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Ripening of pepper (*Capsicum annuum*) fruit is characterized by an enhancement of protein tyrosine nitration

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• **Background and Aims** Pepper (*Capsicum annuum*, Solanaceae) fruits are consumed worldwide and are of great economic importance. In most species ripening is characterized by important visual and metabolic changes, the latter including emission of volatile organic compounds associated with respiration, destruction of chlorophylls, synthesis of new pigments (red/yellow carotenoids plus xanthophylls and anthocyanins), formation of pectins and protein synthesis. The involvement of nitric oxide (NO) in fruit ripening has been established, but more work is needed to detail the metabolic networks involving NO and other reactive nitrogen species (RNS) in the process. It has been reported that RNS can mediate post-translational modifications of proteins, which can modulate physiological processes through mechanisms of cellular signalling. This study therefore examined the potential role of NO in nitration of tyrosine during the ripening of California sweet pepper.
• **Methods** The NO content of green and red pepper fruit was determined spectrofluorometrically. Fruits at the breaking point between green and red coloration were incubated in the presence of NO for 1 h and then left to ripen for 3 d. Profiles of nitrated proteins were determined using an antibody against nitro-tyrosine (NO_2-Tyr), and profiles of nitrosothiols were determined by confocal laser scanning microscopy. Nitrated proteins were identified by 2-D electrophoresis and MALDI-TOF/TOF analysis.
• **Key Results** Treatment with NO delayed the ripening of fruit. An enhancement of nitrosothiols and nitroproteins was observed in fruit during ripening, and this was reversed by the addition of exogenous NO gas. Six nitrated proteins were identified and were characterized as being involved in redox, protein, carbohydrate and oxidative metabolism, and in glutamate biosynthesis. Catalase was the most abundant nitrated protein found in both green and red fruit.
• **Conclusions** The RNS profile reported here indicates that ripening of pepper fruit is characterized by an enhancement of *S*-nitrosothiols and protein tyrosine nitration. The nitrated proteins identified have important functions in photosynthesis, generation of NADPH, proteolysis, amino acid biosynthesis and oxidative metabolism. The decrease of catalase in red fruit implies a lower capacity to scavenge H_2O_2, which would promote lipid peroxidation, as has already been reported in ripe pepper fruit.

Key words: *Capsicum annuum*, confocal laser scanning microscopy, MALDI-TOF/TOF, nitric oxide, nitrosothiols, nitro-tyrosine, NO gas, pepper fruit, protein nitration, proteomics, reactive nitrogen species, ripening, RNS, Solanaceae.

INTRODUCTION

Pepper (*Capsicum annuum*) plants are originally from tropical regions and their fruits are characterized by high levels of antioxidants, such as ascorbic acid (vitamin C), carotenoids, β-carotene (pro-vitamin A) and phenolic compounds (Howard *et al*., 2000; Palma *et al*., 2009, 2011*a*; Martí *et al*., 2011). Due to its wide distribution and culinary value, pepper is the second most consumed vegetable worldwide. In fact, much of the information available on the metabolic (antioxidative) properties of pepper fruits has risen since the influence of pepper consumption on human health was established (Mateos *et al*., 2013). Accordingly, fruit quality has been pursued as a research subject by crop growers and plant biologists.

Based on the shape, bell/sweet peppers are mainly classified into types California, Lamuyo and Dulce italiano. California fruits used in this work commonly change to either red, yellow or orange colour after maturation depending on the cultivar, and show similar sizes of the transverse and longitudinal axes. In most *Capsicum* species, ripening is characterized by important visual and metabolic changes. Thus, besides the typical colour shift occurring at maturation, a highly intense metabolism, emission of volatile organic compounds associated with

respiration, destruction of chlorophylls, synthesis of new pigments (red/yellow carotenoids plus xanthophylls and anthocyanins), formation of pectins, protein synthesis, taste alteration and changes in total soluble reducing equivalents take place (Cámara *et al*., 1995; Markus *et al*., 1999; Howard *et al*., 2000; Manirakiza *et al*., 2003; Palma *et al*., 2011*b*).

These biochemical transformations continue once the fruit is removed from the plant, with certain variations due to interruption of sap movement (Giambanco de Ena, 2006). The ripening of pepper fruit has been the subject of interest of many researchers, not only because of the spectacular change they undergo but also because of the complexity of the mechanisms that take part in the process of biosynthesis of capsanthin, a typical colorant of this fruit (De, 2003). Better understanding of the molecular changes associated with pepper fruit ripening will allow the provision of data on varieties and harvest times to improve fruit quality (Martí *et al*., 2011). In fact, demand for tailor-made pepper fruits to suit diverse product applications and large-scale food processing is increasing. Thus, the provision of products with required levels of aroma, pungency and colour requires additional scientific information and indicates the importance of harvesting stage and primary processing conditions on product quality (De, 2003; Martí *et al*., 2011).

Nitric oxide (NO) is a radical gas from which a series of derived molecules are formed, including *S*-nitrosoglutathione (GSNO) and peroxynitrite ($ONOO^-$), all of them being framed within the term reactive nitrogen species (RNS). The metabolism of RNS and their physiological implications have been thoroughly investigated in animal cells. However, little is known regarding their role in plant cells. It has been reported that RNS can mediate post-translational modifications (PTMs) of proteins, which can modulate physiological processes through mechanisms of cellular signalling (Radi, 2004; Dalle-Donne *et al*., 2005; Ischiropoulos and Gow, 2005; Corpas *et al*., 2007, 2008, 2014). These RNS-mediated PTMs include events such as binding to metal centres, nitrosylation of thiol and amine groups, and nitration of tyrosine and other amino acids (Gow *et al*., 2004).

Nitration of tyrosine is a covalent protein modification resulting from the addition of a nitro (-NO_2) group to one of the two equivalent ortho carbons in the aromatic ring of tyrosine residues (Gow *et al*., 2004), and it is mediated by the formation of peroxinitrite from the reaction between NO and superoxide radicals ($O_2^{\bullet -}$). This event converts tyrosine into a negatively charged hydrophilic nitrotyrosine linked to a change of the local hydroxyl p*K*a (Turko and Murad, 2002). This covalent PTM is considered a selective process, which depends on several factors such as protein structure, the nitration mechanism and the cell loci where the protein is located (Romero-Puertas *et al*., 2007; Corpas *et al*., 2009*a*). Nitration of the tyrosine residues has been broadly described as an irreversible process that can alter the normal protein functions with either loss or gain of function, but also with no change in function (Souza *et al*., 2008; Petre *et al*., 2012; Radi, 2013). Nevertheless, other reports point towards the reversibility of the nitration process (Görg *et al*., 2007). Nitration has been used traditionally as a disease and oxidative stress marker in animal cells (Ischiropoulos, 2003), whereas in plants it has been studied under biotic (Cecconi *et al*., 2009; Chaki *et al*., 2009*a*) and abiotic stresses including salinity (Valderrama *et al*., 2007; Corpas *et al*., 2009*b*; Tanou *et al*., 2012), extreme temperature (Chaki *et al*., 2011; Airaki *et al*., 2012) and high arsenic concentration (Leterrier *et al*., 2012). All these data have led to the proposal of protein nitration as a potential nitrosative stress marker (Corpas *et al*., 2007, 2014). It has also been reported that there is a basal nitration under physiological conditions in different plant organs (Chaki *et al*., 2009*b*; Begara-Morales *et al*., 2013); however, to our knowledge, no information regarding pepper fruits and their ripening process is available.

In the present study, the protein nitration profile in green and red California pepper fruits was studied. Ripening is associated with a higher nitration of proteins and is prevented by treatment of fruits with exogenous NO gas. The pattern of nitrated proteins was investigated using proteomics approaches. Six potentially nitrated proteins participating in redox, oxidative, protein and carbohydrate metabolisms and in the glutamate biosynthesis of pepper fruits were identified.

MATERIALS AND METHODS

Plant materials

Pepper (*Capsicum annuum* L., cv. Melchor) fruit of California type were obtained from Syngenta Seeds, Ltd (El Ejido, Almería, Spain). Fruit were used at three differentiated ripening stages: green (GP), breaking-point (BPP, between red/green) and red (RP; Supplementary Data Fig. S1). In the cultivar used in this work, the total shift from green to red stages commonly lasts 5–6 d.

Treatment of pepper fruits with exogenous NO gas

Pepper fruit at the breaking-point (BPP) stage were used in these experiments. Fruits harvested from plants were immediately placed in a hermetically sealed methacrylate box from which the air had been removed by applying vacuum for 15 min. NO gas was then generated as indicated in Supplementary Data Fig. S2, in a solution containing excess copper and nitric acid. In the initial reaction and under concentrated HNO_3, nitrous oxide is formed according to the reaction:

$$Cu(s) + 4HNO_3(aq) \rightarrow Cu(NO_3)_2(aq) + 2NO_2(g) + 2H_2O.$$

Once the nitric acid is partially consumed, a second reaction occurs:

$$3Cu(s) + 8HNO_3(aq) \rightarrow 3Cu(NO_3)_2(aq) + 2NO(g) + 4H_2O.$$

In the experimental design developed here, the NO_2 is trapped in a flask by concentrated NaOH. NO gas was then pumped into the incubation chamber by a suctioning mechanism integrated in the NO detector (Nitric Oxide Meter, Environmental Sensors Co., Boca Raton, FL, USA). When readings in the detector reached 5 p.p.m. (160 μmol L^{-1}) NO, connections were tightly sealed and the incubation was maintained for 1 h at room temperature. Afterwards the box was opened and the pepper fruits were kept at room temperature for 3 d to test the effect on ripening and then analysed as indicated below. As control

conditions, sets of pepper fruits were subjected to the same protocol but without NO treatment. Thus, fruits were equally placed in an incubation box which was sealed and kept there for 1 h at room temperature without adding any gas. Fruits were then left at room temperature for 3 d and further analysed.

Preparation of samples and protein concentration

All operations were performed at 0–4 °C. Fruits were ground in liquid N_2 with a mortar and pestle, and the resulting powder was resuspended in 0·1 M Tris-HCl buffer, pH 8·0, containing 1 mM EDTA, 10 % (v/v) glycerol to a final ratio 1 : 2 of plant material/buffer. Homogenates were filtered through two layers of nylon cloth and centrifuged at 27 000 *g* for 20 min. Supernatants were immediately used for the assays.

For electrophoresis and Western blotting purposes pepper fruits were homogenized in medium containing 50 mM Tris-HCl, pH 7·5, 0·1 mM EDTA, 0·1 % (v/v) Triton X-100, 5 mM dithiothreitol and 10 % (v/v) glycerol. Homogenates were filtered through four layers of nylon and centrifuged at 27 000 *g* for 15 min. Supernatants were used for protein precipitation with acetone. Three volumes of 100 % acetone at −20 °C were added to the supernatants with stirring for 30 min (70 % final concentration, v/v) and the mixture was centrifuged at 16 000 *g* for 15 min. The precipitate, which contained most of the proteins, was taken up in 5 mL of 50 mM Tris-HCl, pH 7·5 and incubated overnight at 4 °C with stirring. Then, the solution was centrifuged at 39 000 *g* for 20 min. The supernatants were applied onto Sephadex G-25 gel filtration columns (NAP-10; Amersham, Piscataway, NJ, USA) equilibrated and eluted with 50 mM Tris-HCl, pH 7·5 (Begara-Morales *et al*., 2013).

In pepper fruit samples the protein concentration was determined with Bio-Rad Protein Assay reagent (Hercules, CA, USA) using bovine serum albumin (BSA) as a standard.

NO determination by a spectrofluorometric assay

Freshly homogenized fruits were incubated in the presence of 10 μM 4,5-diaminefluorescein (DAF-2; Merck Biosciences, Kenilworth, NJ, USA), at the final concentration. The reaction mixtures were incubated in the dark for 2 h at 37 °C. Then, the fluorescence in the reactions was measured in a Shimadzu RF-540 spectrofluorophotometer (Shimadzu, Columbia, MD, USA) at excitation and emission wavelengths of 485 and 515 nm, respectively (Nakatsubo *et al*., 1998; Airaki *et al*., 2012).

One-dimensional (1-D) SDS–PAGE and Western blot

SDS–PAGE of pepper fruits was carried out on 10/12 % polyacrylamide gels as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250 as reported previously (Distefano *et al*., 1999) for protein detection.

For Western blot analyses, proteins were transferred to PVDF membranes using a semi-dry Trans-Blot cell (Bio-Rad, Hercules, CA, USA). After transfer, membranes were used for cross-recognition assays using polyclonal antibodies against 3-nitrotyrosine (NO_2-Tyr; 1 : 8000 dilution) (Chaki *et al*., 2009*a*) and against catalase. The Sigma-Genosys (Cambridge, UK) service of polyclonal antibody production from a selected peptide was used to obtain the antibody against catalase. A peptide of 14 amino acids was synthesized from a pepper catalase internal consensed sequence (AF227952) also present in several taxonomically related species. The peptide was ERGSPE TLRDPRGFA, which corresponds to the residues between Glu110 and Ala124. The peptide is hydrophilic and contains one predicted β-turn. The selected peptide was conjugated to a carrier protein, keyhole limpet haemocyanin (KLH), which is derived from marine molluscs via the thiol group of a cysteine residue added to the N terminus of the selected peptide using MBS (maleimidobenzoyl-*N*-hydroxysuccinimide ester) chemistry. Thus, the construction KLH-[C]-ERGSPETLRDPRGFA was used for the immunization of two rabbits according to the protocol of six immunizations per rabbit (Sigma-Genosys).

For immunodetection in membranes, a goat anti-rabbit IgG–horseradish peroxidase conjugate (Bio-Rad) as the secondary antibody, and an enhanced chemiluminescence kit (ECLPLUS; Amersham) were used. Nitrated BSA (NO_2-BSA) was used as a positive control for detection of Tyr-nitrated proteins. Immunoblot assays were performed at least three times, all showing similar results.

2-D electrophoresis and immunoblot analysis

Acetone-concentrated samples from green and red peppers were separated by 2-D gel electrophoresis (2DE) (Begara-Morales *et al*., 2013). Isoelectric focusing was carried out with precast immobilized pH gradient gels (pH 3–10), where each gel was loaded with 100 μg of proteins. The second dimension separation was performed by glycine SDS–PAGE. Two replicates for each sample were done. In one of them, the gel was stained with Sypro Ruby, scanned and analysed with Bio-Rad PDQuest software. The second gel was used for immunoblot analysis. Polypeptides were transferred to PVDF membranes and nitroproteins were detected as indicated above. Spots from the first gel that matched those showing immunoblot labelling were used for further proteomic analysis. Three independent assays were carried out and the results shown correspond to a representative trial.

In situ *digestion of 2-D spots and protein identification by MALDI-TOF/TOF analysis*

Identified spots in the Sypro Ruby-stained gels were automatically picked using an Investigator ProPic Protein Picking Workstation (Genomic Solutions, Huntington, UK). They were then distained and digested with trypsin using an Investigator ProGest Protein Digestion Station (Genomics Solutions) as described earlier (Chaki *et al*., 2009*a*; Begara-Morales *et al*., 2013). Peptides were purified using a ProMS station (Genomic Solutions) with a C18 column (ZipTip; Millipore, Billerica, MA, USA) and eluted with α-cyano-4-hydroxycinnamic acid (3 mg mL^{-1}) in 70 % acetonitrile/0·1 % TFA on a MALDI plate (1 μL). After crystallization, samples were analysed by MALDI-TOF/TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry over a mass-to-charge ratio (*m*/*z*) range of 800–4000 Da with the aid of a 4700 Proteomics Analyzer spectrometer (Applied Biosystems, Foster City, CA, USA) in

automatic mode. For internal calibration of the mass spectra, the *m/z* of the peptides from porcine trypsin autolysis was used (mass $MH^+ = 842{\cdot}509$, mass $MH^+ = 2211{\cdot}104$) given an *m/z* ratio precision of 20 p.p.m. The three spectra with the highest *m/z* ratios were selected from each sample. Proteins were identified by combining the MS spectra with the corresponding MS/MS with the aid of the MASCOT program from the MatrixScience database (http://www.matrixscience.com/). The following search parameters were applied limiting the taxonomic category to plants: a mass tolerance of 100 p.p.m. and one incomplete cleavage were allowed, complete alkylation of cysteine by carbamidometylation, and partial oxidation of methionine.

Catalase activity assay: treatment with SIN-1 (peroxynitrite donor)

Catalase (EC 1.11.1.6) activity was determined by following the decomposition of hydrogen peroxide at 240 nm (Aebi, 1984). SIN-1 (3-morpholinosydnonimine; Calbiochem, Billerica, MA, USA) has been shown to generate peroxynitrite, a protein-nitrating compound (Daiber *et al*., 2004). Crude extracts from pepper fruits were incubated at 37 °C for 1 h with 2 mM SIN-1 made up fresh before use. The samples were then eluted through NAP-10 columns to avoid interference with SIN-1 in the activity assay.

Detection of S*-nitrosothiols (SNOs) by confocal laser scanning microscopy (CLSM)*

SNOs were detected using the fluorescent reagent Alexa Fluor 488 Hg-link phenylmercury (Valderrama *et al*., 2007; Corpas *et al*., 2008; Chaki *et al*., 2011). Pepper fruits segments were incubated at 25 °C for 1 h, in the dark, with 10 mM *N*-ethyl-maleimide (NEM) prepared in ethanol, and then washed three times, 15 min each, in 10 mM Tris-HCl buffer, pH 7·4. The pieces were then incubated with 10 μM Alexa Fluor 488 Hg-link phenylmercury (Molecular Probes, Eugene, OR, USA) for 1 h at 25 °C in the dark. After washing three times in the same buffer, pepper segments were embedded in a mixture of 15 % acrylamide–bisacrylamide stock solution as described previously (Corpas *et al*., 2006; Chaki *et al*., 2011). Pieces, 80–100 mm thickness, as indicated by the vibratome scale, were cut under 10 mM phosphate-buffered saline (PBS). They were then soaked in glycerol/PBS (1 : 1, v/v), containing azide, and mounted in the same medium for examination with a CLSM system (Leica TCS SL; Leica Microsystems, Wetzlar, Germany). The pieces were analysed using standard filters for Alexa Fluor 488 green fluorescence (excitation, 495 nm; emission, 519 nm). For background staining, control sections were incubated with β-mercaptoethanol plus Alexa Fluor 488 and without NEM (Corpas *et al*., 2008).

RESULTS

Due to the role reported previously for NO in different physiological processes in plants, the involvement of this RNS was investigated in ripening of pepper fruits following a spectrofluorometric assay using DAF-2 as fluorochrome. A considerable decrease in red fruits was observed with respect to green fruits (Fig. 1A). Furthermore, the profile of RNS-triggered effects at maturation of pepper fruits was also determined. Thus, levels of both nitrosothiols (SNOs) and nitrated proteins were studied in pepper fruits at different ripening stages by CLSM and immunoblot using an antibody against nitro-Tyr, respectively. As indicated in Fig. 1B, high levels of SNOs (green fluorescence colour) were detected in the endocarp of red fruits, whereas negligible labelling was found in green peppers. Additionally, although little differences in the respective total protein patterns were found in green fruits versus red fruits (Fig. 2A), a notable increase in the number and amounts of nitrated proteins was observed in red peppers compared with green peppers (Fig. 2B). Four polypeptide bands visibly changed in fruits at ripening: three of them, of 97, 50 and 32 kDa, were detected only in red fruits, whereas a 34-kDa polypeptide band was more abundant in green fruits (Fig. 2A). Western blotting analysis of nitrated proteins showed up to four polypeptide bands whose amounts were clearly enhanced in red fruits compared with green fruits, with molecular masses of 79, 63, 44 and 25 kDa. Additionally, four bands of 89, 50, 32 and 28 kDa were observed only in red fruits. Three polypeptide bands, with sizes of 70, 72 and above 90 kDa, were detected only in green fruits, as shown in Fig. 2B, which shows representative lanes of the respective treatments taken from several assays.

Given these data on RNS metabolism in pepper fruits during ripening, the effect of the application of exogenous NO gas on this physiological process was investigated. Fruits at early breaking point stage showed negligible visual symptoms of ripening (Fig. 3, upper panels). The pepper variety used in these experiments commonly ripen fully after 5–6 d at room temperature, so the shift from green to red colours is easily followed after that time. Two sets of fruits were either subjected or not subjected to NO gas treatment (see Materials and methods). As observed in Fig. 3, the NO treatment clearly delayed fruits turning red after 3 d as compared with untreated fruits. Fruits not treated with NO followed their natural ripening process with red bright colour observed at the end of the experiments.

Fruits at BPP stage treated and not treated with NO (1 h with/without NO plus 3 d at room temperature, as indicated in Materials and methods) gas were then processed and their respective nitroprotein profiles were analysed and compared with those of green and red peppers. Fruits at BPP stage showed higher levels of labelling for nitrated proteins than green fruits (Fig. 2B). BPP fruits also displayed a banding pattern close to that observed in red fruits. Interestingly, when BPP fruits were subjected to NO, a decrease in nitrated protein labelling was seen with the Western blotting analysis (Fig. 2B).

Accordingly, the nitroproteome of both green and red pepper fruits was investigated by 2DE and further MALDI-TOF/TOF analysis. Each kind of fruit, either green or red, was subjected to double 2-D runs: one of the gels was stained for total proteins, and the other one was processed for Western blotting to detect nitroproteins. Comparisons of both protein-stained gels and nitrated proteins detected in PVDF membranes using the PDQuest software (Bio-Rad) revealed matching spots, which were then analysed by MS. Whereas in green fruits 15 polypeptides that recognized the antibody against NO_2-Tyr were found (Fig. 4A, B, with matched spots being numbered 1–15), 14 were found in red fruits (Fig. 4C, D, matched spots numbered

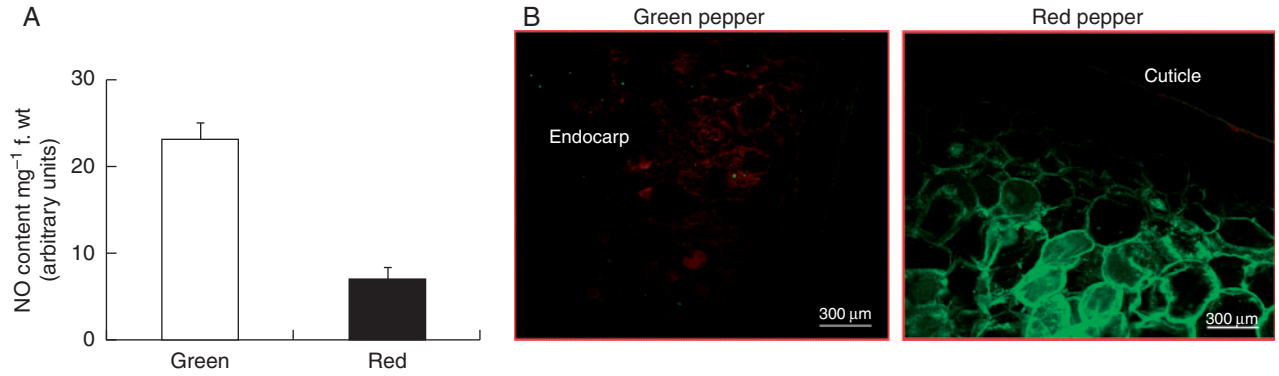

FIG. 1. NO content and detection of *S*-nitrosothiols (SNOs) in green and red pepper fruits. (A) NO was determined by spectrofluorometry in the presence of DAF-2. Values (means ± s.e.m. of three independent experiments) are expressed as arbitrary units for green and red fruit. (B) Cross-section of fruits are shown where the bright green colour corresponds to the detection of SNOs by CLSM, and the red color corresponds to autofluorescence. Representative pictures of three independent experiments are shown.

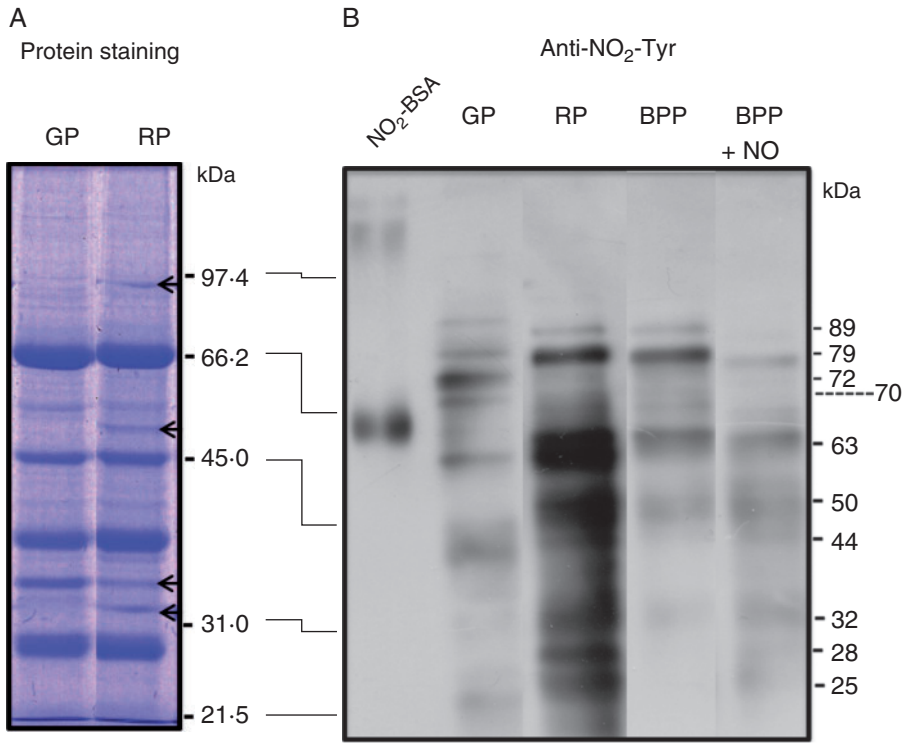

FIG. 2. Detection of nitrated proteins in green and red pepper fruits and after treatment with exogenous NO gas. (A) Proteins were separated in 12 % polyacrylamide gels and stained with Coomassie blue. Polypeptides that were differentially expressed in green and red fruits are indicated by arrows. (B) Proteins were separated in 10 % polyacrylamide gels. Then, nitrated proteins were detected by Western blotting using an antibody against NO_2-Tyr and NO_2-BSA as positive control. Values of molecular weight markers are indicated between the two panels. Values shown on the right of B correspond to estimated sizes of major bands detected in green and red fruits. To study the effect of NO on the pattern of nitrated proteins, breaking point pepper fruits were either untreated (BPP) or treated with 5 p.p.m. NO (BPP + NO) as indicated in the Materials and methods. GP, green fruits; RP, red fruits; M, molecular weight markers.

1–14). Nevertheless, similar to what was obtained in 1-D Western blotting (Fig. 2B), greater labelling was also observed in red fruits after 2DE and immunoblot analysis. After matching images of both protein-stained gels with Sypro Ruby and Western blots with anti-NO_2-Tyr, spots from green and red fruits, respectively, were subjected to MALDI-TOF/TOF analysis (Fig. 4). Nine potentially nitrated proteins were clearly identified in both green and red pepper fruits, although four of them corresponded to catalase variants. As indicated in Table 1, an NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, a transketolase 1, a 20S proteasome alpha 6 subunit (spots 9, 14 and 15, respectively in Fig. 4A, B) and catalase (spots 10–13, Fig. 4A, B) were identified only from green fruits, whereas a putative ferredoxin-dependent glutamate synthase 1

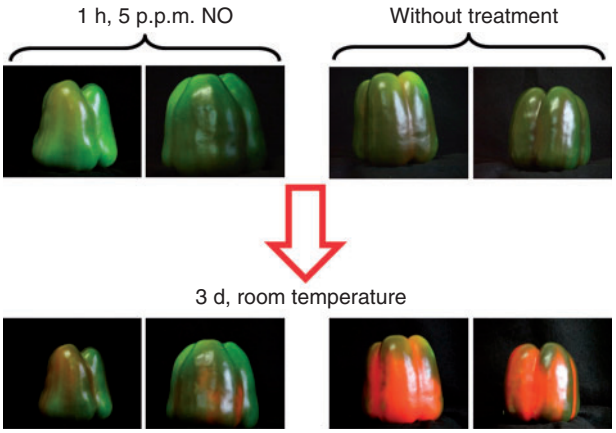

FIG. 3. Effect of exogenous NO gas on the ripening of pepper fruits. NO (5 p.p.m.) was either applied or not applied to fruits at an early breaking point stage for 1 h. Then, peppers were incubated at room temperature for 3 d.

and a putative carrier protein, similar to that associated with Graves disease, a human autoimmune disorder (spots 1, 2 and 14, respectively in Fig. 4C, D), were detected in red fruits exclusively. The only identified protein shared by both types of fruits was catalase, which in red fruits corresponds to spots 9–12 in Fig. 4C, D).

Given the importance of the redundant presence of catalase as a potential nitrated protein in both green and red fruits, assays on nitration and its influence in the activity of this enzyme were performed in pepper crude extracts. Catalase activity, as demonstrated previously (Mateos *et al.*, 2003), was much higher in green than in red pepper fruits (Fig. 5), and this was coincident with the lower amount of this enzyme in the red fruits, as shown by immunoblot analysis carried out with an antibody against catalase (Fig. 5, top). In the presence of SIN-1, catalase activity was much lower in both red and green fruits, and this was notable in green pepper, where the values decreased about three-fold (Fig. 5, bottom).

DISCUSSION

A growing body of information has recently contributed to better understanding of fruit ripening, mainly in those species destined for human consumption. Thus, research from nutritional, physiological and genetic point of views has gained particular attention with overlapping demands from different sectors such as agriculture, economics and even politics. Pepper is a dominant agricultural species worldwide as it is consumed in practically all countries under different forms. The study of many aspects of pepper culture, including breeding, pathology, ripening control, post-harvest strategies and fruit quality with added value, is accordingly growing, although information remains scarce. In the present study, the potential role of the radical gas NO and its subsidiary compounds, namely RNS, was investigated during ripening of pepper fruits to gain knowledge that may have use for crop strategies in the future.

At present, there is a consensus within the plant biology community that NO is a key signal molecule involved in the defence response to both biotic and abiotic stress (Delledonne *et al.*, 2001; Neill *et al.*, 2008; Airaki *et al.*, 2012), and its role as a regulator of growth, development, immunity and environmental interactions has been also considered (Yu *et al.*, 2014). Thus, NO participates in processes such as germination (Beligni *et al.*, 2002), flower setting and flower development (He *et al.*, 2004; Lee *et al.*, 2008; Kwon *et al.*, 2012), growth and development of roots (Corpas *et al.*, 2007; Fernández-Marcos *et al.*, 2011; Kwon *et al.*, 2012; Yu *et al.*, 2014), and senescence (Corpas *et al.*, 2004, 2006; Prochazkova and Wilhelmova, 2011; Begara-Morales *et al.*, 2013; Khan *et al.*, 2014). In this work, the delaying effect of NO on the ripening of the non-climateric pepper fruits is reported. It is known that NO affects ethylene biosynthesis, and the antagonism between both gases has been described (Prochazkova and Wilhelmova, 2011). NO influences ethylene production in *Arabidopsis thaliana* through nitrosylation of methionine adenosyltransferase, an enzyme involved in the synthesis of the ethylene precursor *S*-adenosymethionine (Lindermayr *et al.*, 2006). Early reports showed that NO added exogenously in liquid preparation (either sodium nitroprusside, NONO-ate or GSNO), but not as a gas, delayed flower and leaf senescence as well as fruit ripening (Leshem and Haramaty, 1996; Leshem, 2000; Leshem and Pinchasov, 2000). In rice (*Oryza sativa*) leaves, the application of exogenous NO counteracts abscisic acid-induced senescence (Hung and Kao, 2003) and it can retard programmed cell death in barley (*Hordeum vulgare*) aleurone layers (Beligni *et al.*, 2002). Furthermore, it has been found that the use of (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl) amino] diazen-1-ium-1, 2-diolate (DETANO), a compound that releases NO, can prolong the lifespan of certain vegetables (Wills *et al.*, 2007).

After ripening there is a considerable reduction in the endogenous NO content in pepper fruits, so it might be assumed that this radical is somehow scavenged or changed to other RNS such as by the formation of nitrosothiols through a nitrosylation process or the generation of nitroproteins by nitration events. Our results indicate that both pathways occur in pepper fruits. Nitrosothiol metabolism, as indicated herein, needs deeper investigation and, in fact, is now under study in our laboratory. Consequently, the present work concerns the nitration taking place in pepper fruits and its regulation during ripening. Protein nitration as a potential nitrosative stress marker has recently been proposed (Corpas *et al.*, 2007, 2014), but its role in the development and senescence processes in higher plants has been little reported (Chaki *et al.*, 2009*b*; Lozano-Juste *et al.*, 2011; Begara-Morales *et al.*, 2013). Nevertheless, information on protein nitration as a modulating process in the ripening of fruits is virtually absent.

Fruit ripening is a genetically controlled process commonly associated with plant senescence, in which an increase in generation of reactive oxygen species (ROS) usually takes place. In fact, mature pepper fruits display higher lipid peroxidation levels than green fruits (Martí *et al.*, 2011). However, in this work, we report the association of ripening of pepper fruits with an enhancement of nitrated proteins. After exogenously adding NO to BPP fruits one might expect a parallel increase in the nitration process. But this event did not occur. In fact, the addition of NO reversed the physiological process, so immature fruit stages appear to be associated with higher NO contents, and once ripening symptoms appear NO is driven to the formation of both nitrosothiols and nitroproteins. A proposed model

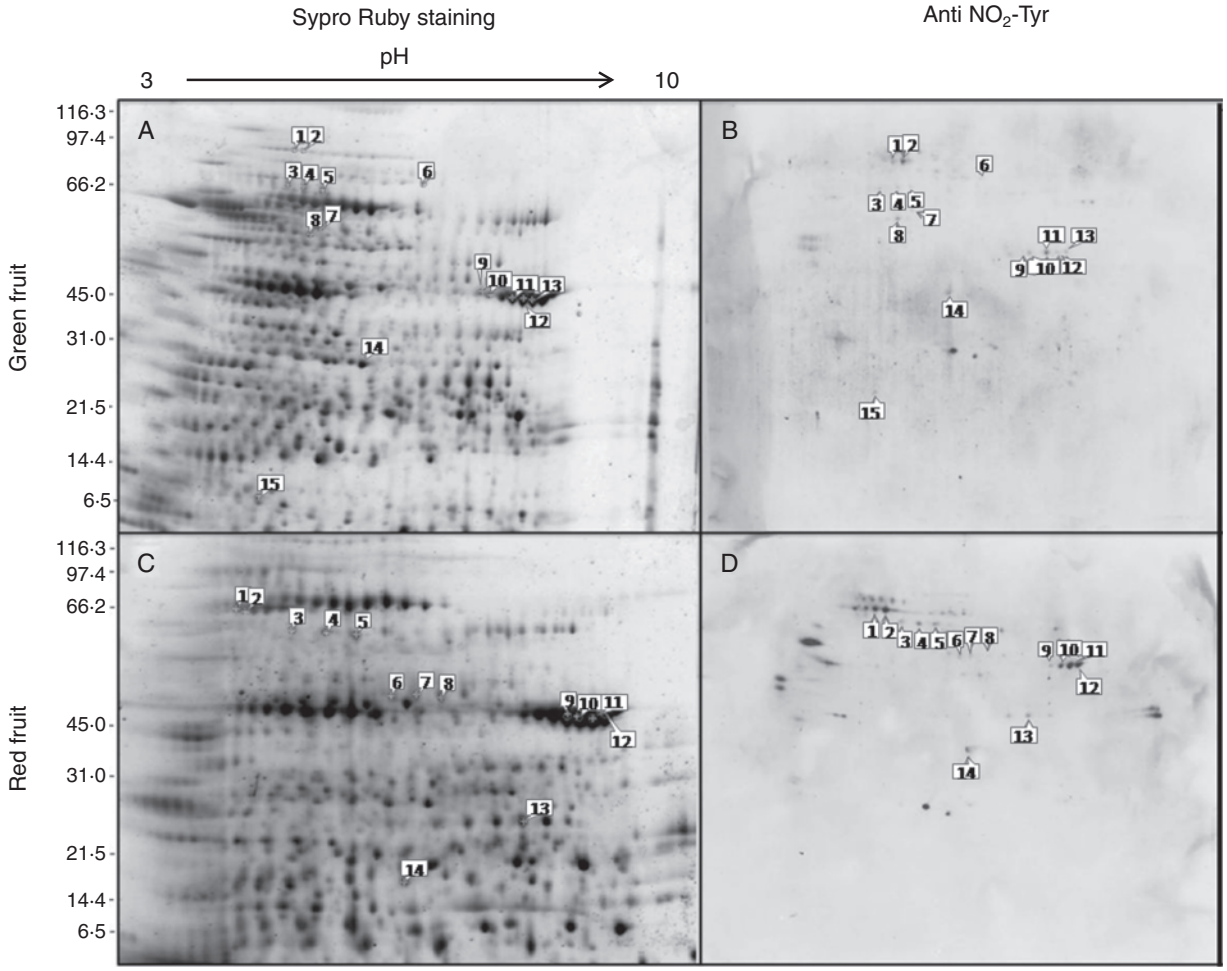

FIG. 4. 2DE of proteins from pepper fruits. Two replicates for each sample were done. In one of them, the gel was stained with Sypro Ruby for total protein detection. The second gel was used for immunoblot analysis. Polypeptides were transferred to PVDF membranes and nitroproteins were detected using an antibody against NO_2-Tyr. Spots from the protein-stained gels that matched with those showing immunoblot labelling (A and B for green fruits, and C and D for red fruits) were used for further proteomic analysis. Fifteen and 14 spots from green and red fruits, respectively (indicated by arrows), were selected for MALDI-TOF/TOF analysis. Molecular weight markers are indicated on the left.

TABLE 1. *Identification of proteins from green and red pepper fruits through proteomic analysis combining 2DE and MALDI-TOF*

Identified protein	Type of pepper	Accession number/ UniProt	Plant species	Protein score CI %/ peptide count	MW/pI	Functional grouping
NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	Green	D7U1A1	*Vitis vinifera*	100/7	53 824·7/6·76	Redox metabolism
Transketolase 1	Green	O78327	*Capsicum annuum*	100/18	80 397·7/6·16	Carbohydrate metabolism
20S proteasome alpha 6 subunit	Green	Q8H1Y2	*Nicotiana benthamiana*	100/8	30 142·9/5·07	Protein metabolism
Catalase isoenzyme 1	Green	P49319	*Nicotiana tabacum*	100/14	57 301·4/6·6	Oxidative metabolism
Catalase isoenzyme 2	Green	P49316	*Nicotiana plumbaginifolia*	100/9	57 317·6/6·75	Oxidative metabolism
Catalase	Green, red	Q9M5L6	*Capsicum annuum*	100/8	56 957·4/7·31	Oxidative metabolism
Catalase	Red	P55311	*Solanum melongena*	100/26	57 097·5/6·86	Oxidative metabolism
Putative ferredoxin-dependent glutamate synthase 1	Red	B5LAU8	*Capsicum annuum*	99·9/17	179 205·1/6·11	Glutamate biosynthesis
Graves' disease carrier protein, putative	Red	B9RQR8	*Ricinus communis*	99·9/11	37 639·8/9·84	Transport across membrane (Redox metabolism)

Concentrated green and mature pepper (*Capsicum annuum* L.) extracts were subjected to 2DE and inmunoblot probed with an antibody against 3-nitrotyrosine. The identified spots were analysed by MALDI-TOF MS after trypsin digestion. The MASCOT search engine was used to parse MS data to identify proteins from primary sequence databases. The closer the protein score confidence interval (CI) to 100 % indicates a strong likelihood that the protein is correctly matched. Peptide count, number of identified peptides; MW, molecular weight; pI, isoelectric point.

for the relationship among RNS and ripening of pepper fruits is depicted in Fig. 6. Pepper fruits usually ripen within 3–8 d depending on the variety. Once they overtake the BPP stage, ripening proceeds following one direction irreversibly. Administration of NO gas delays ripening of fruits (V. Codesido, C. Ruiz, F. J. Corpas and J. M. Palma, unpubl. res.; see also Fig. 3) and after this early NO effect is over (NO is a radical with high reactivity) the nitration of proteins proceeds. It has recently been reported that treatment with NO enhances chilling tolerance through improvement to the antioxidative enzymatic battery of banana fruits (Wu *et al.*, 2014) and cabbage seedlings (Fan *et al.*, 2014). Similarly, the exogenous addition of NO ameliorated the oxidative stress provoked by salinity in tomato plants (Manai *et al.*, 2014). A detailed study of the potential effect of NO treatment on the antioxidative systems of pepper fruits is currently underway in our laboratory.

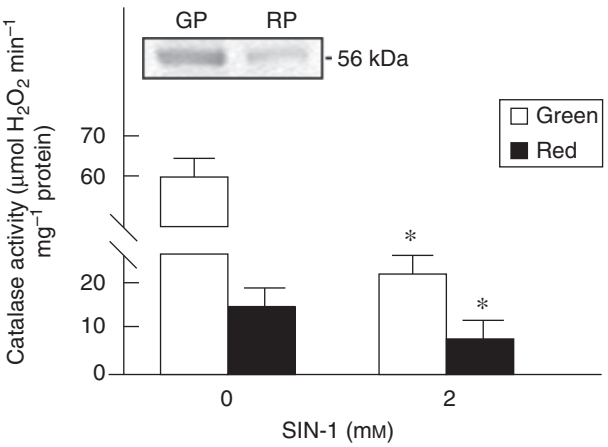

FIG. 5. Effect of SIN-1 on catalase activity from pepper fruit. Crude extracts from green and red fruits were incubated in the absence/presence of 2 mM SIN-1, and catalase activity was determined. Western blotting of green (GP) and red (RP) fruits using an antibody against a pepper catalase internal consensed sequence is shown. *Significant difference between SIN-1 treatment ($P < 0{\cdot}05$).

Only a few nitrated proteins were eligible to be characterized by proteomic analysis (four in green fruits and three in red fruits), the most abundant being involved in redox, proteins, carbohydrates, glutamate and oxidative metabolism. Detection handicaps may be responsible for this paucity, although the still scarce information on the sweet pepper genome with which to match our results cannot be ruled out. The NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9; also known under four other alternative names) found in green fruits is an enzyme belonging to the aldehyde dehydrogenase family with an important function as a means of generating NADPH for biosynthetic reactions (http://www.uniprot.org/uniprot/Q1WIQ6, and references therein). In pepper fruits this enzyme may also play a crucial role as NADPH and other dehydrogenases have been demonstrated to be involved in the ripening process (Mateos *et al.*, 2009). This enzyme has been also reported to be nitrated in *A. thaliana* seedlings (Lozano-Juste *et al.*, 2011). Transketolase (EC 2.2.1.1) catalyses two opposite reactions in the Calvin cycle and in the oxidative pentose phosphate pathway, but it also produces erythrose-4-phosphate, which is a precursor for the shikimate pathway leading to phenylpropanoid metabolism (Flechner *et al.*, 1996; Henkes *et al.*, 2001). Phenylpropanoids are an important class of secondary metabolites that participate in the plant cell structure, defence and signalling mechanisms (Dixon and Paiva, 1995). They derive from aromatic amino acids, which are synthesized via the shikimate pathway in plastids (Henkes *et al.*, 2001). Given the role of transketolase in photosynthesis, our results indicate that this might be a regulation point in the transition from chloroplasts to chromoplasts with loss of the photosynthetic apparatus. Transketolase has also been reported to be

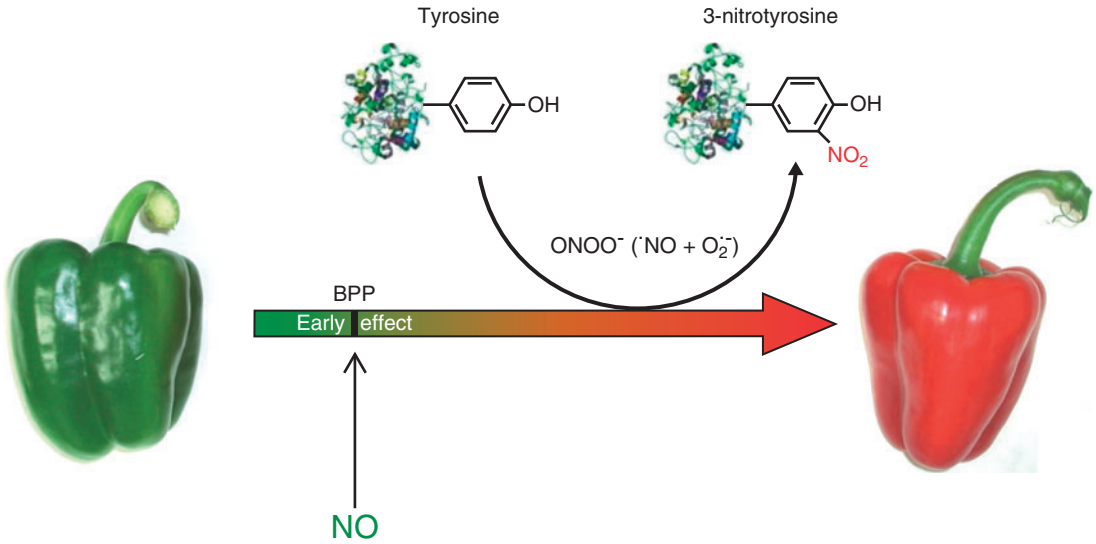

FIG. 6. Proposed model for the involvement of reactive nitrogen species (RNS) on the ripening of pepper fruits. Full ripening lasts about 3–8 d depending on the variety (5–6 d in the variety used in this work) once fruits overtake the breaking point stage (BPP). As an early effect, exogenously added NO gas delays fruit ripening, but it does not promote protein nitration. Once the early effect of NO is over, fruit ripening proceeds and the conversion of certain protein tyrosines takes place generating 3-nitrotyrosine in a process which involves peroxynitrite through the reaction of NO and superoxide radicals. This nitration reaction is a simplification of the one reported elsewhere (Corpas *et al.*, 2013).

potentially nitrated in *A. thaliana* seedlings (Lozano-Juste *et al*., 2011).

Another polypeptide whose nitration has been detected in green fruits is the 20S proteasome alpha 6 subunit. Plant cells harbour a mix of both 26S and 20S proteasomes that mediate proteolysis ubiquitin-dependently and ubiquitin-independently, respectively. The 26S proteasome includes the 20S proteasome plus one or two regulatory particles required for ubiquitin-dependent degradation. Loss of 26S proteasome function leads to increased 20S proteasome biogenesis, and this enhances the cellular capacity to degrade oxidized proteins, thus increasing oxidative stress tolerance. These findings indicated that 26S and 20S proteasome activities are regulated to control plant development and stress responses (Kurepa *et al*., 2009). Nitration of the 20S proteasome occurring in green pepper fruits highlights the role of this system in the ripening of pepper fruits and suggests that proteolysis, which commonly takes place during this process (Palma *et al*., 2011*b*), does not involve ubiquitin labelling of oxidized proteins but a direct action on them.

Recently, it was shown that protein oxidation proceeds as pepper fruits ripen (Martí *et al*., 2011). The ferredoxin-dependent glutamate synthase 1 (EC 1.4.7.1; also named Fd-GOGAT; http://www.uniprot.org/uniprot/Q9ZNZ7) found in red fruits is a chloroplastic enzyme responsible for glutamate biosynthesis in leaves and required for the re-assimilation of ammonium ions generated during photorespiration, as well as for primary nitrogen assimilation (Coschigano *et al*., 1998; Jamai *et al*., 2009; Potel *et al*., 2009). The photorespiratory pathway occurs in three organelles, namely chloroplasts, peroxisomes and mitochondria. Although photorespiration is commonly linked to green tissues in plants, this metabolic route was also functional in red pepper fruits, as was demonstrated following investigation in purified peroxisomes of the profile of the photorespiratory enzymes glycollate oxidase and hydroxypiruvate reductase (Mateos *et al*., 2003). Therefore, the results shown here provide evidence for modulation of the provision of metabolites from the photorespiration in ripe pepper fruits at the level of Fe-GOGAT. The Graves disease carrier protein is an inner mitochondrial membrane-bound protein that belongs to the metabolite carrier family (includes an ADP/ATP translocator, a phosphate carrier and a hydrogen ion uncoupling protein) and is required for the accumulation of coenzyme A in the mitochondrial matrix (Prohl *et al*., 2001). This protein is recognized by IgG from patients with active Graves disease, a human autoimmune disorder, but its homologue in plant tissues has not yet been reported.

Catalase appears to be the most abundant nitrated protein. Catalase is one of the main peroxisomal proteins involved in the detoxification of hydrogen peroxide in eukaryotic cells under normal conditions, but it also behaves as an antioxidant in many situations where oxidative stress operates (Palma *et al*., 2013). In early reports on the profile of catalase from peroxisomes isolated from both green and red pepper fruits, a lower activity was found in red mature fruits (Mateos *et al*., 2003). The results reported here provide evidence that this reduction is due not only to a reduced protein content of catalase in red fruits but also to the potential nitration occurring during ripening in this species. The decrease of catalase in red fruits implies a lower capacity to scavenge H_2O_2, thus promoting the lipid peroxidation already reported in ripe pepper fruits (Martí *et al*., 2011).

This is the first report of RNS metabolism in fruits from a higher plant during the ripening process. Important cues are proposed here to establish a clear relationship between ripening and protein nitration. However, additional research is necessary to understand the exact stoichiometry and, overall, to identify the partners that drive the shift from green to red fruits in pepper plants.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: images of pepper fruit at the different ripening stages. Figure S2: illustration of the experimental system used to incubate fruit with NO gas.

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