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NADP-malic enzyme and glutathione reductase contribute to glutathione regeneration in *Fragaria vesca* fruit treated with protective high CO_2 concentrations

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ABSTRACT

Treatment of harvested strawberries (*Fragaria vesca* L. Mara des Bois) with high concentrations of CO_2 is an effective means of limiting fungal decay and avoiding disorders caused by low temperature storage. In the present study, we investigated the role of NADP-ME gene expression and activity in lowering malic acid contents and in the provision of NADPH required for the regeneration of the reduced form of glutathione (GSH). We also measured glutathione reductase (GR: EC 1.6.4.2) activity in strawberries treated with different high CO_2 concentrations (0, 20 and 40% for 3 days) during storage at 0 °C. A decrease in malic acid content in fruit exposed to 20% CO_2 was primarily mediated by the stimulation of NADP-ME activity, rather than associated with changes in the expression of cytosolic NADP-ME genes. Moreover, malic acid decarboxylation was associated with a marked increase in GR activity, which may account for the increased levels of glutathione in fruit following exposure to 20% CO_2. This chain of events was not observed in untreated fruit stored in air or in fruit treated with 40% CO_2, suggesting that the unique cellular redox status of 20% CO_2-treated fruit plays an important role in detoxification and protection from damage during storage. Based on these findings, we propose that NADP-ME activation in fruit exposed to 20% CO_2 provides NADPH for glutathione regeneration by GR, thereby conferring protection against the cellular damage caused by low temperatures or excessive high CO_2 levels.

1. Introduction

Brief exposure to high concentrations of CO_2 after harvest has long been recognized as an effective means of restricting fungal decay and extending the postharvest life of strawberries (Ke et al., 1991; Harker et al., 2000; Blanch et al., 2012a). These beneficial effects are produced by CO_2 concentrations of 20% and damage can occur if this concentration threshold is exceeded. Thus, it is important to identify the metabolites and metabolic pathways in strawberries that mediate the protective effects of high CO_2 concentrations. Changes in the levels of specific organic acids such as malic and succinic acid in CO_2-treated fruit and vegetables have been well documented (Ke et al., 1993; Yang et al., 1998; Fernández-Trujillo et al., 2001; Maldonado et al., 2004; Blanch et al., 2012b). In the case of malic acid, these changes may be mediated by its oxidative decarboxylation to yield pyruvate, CO_2 and $NADPH+H^+$, which is catalyzed by NADP-ME or L-malate: NADP oxidoreductase (decarboxylating: EC 1.1.1.40). However, the effects of high CO_2 levels on the activity and transcriptional regulation of NADP-ME are unclear (Ponce-Valadez and Watkins, 2008). NADP-ME is a widely distributed enzyme, and apart from some specialized roles in photosynthetic and non-photosynthetic plant tissues (Edwards and Andreo, 1992), the housekeeping functions of NADP-ME in fruit have not been fully characterized. NADP-ME is known to function as a pH-stat (Davies and Patil, 1974) and it may also play a role in defense by providing NADPH for the biosynthesis of defensive compounds such as lignin and flavonoids (Martinoia and Rentsch, 1994; Casati et al., 1999; Drincovich et al., 2001).

In the present study, we investigated the protective role of NADP-ME in the regeneration of glutathione (GSH). NADPH generated by the catalytic activity of NADP-ME is essential for the regeneration of glutathione by the enzyme glutathione reductase (GR: EC 1.6.4.2), a ubiquitous NADPH-dependent enzyme that catalyzes the reduction of oxidized glutathione (GSSG). The reduced form of glutathione, the main non-protein thiol in most plant species, plays an important role in maintaining cell redox status (Rennenberg, 1980). Moreover, glutathione and other endogenous

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protective enzymatic and non-enzymatic compounds effectively neutralize, remove or scavenge the reactive oxygen species (ROS) generated during normal metabolic activity or after exposure to different kinds of stress. In addition to acting as a scavenger of peroxides, GSH also fulfills an important role in enzyme stabilization (Hodges, 2003). GSH is also one of the major forms of reduced sulfur (Bourgis et al., 1999), and it serves as a defense compound against biotic and abiotic stresses (Noctor and Foyer, 1998; Parisy et al., 2006), as well as a regulator of stress-related gene expression (Ball et al., 2004). Given the importance of GSH, its regeneration by GR may be crucial in protecting labile macromolecules and cell structures against low-temperature-induced damage. Furthermore, as GR requires NADPH as an electron donor, its protective effects depend on the availability of NADPH, in part generated by NADP-ME activity.

We have investigated the effects of different CO_2 concentrations (0%, 20% and 40%) on the activity and gene expression of NADP-ME in strawberries (*Fragaria vesca* L.) stored at 0 °C. In addition, we examined the role of NADP-ME in the lowering of malic acid contents and generation of reducing equivalents necessary for the regeneration of GSH by the NADP-dependent enzyme GR. Our findings demonstrate that these pathways are activated by 20% CO_2 conferring a different cellular redox status that could be essential in the detoxification and protection from damage induced during postharvest storage.

2. Materials and methods

2.1. Plant material

Ripe, full size organic strawberries (*F. vesca* L. cv. Mara des Bois), with a total soluble solid/titratable acidity ratio of 12, were harvested from the Monjarama orchard in San Sebastian de los Reyes (Madrid). Two hours after harvest, fruit of uniform size and color were stored at 0 °C (±0.5) and >95% RH in three sealed containers with a capacity of 1 m^3. Fifteen plastic boxes each containing approximately 0.5 kg of fruit were stored in each container and exposed to either a continuous flow (100 mL/min) of air (untreated) or a gaseous mixture containing 20% CO_2 + 20% O_2 + 60% N_2, or 40% CO_2 + 20% O_2 + 40% N_2 for three days. Carbon dioxide and oxygen concentrations were measured using a gas analyzer (PBI Dansensor mod, Checkmate 9900). After harvest (pre-stored fruit) and from each treatment group (untreated, 20% and 40% CO_2-treated fruit), 45 strawberries were selected for quality analysis, and another 45 were removed at random from each group and divided into three batches of 15 strawberries. The 15 strawberries from each batch, which served as a biological replicate, were mixed, frozen in liquid nitrogen and stored at −80 °C for further analysis. At least two different measurements were taken from each of the three biological replicates.

2.2. Relative gene expression of NADP-ME by quantitative RT-PCR

The relative expression of two *F. vesca* genes, encoding NADP-dependent malic enzyme-like (GeneBank accession no. XM_004287420 and XM_004291116), was assayed by quantitative RT-PCR (RT-qPCR) using whole strawberries from untreated and CO_2-treated (20% or 40% CO_2) groups. Total RNA was extracted from each biological replicate as described previously (Yu et al., 2012), and the purity and quality of total RNA was evaluated by agarose gel electrophoresis and spectrophotometry (NanoDrop 2000, Thermo Scientific). The total RNA was treated with DNase I recombinant RNase-free (Roche) to remove any genomic DNA and then cDNA was synthesized from 1 μg of each sample using the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad). Amplifications were carried out in a 96 well-plate iCycler iQ thermal cycler (Bio-Rad) and quantified using iCycler iQTM-associated software (Real Time Detection System Software, version 2.0). Each gene was evaluated in at least two independent runs. The primer pairs used in the RT-qPCR for XM_004287420 were FraME1-QFw: 5′-GTGGGGAAATTGGCTTTGTA-3′ and FraME1-QRv: 5′-GCGTGAGCCCAATGTAGAAT-3′; and for XM_004291116 were FraME2-QFw: 5′-CCATCAGGGAACTTGTCGAT-3′ and FraME2-QRv: 5′-CATGGCCTCAACCACATCCT-3′. To calculate the efficiency of the reaction (optimal range 90–110%) and to establish the most suitable template concentration, cDNAs synthesized from serial dilutions of total RNA were amplified (from 40 ng to 2.5 ng). Standard curves and linear equations were determined by plotting cycle threshold (*Ct*) values (*y*-axis) against logs of the total RNA (*x*-axis). The efficiency of each individual run was calculated based on the raw fluorescence data (ΔR_n), exported as an output file and subsequently imported into the LinReg PCR program. The specificity of the products was validated by dissociation curve analysis and agarose gel electrophoresis, and their sequences were confirmed at the Genomics Department of the CIB-CSIC. Transcript levels were normalized to those of the ubiquitin gene from *F. vesca* (*FUBI1*; GeneBank accession no. XM_004293212), which was amplified using the oligonucleotides FaUbq-F: 5′-CAGACCAGCAGAGGCTTATCTT-3′ and FaUbq-R: 5′-TTCTGGATATTGTAGTCTGCTAGGG-3′, as described previously (Mouhu et al., 2009). The results were calculated relative to a calibrator sample (pre-stored sample) using the $2^{-\Delta\Delta Ct}$ method.

2.3. Measurement of NADP-ME (EC 1.1.1.40) and glutathione reductase (GR: EC 1.6.4.2) activity

Strawberry tissue (1 g) was homogenized in 1 mL of 0.1 M Tris–HCl buffer (pH 8.0) containing 0.1% Triton X-100, 2 mM dithiothreitol (DTT), 0.8 mM phenylmethanesulphonyl fluoride (PMSF) and 0.02 g polyvinylpolypyrrolidone. The homogenate was centrifuged at 27,000 × *g* for 30 min (Ponce-Valadez and Watkins, 2008) and the supernatant was used directly for the NADP-ME and GR assays.

NADP-ME activity was measured by monitoring NAPDH production at 340 nm using a Synergy Mx (BioTek®) plate reader. The reaction mixture contained 112 mM Tris–HCl (pH 8.0), 5 mM $MnSO_4$, 10 mM L-malate, 0.6 mM NADP and 50 μL of enzyme extract. All assays were carried out at 30 °C in a final volume of 220 μL. One unit of enzyme activity (*U*) was defined as the amount that catalyzes the production of 1 μmol of NADPH per minute. The reaction was initiated by the addition of 50 μL of enzyme extract and the rate of oxidation was calculated using the extinction coefficient of NADPH (6.22 $\mu mol^{-1}\ mL\ cm^{-1}$). The increase in absorbance (*A*) was recorded at 340 nm for 10 min. NADP-ME activity was expressed as μmol NADPH per mg protein per min, and all the activity values presented represent the means of at least three replicates.

Determination of GR activity was carried out by measuring the decrease in absorbance due to NADPH oxidation during the reduction of oxidized glutathione (GSSG) (Rodríguez-Ramiro et al., 2011). Enzyme activity was measured in a mixture of 50 mM potassium phosphate buffer (pH 7.0), 50 mM GSSG and 9.6 mM NADPH. The decrease in A was recorded at 340 nm for 10 min using a Beckman DU-640 spectrophotometer. One unit of GR activity was calculated as 1 μmoL of NADPH consumed per minute under standard conditions. Glutathione reductase activity was expressed as nmol GR oxidized per milligram of protein per min. Protein concentration was determined by the Bradford method (1976) using BSA as a standard.