 50 °C for 5 min, then increasing at 10 °C min⁻¹ to 180 °C, then 3 °C min⁻¹ to 250 °C, and then 5 °C min⁻¹ to 320 °C, where the temperature was held for 2 min. The mass spectrometer worked in electron ionization mode and mass spectra were acquired between 50 and 1000 arbitrary units of mass. Compounds were identified on the basis of similarity with the NIST08 mass spectral library and using standards injected in the same conditions.

Results and discussion

From the point of view of growers and consumers, fruit ripening involves a series of organoleptic changes that lead to products of good or high quality (i.e. fruits that have a desirable color, attractive flavor, palatable nature and other textural properties). On the basis of the physiological behavior of ripening, fruits are classified as either climacteric or non-climacteric. Climacteric fruits continue to ripen after harvesting; for example, harvested green tomatoes can ripen to red because green tomatoes emit ethylene and also have an increased rate of respiration. By contrast, non-climacteric fruits, such as pepper, do not ripen further once they have been harvested and do not respond to ethylene treatment. In previous studies, we demonstrated that endogenous NO was down-regulated during the transition of sweet pepper from green to red (Chaki *et al.*, 2015; Muñoz-Vargas *et al.*, 2018). Consequently, the main goal of the present study was to discover whether exogenously applied NO gas can modulate pepper fruit ripening as far as the whole transcriptome is concerned, and to evaluate at the biochemical and metabolic levels the functional effects of NO application. There was particular interest in the analysis of the transcriptome, which might provide routes and events that can be modulated during ripening of non-climacteric fruits.

De novo assembly of the sweet pepper fruit transcriptome

The first step in identifying the genes and functions affected by NO treatment was to construct a transcriptome that

incorporates the sweet pepper fruit stages analyzed. Fruits were collected at the standard ripening stages: immature fully green (G), breaking point 1 (BP1), and ripe fully red (R). In addition, to study the effect of NO gas on fruit ripening, two further groups at a breaking point 2 (BP2) stage were included: fruits treated with 5 ppm NO for 1 hour (BP2+NO) and a parallel group of fruits not treated with NO (BP2–NO) followed by storage for 3 d at room temperature. Fig. 1 illustrates the phenotype of pepper fruits at the different ripening stages. In total, 24 cDNA libraries, comprising four independent replicates belonging to the green stage and five to each of the other stages, were generated from these samples. The libraries were sequenced and produced a total of 1 204 589 625 raw reads, which were then trimmed, resulting in 1 153 185 684 (95.73%) useful reads (Supplementary Table S1). Owing to the large number of reads, only two replicates from each ripening stage were loaded into TransFlow (Seoane *et al.*, 2018) to obtain the best affordable *de novo* assembly using these reads. The best transcriptome consisted of 564 642 TTs with a mean length of 791 bp, of which 42.19% were over 500 bp in length (Supplementary Table S2).

After annotation on the Full-LengtherNext platform, 31.13% of TTs had a clear ortholog and 34 071 were non-redundant according to their UniProtKB identifier (ID). Remarkably, 66 847 TTs encoded complete proteins, 18 397

of which had unique IDs. This suggests that, despite the clear overestimation of the total number of transcripts, full-length open reading frames (ORFs) were reconstructed for a major set of genes expressed in sweet pepper fruit. Annotation also revealed that 67.93% of TTs had no significant homology to any other plant gene, even though 49 708 TTs were predicted to code for complete, unknown proteins by TransDecoder as part of a Full-LengtherNext analysis (Supplementary Table S2). These TTs could be sweet pepper-specific transcripts, small splicing intermediates, or simply assembling artifacts (Benzekri *et al.*, 2014; Ocaña *et al.*, 2015). Although the libraries were not designed to detect any non-coding RNA, due to the deep sequencing analysis by the NextSeq500 platform, 4903 TTs presented significant homology to small non-coding RNA precursors (over 40 nt in length) derived from the RNACentral database (The RNACentral Consortium, 2017).

Suitability of the sweet pepper fruit reference transcriptome

The 564 642 TTs can be clearly inferred to include many redundancies and significant transcript fragmentation, which will hinder their further use. Because of this, we used Full-LengtherNext to select a subset of TTs that have a representative sweet pepper fruit transcriptome. This resulted in 63 359 TTs, which include the longest TT for each unique ortholog and all non-redundant, unknown TTs that code for a complete protein (Supplementary Table S2). The mean TT length of this representative transcriptome was 1560 bp, with 55 172 TTs of over 500 bp (results not shown). Quality enhancement was first evaluated by mapping the original set of reads (in Supplementary Table S1) on to the reference transcriptome. Given that the representative transcriptome accounts for only 11.22% of the full set of TTs, a mean of 56.23% reads was found to be mapped (Supplementary Table S1, last column), indicating a 5-fold mapping enrichment, which demonstrates the suitability of the representative transcriptome.

The representative transcriptome was further evaluated according to the annotation level of different protein sets (Table 1). First, annotation in the Plant division of UniProtKB revealed that the reference transcriptome contained 29 716 TTs for different proteins, 17 281 of which coded for a complete protein, and included 13 631 TTs that had no ortholog but were predicted to code for an unidentified protein. These data show that the quality of the representative transcriptome was enhanced with respect to the complete set of TTs. Another independent source of annotation was *C. annuum* (bell pepper), which has the closest sequenced genome to sweet pepper (Table 1); it was not included as a source of annotation in the Plant division of UniProtKB, as its proteome status has not been reviewed. A total of 46 924 TTs were homologous to bell pepper proteins, but covered only 18 674 different UniProtKB IDs (52.5% of the bell pepper proteome), 12 726 of which contain the complete ORF. The pepper fruit transcriptome was not expected to contain fewer different transcripts than a complete transcriptome. However, surprisingly, the Plant division of the UniProtKB database provided more orthologs than the bell pepper proteome,

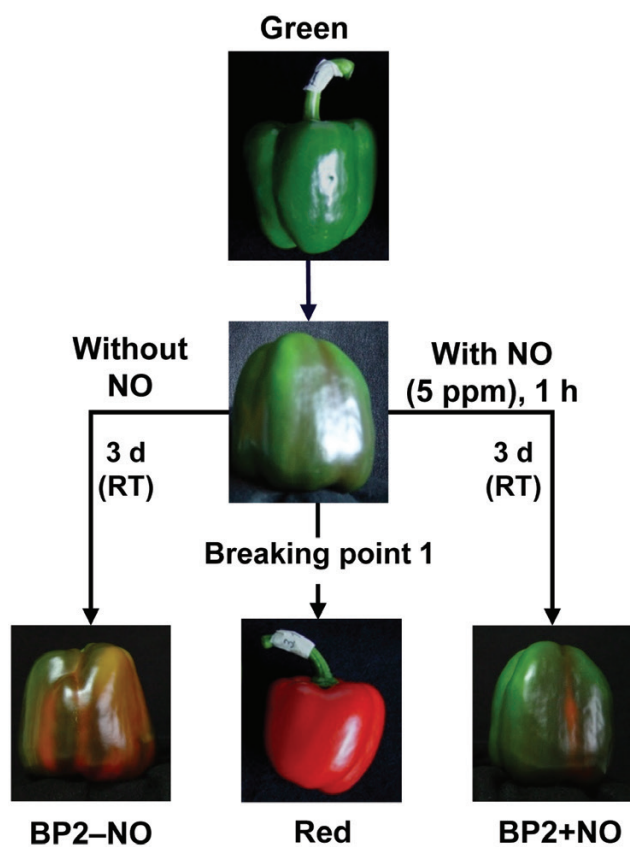


Fig. 1. Visual phenotype of sweet pepper (*Capsicum annuum* L.) fruits at different stages and treatments: immature green (G), breaking point 1 (BP1), breaking point 2 without NO-treatment (BP2–NO), breaking point 2 with NO-treatment (BP2+NO), and ripe red (R). Fruits were subjected to an NO-enriched atmosphere (5 ppm) in a hermetic box for 1 h and were then stored at room temperature (RT) for 3 d.

Table 1. Statistical characterization of the representative transcriptome annotations using different sets of proteins: plant division of UniProtKB, *Capsicum annuum* (bell pepper), *Arabidopsis thaliana*, *Solanum lycopersicum* (tomato), and *Solanum tuberosum* (potato)

Property	Plant division	Bell pepper	Arabidopsis	Tomato	Potato
TTs with annotation	49 701	46 924	36 481	47 270	50 324
Unique IDs	29 716	18 674	14 060	18 051	23 030
TTs including a complete ORF	26 968	24 878	19 482	25 357	30 345
Unique IDs for complete ORFs	17 281	12 726	10 081	12 754	16 416
TT without ortholog	13 631	16 404	26 874	16 080	13 022

ID, Identifier; ORF, open reading frame; TT, tentative transcripts.

suggesting that the representative sweet pepper fruit transcriptome contained TTs not yet described in the bell pepper proteome. It would therefore appear to be more appropriate to use our *de novo* transcriptome than the unreviewed bell pepper proteome/transcriptome.

To facilitate subsequent functional analysis, the reference transcriptome was also annotated with curated proteomes of the model species *A. thaliana* and other species closely related to sweet pepper, such as tomato and potato. The *A. thaliana* proteome, only 45.8% of whose proteins have an ortholog in sweet pepper, was the poorest source of orthologs (Table 1). However, despite the missing information on 26 874 TTs, many bioinformatic tools for functional analysis capable of handling *A. thaliana* annotations can be very useful. Superior results were obtained with the *S. lycopersicum* proteome, 53.2% of whose proteins (a proportion similar to that for bell pepper proteins) were able to annotate 18 051 different TTs, with only 16 060 remaining unannotated (Table 1). The results for the *S. tuberosum* proteome, which provided the largest number of unique orthologs using one single proteome, were even more striking (Table 1). For this species, 23 030 different UniProtKB IDs were actually used, with only 13 022 sweet pepper fruit TTs remaining unannotated (the lowest figure of all; Table 1). This, together with the large number of TTs coding for a complete ORF, clearly indicates that our reference transcriptome provides a suitable representation of the sweet pepper proteome/transcriptome, and it was therefore used for the RNA-Seq studies.

Differentially expressed genes under NO gas treatment

Although the effect of NO treatment could be studied by comparing the differences in gene expression between BP2–NO and BP2+NO pepper fruits, our experimental design (Fig. 1) facilitated a more exhaustive analysis by using the previous ripening stage (BP1) as a reference point. We identified a total of 5153 genes (2413 up-regulated and 2740 down-regulated) expressed differentially in BP1 and BP2–NO; the comparison between BP1 and BP2+NO involved 2664 genes (1556 up-regulated and 1108 down-regulated) (Fig. 2A). In addition, the genes could be divided into groups sharing different expression patterns. Table 2 summarizes eight such groups of genes; these groups enabled us to reduce the complexity of the analysis and to obtain useful biological information (Khatri *et al.*, 2012) (Fig. 2B). In all the gene sets analyzed, a functional enrichment analysis based on GO was carried out, and the

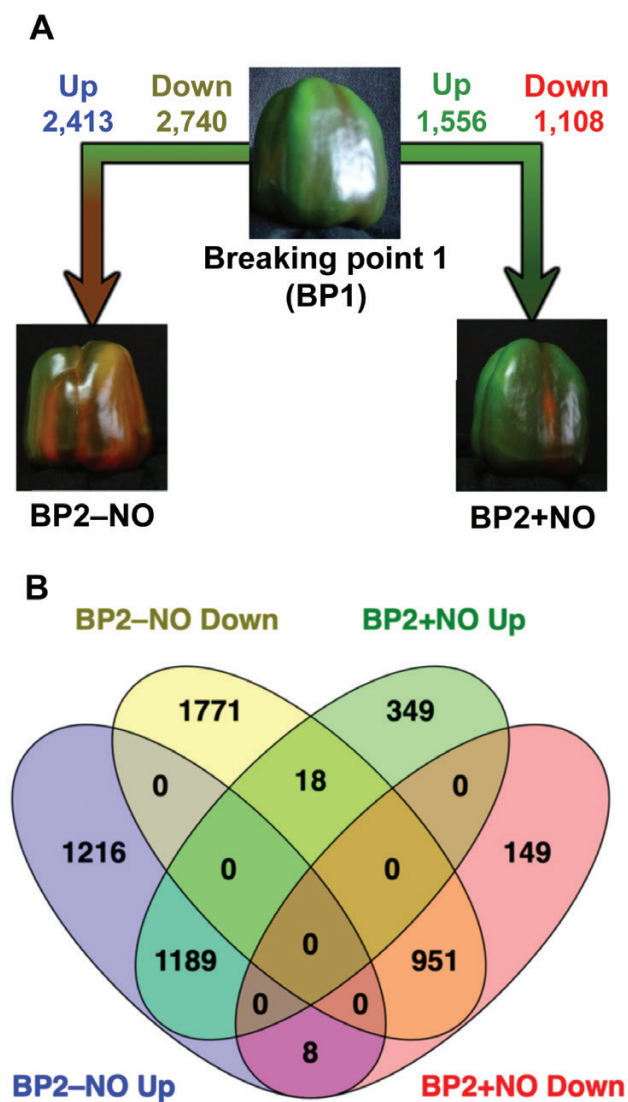


Fig. 2. Analysis of gene expression during ripening and modulated by NO in sweet pepper fruits. (A) Analysis of differentially expressed genes (DEGs) between pepper fruits at breaking point 1 (BP1), breaking point 2 in untreated fruits (BP2–NO), and breaking point 2 in fruits treated with NO (BP2+NO). (B) Venn diagrams combining DEGs (up- and down-regulated) among pepper fruits at different stages of ripening and under NO treatment.

assigned GO categories together with their *P* values were then filtered using the REViGO web application to gather relevant information.

Table 2. Groups of genes modulated by NO gas treatment observed in Fig. 2B

Group	Description	Tendency	Gene number
1	Genes not affected by the NO treatment	Up-regulated	1189
2		Down-regulated	951
3	Genes affected by the NO treatment	Up-regulated	1216
4		Down-regulated	1771
5	Genes specifically affected by the NO treatment	Up-regulated	349
6		Down-regulated	149
7	Genes whose pattern of expression was changed by the NO treatment	From up- to down-regulated	8
8		From down- to up-regulated	18

Functions and processes influenced by NO gas treatment

Depending on the comparison between gene groups, the effects of NO gas treatment could be masked by genetic changes caused by the natural ripening process itself (Fig. 2A, left side). Up- or down-regulated genes that share the same tendency in both comparisons (BP1 versus BP2–NO and BP1 versus BP2+NO) are considered to be genes whose expression is not modulated by NO; this was the case for a total of 2140 genes (1189 up-regulated and 951 down-regulated) (Fig. 2B). With respect to biological processes where genes were up-regulated regardless of the NO gas treatment, we focused on responses to stimuli such as temperature, redox state, bacteria, oxidative stress, and ABA. We also detected primary metabolic processes including photosynthesis and cellular lipid metabolism, as well as other important mechanisms such as wax biosynthesis, leaf senescence, and protein stabilization. Conversely, in the group of down-regulated genes, we identified different categories: immune systems, developmental processes, ribosome biogenesis, and cell death.

The genes identified when comparing BP1 and BP2–NO (1216 up-regulated and 1771 down-regulated) were those whose overexpression and repression, respectively, were impeded by NO. Treatment with NO masked the overexpression of genes involved in processes such as osmotic stress responses, development, lipid oxidation, and proteolysis. Furthermore, NO prevented the repression of genes involved in methylation, respiratory burst, chloroplast protein targeting, and auxin influx.

Genes specifically affected by NO treatment

The groups of up- and down-regulated genes exclusively found in BP2+NO (349 and 149, respectively) are those whose expression is exclusively modulated by NO. Fig. 3A shows a gene set enrichment analysis of the 349 genes that were up-regulated by NO treatment. We then carried out a more precise classification to highlight genes encoding proteins, such as calmodulin, related to the regulation of NO metabolism; responses to H₂O₂, such as heat shock and ubiquitination proteins; and oxidoreductase activity, including thioredoxins, APX, and glutaredoxin (Wang *et al.*, 2013; Mata-Pérez *et al.*, 2016; Zhou *et al.*, 2016; Niu *et al.*, 2017; Zhang *et al.*, 2018). We also classified the 149 down-regulated genes (Fig. 3B), among which we found genes encoding proteins with roles in

biological processes involving arginylation and protein folding. Additionally, we detected molecular functions such as cysteine synthase, connected with sulfur metabolism.

Beyond the simple arithmetic analysis, it could be concluded that the NO gas treatment had less effect on the gene expression pattern than the post-harvest storage for 3 d. In other words, NO seems to modulate the profound genetic changes that take place throughout the ripening process of pepper fruits—a condition that perhaps also occurs in other crop fruits.

Time-course analysis of NO-treated and untreated fruits

We carried out two different time-course analyses to study variations in transcriptome profiling during fruit ripening and after exposure to NO gas. We first analyzed the natural ripening process in fruits at the G, BP1, BP2–NO, and R stages, and then examined the NO-treated fruits at the stages evaluated in the first time-course analysis. In the second analysis, the BP2–NO sample was replaced by the BP2+NO sample, as both samples had similar time intervals. The maSigPro tool revealed profound temporal changes in the expression of 8805 genes grouped into nine clusters. Fig. 4 shows that NO treatment caused a significant variation in the time course of expression of many genes, especially in clusters 1, 2, 3, 4, 7, and 9. Overall, we observed that the genes in BP2+NO fruits clearly behaved differently from those in BP2–NO fruits, with the possible exception of genes in clusters 5, 6, and 8. Cluster 1 included several down-regulated genes coding for key enzymes involved in ROS and RNS metabolism, including L-galactono-1,4-lactone dehydrogenase (GalLDH), an enzyme that catalyzes the terminal step of the ascorbate biosynthesis pathway (Scherlt *et al.*, 2012); S-nitrosoglutathione reductase, which catalyzes the NADH-dependent reduction of S-nitrosoglutathione, a mobile reservoir of NO (Letierri *et al.*, 2011); Fe-superoxide dismutase (Fe-SOD), which catalyzes the dismutation of the superoxide radical into H₂O₂; and 6-phosphogluconate dehydrogenase (6PGDH), which is part of the oxidative pentose phosphate pathway that generates the NADPH necessary for FA synthesis (Hutchings *et al.*, 2005). Some of these enzymes have previously been found to be modulated during ripening (Mateos *et al.*, 2013; Rodríguez-Ruiz *et al.*, 2017a, b). Moreover, *cue1/nox1* (chlorophyll *a/b* binding protein underexpressed 1/NO overproducer 1), which codes for a phosphoenolpyruvate/phosphate translocator of

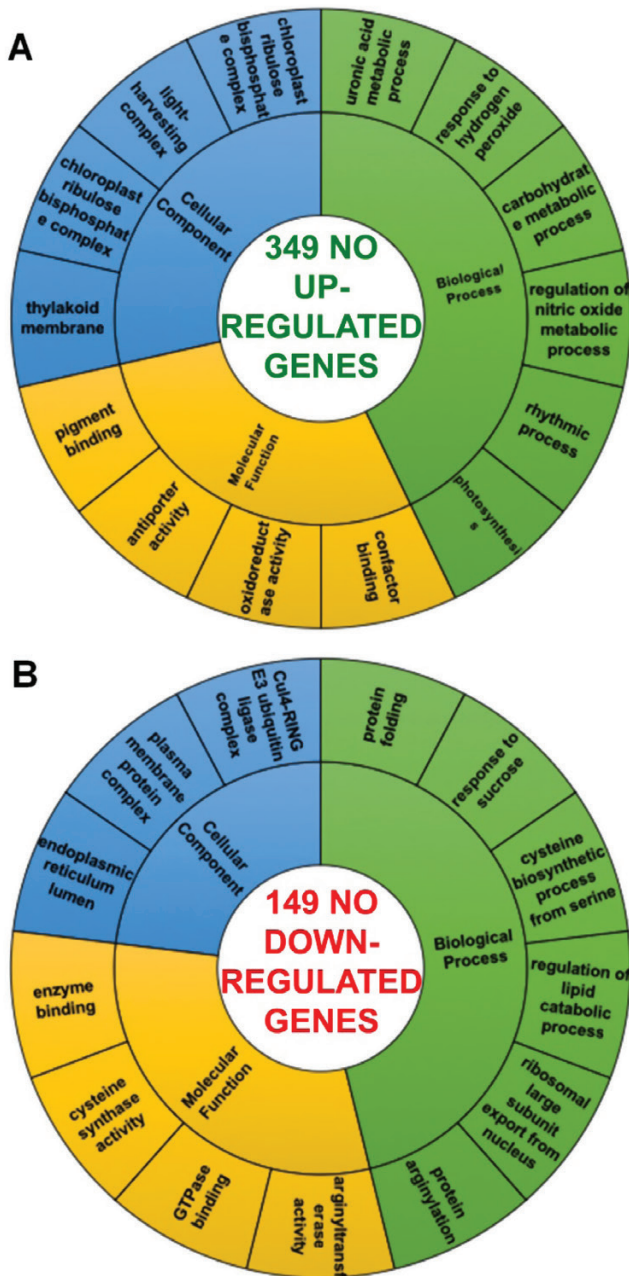


Fig. 3. Functional classification of sweet pepper fruit genes specifically affected by NO gas treatment. Gene ontology (GO) enrichment analysis was performed using PlantRegMap. Genes were classified according to biological processes, molecular functions, and cellular component categories. GO categories with their associated *p*-values were submitted to REVIGO to remove redundant information. (A) Genes up-regulated by NO. (B) Genes down-regulated by NO.

the inner plastid envelope membrane, was present in cluster 1 (Voll *et al.*, 2003). The Arabidopsis knockout mutant (*nox1*) is characterized by L-arginine and citrulline accumulation and higher NO content compared with wild-type Arabidopsis (He *et al.*, 2004). This suggests that high NO content is caused by NOS-like activity, as *nox1* has high L-arginine content. Given this possibility in pepper fruits, the down-regulation of *NOX1* during ripening could restore endogenous NO levels, as we previously showed that green pepper fruits have a higher NO

content than red fruits (Chaki *et al.*, 2015; Muñoz-Vargas *et al.* 2018). This hypothesis is further corroborated by the presence of the *NOA1/RIF1* (nitric oxide-associated protein 1/resistant to inhibition with fosmidomycin 1) ortholog in cluster 9, which could restore endogenous NO levels. On the other hand, although the Arabidopsis null mutant *noa1* is reported to have lower NO content than wild-type Arabidopsis, this gene does not encode an NO-generating protein but instead plays an indirect role in NO accumulation (Van Ree *et al.*, 2011). Interestingly, orthologous expression of *NOA1* in pepper appears to increase in cluster 9 in the presence of exogenous NO gas, which correlates with the NO metabolism. These findings suggest that the *NOA1/RIF1* protein is not essential for NO production and probably plays only an indirect role in NO accumulation.

Moreover, genes in the first three clusters showed enriched GO terms associated with lipid metabolism, including long-chain acyl-CoA synthetase 1, peroxisomal acyl-coenzyme A oxidase 1, and biotin carboxyl carrier protein of acetyl-CoA carboxylase 1, as well as lipoxygenase 6 and phospholipase D. We therefore decided to analyze the FA profile of fruits at all stages of ripening, as well as lipid peroxidation and proline content, which are two parameters associated with different types of oxidative stress.

NO gas modulates the profiles of proline, lipid peroxidation, and glutathione, FA content, APX and LOX activity during ripening

Fig. 5A shows that proline content gradually increased at the different stages of fruit ripening that were analyzed, with a maximum 2.4-fold increase in red fruit compared with green fruit. Significantly, fruit with and without NO treatment (BP2+NO and BP2-NO, respectively) both showed proline content values similar to those for green pepper; values were slightly lower in NO-treated fruits compared with untreated fruits, but the difference was not statistically significant. An increase in proline content is usually associated with a stress response mechanism (Szabados and Savouré, 2010), which is normally associated with oxidative stresses such as salinity, drought, and heavy metals (Signorelli *et al.*, 2013; Bouthour *et al.*, 2015; Rodríguez-Ruiz *et al.*, 2019). However, an increase in proline content has also been linked to other physiological processes, such as senescence, as has been observed in flowers and leaves (Zhang and Becker, 2015). Previous studies of grapevine and tomato fruits also report an increase in proline content during ripening (Stines *et al.*, 1999; Claussen *et al.*, 2006), suggesting that it is an indicator of fruit quality. Our findings corroborate these observations of proline accumulation during ripening but also indicate that NO may negatively regulate proline biosynthesis, which opens up new questions concerning this process. These data are well correlated with the down-regulation of 6PGDH, considering that the biosynthesis of proline from glutamate requires NADPH (Signorelli and Monza, 2017).

Lipid peroxidation was analyzed because this parameter is a recognized marker of oxidative stress (Yamauchi *et al.*, 2008) characterized by an increase of MDA. Fig. 5B shows

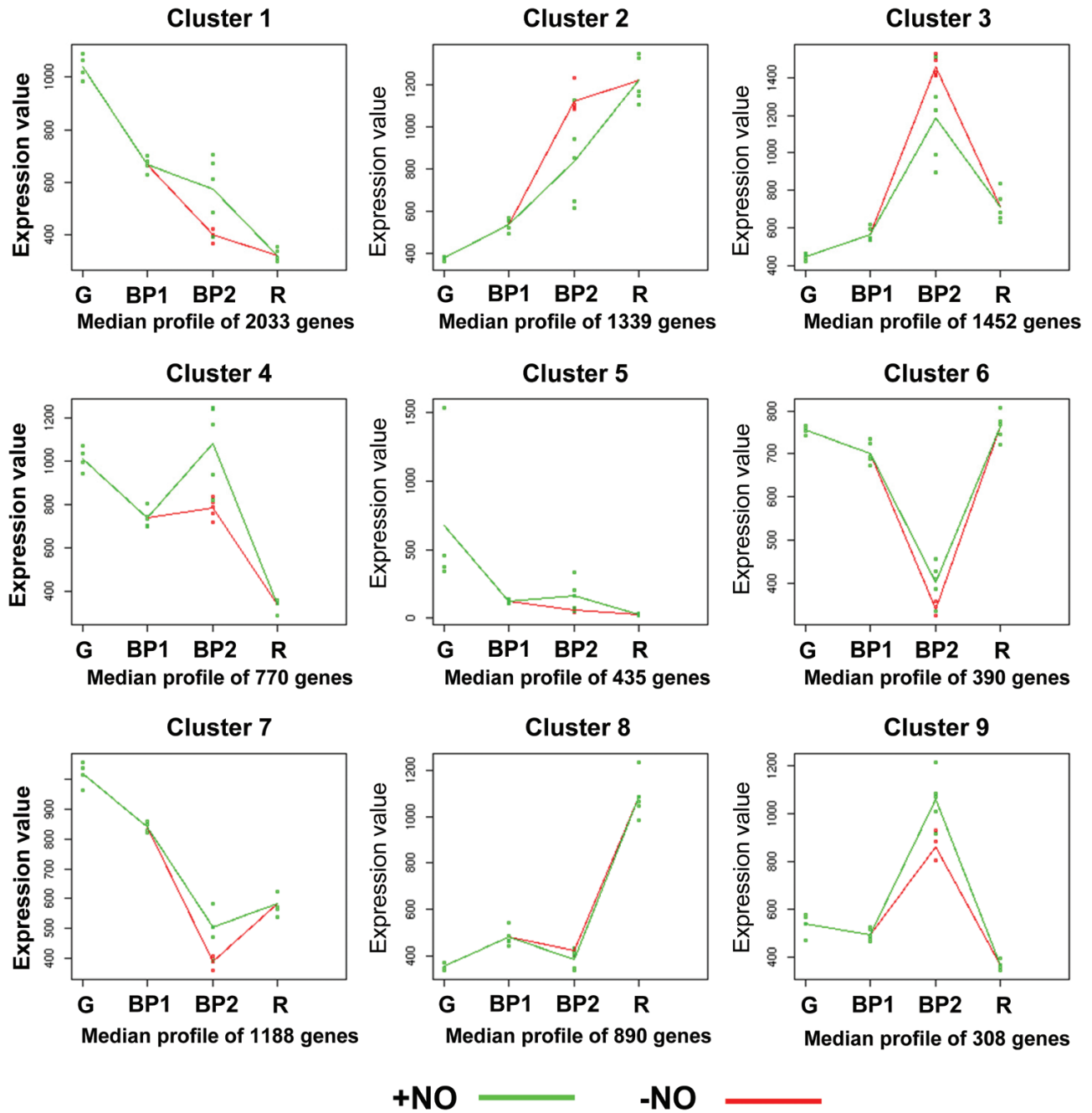


Fig. 4. Time-course analysis of genes showing statistically significant changes in expression throughout ripening in sweet pepper fruits exposed to NO gas. Genes were grouped into nine clusters according to their expression profiles at different ripening stages. Expression values corresponding to the ripening stages G, BP1, and R are shared by both time-courses. However, at stage BP2, the expression profile of the clustered genes is represented as either untreated fruits (red line) or fruits treated with NO (green line). The solid line indicates the mean and dots represent the SD of the expression values at each ripening stage. In all clusters the treatment with NO altered the natural post-harvest evolution of gene expression represented by the sequence G → BP1 → BP2 → R.

that MDA content progressively increased across the different ripening stages, with a 3.7-fold increase in red fruit compared with green fruit. Again, it is worth noting that fruit with and without NO treatment showed similar and intermediate values for MDA content, with a slightly lower value observed for NO-treated fruits compared with untreated fruits, although, again, the difference was not statistically significant. These findings confirm that the ripening process is associated with oxidative stress and that NO treatment has the capacity to reduce

MDA content. An increase in lipid peroxidation has also been described during ripening of the fruit of other pepper cultivars (Martí *et al.*, 2011) and species such as blackberry (Wang and Jiao, 2001), tomato (Jiménez *et al.*, 2002), and peach (Huan *et al.*, 2016), which are associated with lower antioxidant enzyme activities. These data are in good agreement with the down-regulation of *Fe-SOD* found in gene cluster 1, described earlier. In previous studies of pepper ripening, catalase activity was found to diminish, and this was associated with an increase

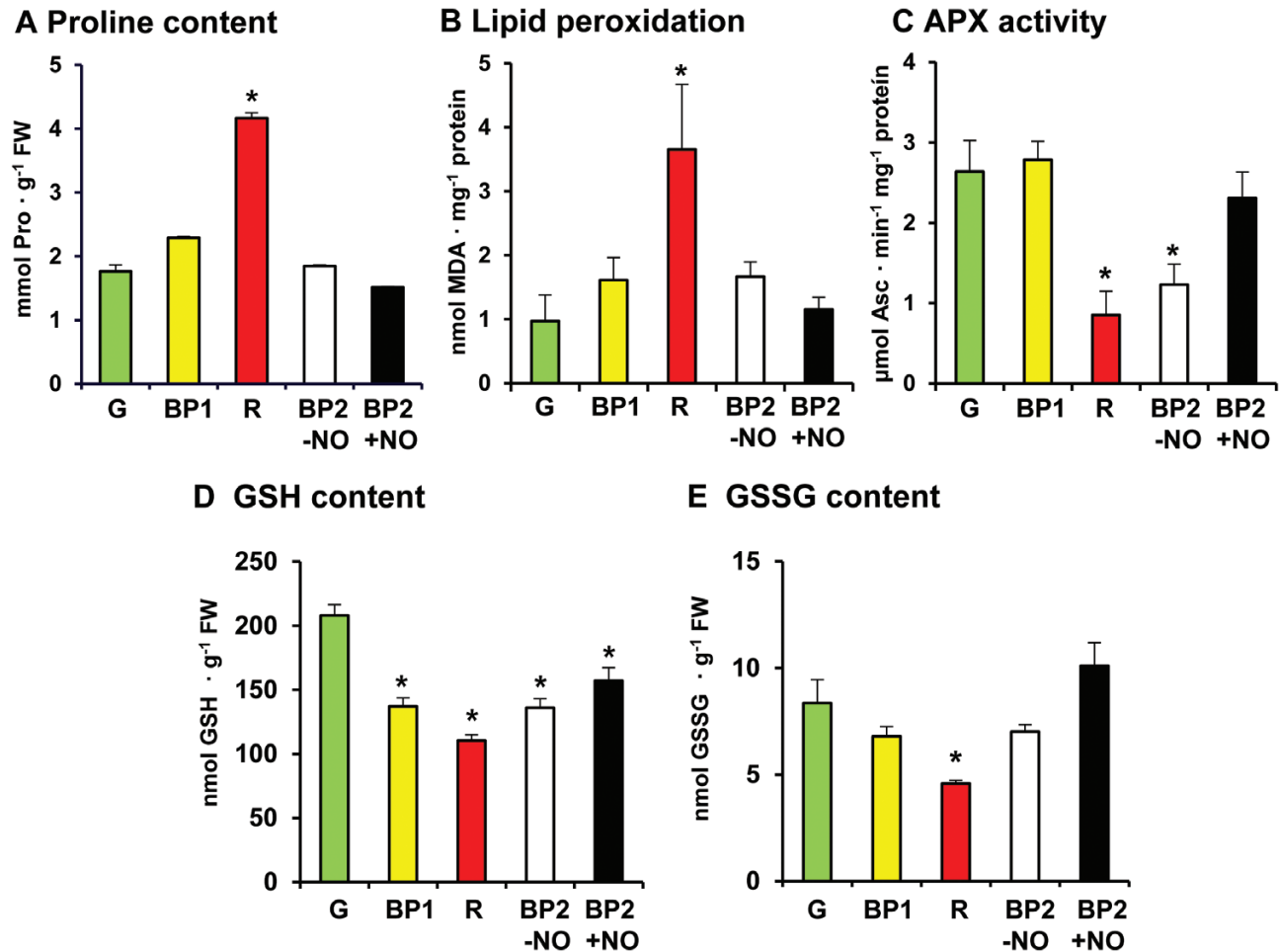


Fig. 5. (A) Proline content, (B) lipid peroxidation level, (C) ascorbate peroxidase (APX) activity, (D) reduced glutathione (GSH) content, and (E) oxidized glutathione (GSSG) contents of sweet pepper fruits at different stages of ripening: immature green (G), breaking point 1 (BP1), breaking point 2 with and without NO treatment (BP2+NO and BP2-NO, respectively), and ripe red (R). Lipid peroxidation was determined by the thiobarbituric acid reactive substances method, using malondialdehyde as standard. Pairwise analysis of variance was used to detect differences in comparison to green fruits. * $P < 0.05$. (This figure is available in colour at *JXB* online.)

in tyrosine protein nitration (Chaki *et al.*, 2015), a recognized marker of nitro-oxidative stress (Corpas *et al.*, 2009). Recently, we found that $O_2^{\cdot-}$ -generating NADPH oxidase activity is up-regulated during pepper fruit ripening (Chu-Puga *et al.*, 2019), which correlates with the increase observed in lipid peroxidation. All these data connect well with earlier observations that NO-treated pepper fruit showed a significant increase in the activity and gene expression of GalLDH, which catalyzes the last step in the biosynthesis of ascorbic acid, whose content increased by 40% compared with untreated pepper fruit (Rodríguez-Ruiz *et al.*, 2017a). Data pertaining to other plant species under diverse stress conditions show that exogenous NO treatment stimulates the activity of different antioxidant enzymes (Manai *et al.*, 2014; Kharbech *et al.*, 2017), including those in fruits; for example, during peach fruit ripening, NO treatment stimulates SOD activity (Kang *et al.*, 2016; Corpas and Palma, 2018).

To corroborate this potential effect on antioxidant enzymes, we analyzed APX activity. Fig. 5C shows a 2.8-fold decrease in this activity in red fruits compared with green fruits. As with proline and lipid peroxidation, APX activity showed

intermediate values both with and without NO treatment; in this case, APX activity was 2.7-fold higher in NO-treated fruits compared with red fruits. This positive effect of NO on APX activity closely correlates with findings of previous studies that demonstrated that APX activity is positively regulated by S-nitrosation in pea and Arabidopsis (Begara-Morales *et al.*, 2014; Yang *et al.*, 2015).

Fig. 5D shows that GSH content gradually decreased as ripening proceeded, with a 1.9-fold decrease in red fruit compared with green fruit. Fruits with and without NO treatment showed an intermediate GSH content, with a slightly higher value for NO-treated fruits. GSSG followed a similar pattern to that of GSH, but with values 25-fold lower (Fig. 5E). This GSH enhancement indicates that exogenous NO provides a higher antioxidant capacity, confirming what was previously reported with respect to ascorbate content (Rodríguez-Ruiz *et al.*, 2017a). Similar increases in GSH and ascorbate levels have been described recently in sweet cherry fruit treated with NO-releasing chitosan nanoparticles (Ma *et al.*, 2019).

Taken together, the data suggest that exogenous NO treatment has a regulatory effect on FAs by reducing the degree of

lipid peroxidation, with a concomitant increase in GSH content and APX activity, which is expected to affect the mechanism involved in palliating oxidative stress associated with the ripening process.

Plant FAs are involved in various functions, such as cell membrane mechanisms and acting as energy reserves, as well as providing jasmonic acid and oxylipin signaling molecules for plant defense (Kachroo and Kachroo, 2009). Given that the composition of FAs can be modulated by physiological processes and in response to biotic and abiotic stresses, FAs in pepper fruits at the different stages of ripening were identified and quantified (Fig. 6). Polyunsaturated linoleic (C18:2) and linolenic (C18:3) acids were the most abundant FAs in the pepper fruits, followed by saturated palmitic (C16) and stearic (C18) acids. We also identified other less abundant FAs ranging from C12 to C26. These data are very much in line with findings of previous studies of pepper fruits of various origins (Pérez-Gálvez *et al.*, 1999; Sora *et al.*, 2015; Ananthan *et al.*, 2018) and also

studies of tomato (Saini *et al.*, 2017). During the fruit ripening process, the content of these FAs was differentially regulated, with the content of linolenic and stearic acids being observed to increase and that of linoleic acid to decrease. Moreover, as previously observed with respect to proline and MDA content (see Fig. 5), pepper fruits at BP1 generally showed intermediate values for the majority of FAs. The FA content of fruits at BP2 with and without NO treatment also showed intermediate values, with the content of specific FAs being observed to either decrease or increase in NO-treated fruits. Although the content of different FAs either increases or decreases during the ripening process, to our knowledge, no studies have found that exogenous NO treatment affects either the biosynthesis or the breakdown of these FAs in non-climacteric fruits. In contrast, climacteric peach fruits fumigated with NO and then stored at 5 °C showed an increase in the content of palmitoleic (16:1), oleic, and linolenic acids, while the linoleic acid content decreased (Zhu and Zhou, 2006). These data are in accordance

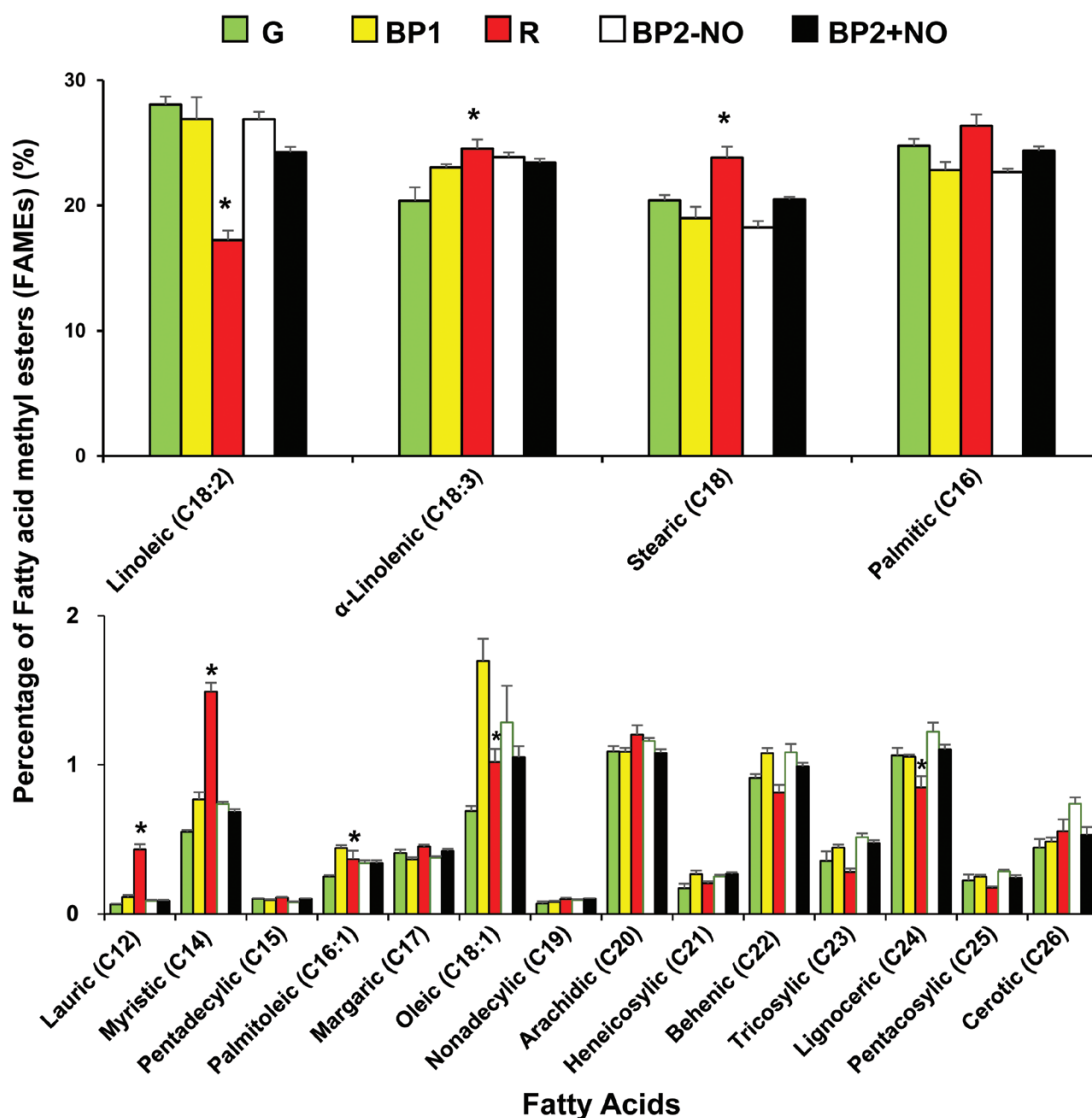


Fig. 6. Fatty acid content and profile in sweet pepper fruits at different stages of ripening: immature green (G), breaking point 1 (BP1), breaking point 2 with and without NO treatment (BP2+NO and BP2-NO, respectively), and ripe red (R). Pairwise analysis of variance was used to detect differences in comparison to green fruits. * $P < 0.05$. (This figure is available in colour at JXB online.)

A Lipoxygenase (LOX) activity

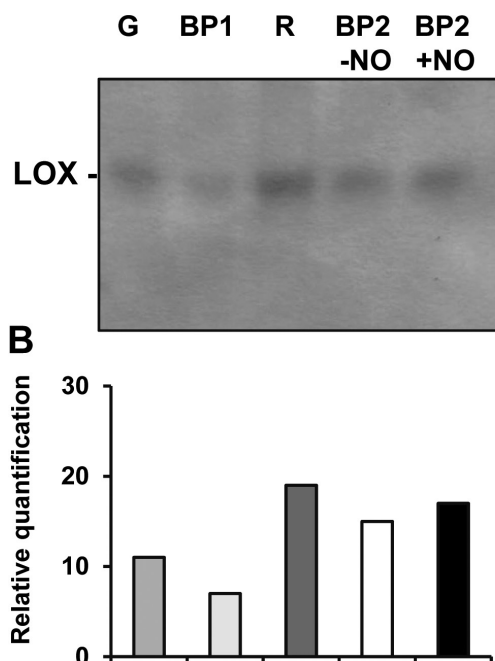


Fig. 7. Lipoxygenase (LOX) activity staining in non-denaturing polyacrylamide gel electrophoresis of sweet pepper fruits at different stages of ripening: immature green (G), breaking point 1 (BP1), breaking point 2 with and without NO treatment (BP2+NO and BP2-NO, respectively), and ripe red (R). (A) Representative picture of a gel stained for LOX activity. (B) Relative quantification of the band activities were determined using ImageJ software. (This figure is available in colour at *JXB* online.)

with some of the FAs affected by NO in pepper fruit, supporting the assertion that FA metabolism is modulated by NO.

Given that linoleic and linolenic acids are the principal substrates of LOX (Siedow, 1991; Porta and Rocha-Sosa, 2002), which catalyzes the first oxygenation step of polyunsaturated FAs, we assayed LOX activity in pepper fruits at different stages of ripening by non-denaturing gel electrophoresis. LOX activity was detected as a single band (Fig. 7A). Relative quantification indicated a 1.7-fold increase in red fruit compared with green fruit (Fig. 7B). LOX activity in BP2 stage fruits with and without NO treatment showed intermediate values, with a slightly higher level of activity being observed in NO-treated fruits, although the difference was not statistically significant; this tendency was similar to that observed in the analyses of proline and MDA content in BP2-NO fruits (see Fig. 5). Several studies have indicated that LOX activity is involved in fruit ripening, which appears to be modulated by two phytohormones (ABA and ethylene) associated with aroma production and fruit quality (Zhang *et al.*, 2009; Lv *et al.*, 2014; Zhang *et al.*, 2017; Del Ángel-Coronel *et al.*, 2018).

Conclusions

This study provides the first transcriptome of sweet pepper fruit, which could be a valuable tool for developing

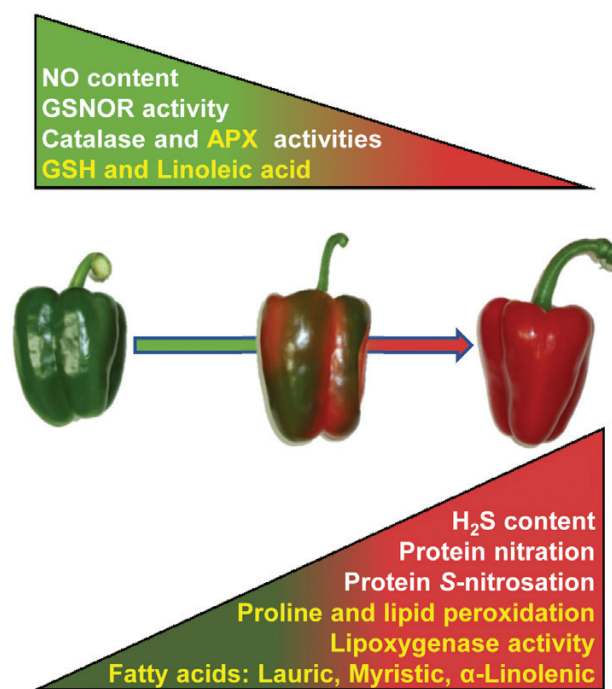


Fig. 8. Working model of the metabolism of pepper fruit during ripening. Yellow text refers to the parameters reported in the present study, whereas white text refers to previously published data. APX, ascorbate peroxidase; GSH, reduced glutathione; GSNOR, S-nitrosoglutathione reductase; H₂S, hydrogen sulfide.

comparative studies of available transcriptomes of hot pepper fruits characterized to have a biosynthetic pathway for capsaicin, which is responsible for the ‘heat’ and pungency associated with hot peppers (Zhang *et al.*, 2016; Tanaka *et al.*, 2017). Likewise, this transcriptomic analysis will allow knowledge to be gained on the pathways that are modulated during ripening in non-climacteric fruits. On the other hand, exogenous NO application has a strong regulatory effect on a wide variety of processes during sweet pepper fruit ripening, which are regulated at the levels of gene and protein expression, enzymatic activity, and, thus, metabolite content. During ripening of sweet pepper fruits from the green to the red stage, the major effects of NO were: (i) delayed ripening, with phenotypic (color) and gene expression changes; and (ii) a regulatory effect on FAs, by reducing the degree of lipid peroxidation with a concomitant increase in antioxidant capacity (GSH, APX activity), which palliates the oxidative stress associated with the ripening process. Fig. 8 shows a working model of natural pepper fruit ripening during the transition from green to red, in which multiple parameters of nitro-oxidative metabolism are modulated. Finally, in addition to the relevance of NO as a regulator of pepper fruit ripening, which is independent of ethylene, the potential involvement of other endogenous molecules, such as H₂O₂, ABA, H₂S, or melatonin, which could exert synergistic or antagonistic actions to NO, establishing a complex network of signals, as has been reported elsewhere (Corpas *et al.*, 2019; Mukherjee 2019; Vithana *et al.*, 2019), should be mentioned. The mechanisms that govern these interactions, and how they modulate the whole ripening process, should be deciphered in future research.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Summary of the data obtained after sequencing of the 24 cDNA libraries.

Table S2. Statistics of the full set of tentative transcripts for the best *de novo* assembled transcriptome.

Fig. S1. Flow chart of the experimental procedure to analyze the transcriptome of sweet pepper fruit.

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