Redox Biology 12 (2017) 171–181

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research paper

Characterization of the galactono-1,4-lactone dehydrogenase from pepper fruits and its modulation in the ascorbate biosynthesis. Role of nitric oxide☆

Marta Rodríguez-Ruiz[a,1], Rosa M. Mateos[b,1], Verónica Codesido[c], Francisco J. Corpas[a], José M. Palma[a,*]

[a] Group of Antioxidants, Free Radicals and Nitric Oxide in Biotechnology, Food and Agriculture, Dept. Biochemistry, Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, CSIC, C/ Profesor Albareda, 1, 18008 Granada, Spain
[b] University Hospital Puerta del Mar, Avenida Ana de Viya, 21, Cádiz 11009, Spain
[c] Phytoplant Research S.L, Rabanales 21 - The Science and Technology Park of Córdoba, C/ Astrónoma Cecilia Payne, Edificio Centauro, módulo B-1, 14014 Córdoba, Spain

ARTICLE INFO

Keywords:
Ascorbate metabolism
Cloning
Galactono-1,4-lactone dehydrogenase
Nitric oxide
Pepper fruit ripening
Reactive nitrogen species

ABSTRACT

Pepper fruit is one of the highest vitamin C sources of plant origin for our diet. In plants, ascorbic acid is mainly synthesized through the L-galactose pathway, being the L-galactono-1,4-lactone dehydrogenase (GalLDH) the last step. Using pepper fruits, the full *GalLDH* gene was cloned and the protein molecular characterization accomplished. GalLDH protein sequence (586 residues) showed a 37 amino acids signal peptide at the N-terminus, characteristic of mitochondria. The hydrophobic analysis of the mature protein displayed one transmembrane helix comprising 20 amino acids at the N-terminus. By using a polyclonal antibody raised against a GalLDH internal sequence and immunoblotting analysis, a 56 kDa polypeptide cross-reacted with pepper fruit samples. Using leaves, flowers, stems and fruits, the expression of GalLDH by qRT-PCR and the enzyme activity were analyzed, and results indicate that GalLDH is a key player in the physiology of pepper plants, being possibly involved in the processes which undertake the transport of ascorbate among different organs.

We also report that an NO (nitric oxide)-enriched atmosphere enhanced ascorbate content in pepper fruits about 40% parallel to increased GalLDH gene expression and enzyme activity. This is the first report on the stimulating effect of NO treatment on the vitamin C concentration in plants. Accordingly, the modulation by NO of GalLDH was addressed. *In vitro* enzymatic assays of GalLDH were performed in the presence of SIN-1 (peroxynitrite donor) and *S*-nitrosoglutahione (NO donor). Combined results of *in vivo* NO treatment and *in vitro* assays showed that NO provoked the regulation of GalLDH at transcriptional and post-transcriptional levels, but not post-translational modifications through nitration or *S*-nitrosylation events promoted by reactive nitrogen species (RNS) took place. These results suggest that this modulation point of the ascorbate biosynthesis could be potentially used for biotechnological purposes to increase the vitamin C levels in pepper fruits.

1. Introduction

Ascorbic acid (vitamin C) is one of the most powerful antioxidants synthesized in the majority of living beings, excepting primates (including humans), guinea pigs, bats and some birds [32,55,74,79,96,97]. Within plant cells, this molecule is ubiquitous and can be detected in many subcellular loci, although it is also found in the apoplast ([46,47,79,80,88]). As an antioxidant, it can directly interact with hydroxyl radicals (·OH), superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and singlet oxygen [21,22,44,89]. Furthermore, ascorbate is an electronic donor for the ascorbate peroxidase (APX; EC. 1.11.1.11) activity to remove the hydrogen peroxide, either as an individual reaction but also as the first stage of the ascorbate-glutathione cycle in plants (Foyer-Halliwell-Asada path-

☆ Note: Sequence data from this article have been deposited in the EMBL/GenBank data libraries under accession number AY547352 and AY572427 for the *Capsicum annuum* L GalLDH complete cDNA and actin mRNA, partial cDNA, respectively.

* Corresponding author.
E-mail addresses: marta.rodriguez@eez.csic.es (M. Rodríguez-Ruiz), mateosrosa@hotmail.com (R.M. Mateos), v.codesido@phytoplant.es (V. Codesido), javier.corpas@eez.csic.es (F.J. Corpas), josemanuel.palma@eez.csic.es (J.M. Palma).
[1] These authors equally contributed to this work.

http://dx.doi.org/10.1016/j.redox.2017.02.009
Received 5 December 2016; Received in revised form 15 January 2017; Accepted 12 February 2017
Available online 20 February 2017
2213-2317/

way). This metabolic route, besides APX, involves the participation of the mondehydroascorbate reductase (MDAR; EC 1.6.5.4), the dehydroascorbate reductase (DAR; EC 1.8.5.1), and the glutathione reductase (GR; EC 1.8.1.7), in a process which needs the continuous provision of NADPH [27,35,39,53,8,80,91]. Moreover, ascorbate is implicated in the regeneration of α-tocopherol and in the xanthophylls cycle, and it serves as a co-factor for many enzymes [28,33,7].

At physiological level, it has been found that, in plants, ascorbic acid participates in the mechanisms of cell signaling playing a role in the responses to pathogens through a complex signal transduction network which implies the modulation of the content of the signal molecules salicylic acid, abscisic acid, ethylene and gibberellins [101,11,22]. Moreover, ascorbic acid has been reported to be linked to hormone synthesis, gene expression, cell division and growth, flowering, developmental senescence, programmed cell death or apoptosis, and stomata movement of guard cells, among others [19,38,64,79]. Overall, this multiplicity of functions makes ascorbate to be considered as a redox buffer in the plant cell [38,91]. Thus, in a very recent report, it has been postulated that ascorbate buffers the important metabolic changes which occur at ripening of pepper acting as a preservative to expand the shelf life of fruits [80].

The biosynthesis of ascorbic acid in plants has been the aim of many investigations, basically in species destined for human consume. Thus, although the main pathways involved in such event have been well established so far [100,103,23,52,64,90,97], transcription and expression studies were achieved during last years in order to understand the complex regulation of those routes [104,107,108,29,49,62,65].

Three pathways for the ascorbic acid biosynthesis were initially proposed in plants: one *via* L-galactose (the L-galactose pathway) [99]; another one from myo-inositol [51,66,67]; and a third one through L-galacturonic acid [2]. An alternative L-gulose pathway sharing some stages with that occurring in animal cells, and implying the involvement of an L-gulono-1,4-lactone oxidase as the last step of this metabolic channel, has been also hypothesized (see reviews in Wolucka et al., 2007 and [64]). Both the linear L-galactose pathway and the L-gulose pathway were lately associated to a VTC2 cycle (GDP-L-galactose phosphorylase, GGP; *VTC2* gene) which provides phosphorylated galactose and phosphorylated mannose, respectively, for the final synthesis of ascorbate ([100,103,57,63,64]).

Thus far, the most consensual route for ascorbate biosynthesis is the L-galactose pathway, with the final step requiring the oxidation of L-galactono-1,4-lactone (GalL) to ascorbic acid, in a reaction which is catalyzed by the L-galactono-1,4-lactone dehydrogensase (GalLDH; EC. 1.3.2.3). This reaction is not coupled to any coenzyme pair, so the electrons from the GalL are directly transferred to the cytochrome *c* located at the inner mitochondrial membrane [12,45,73,92,99]. cDNAs encoding *GalLDH* have been characterized from cauliflower, sweet potato, strawberry, tomato, tobacco, *Arabidopsis*, peach, and rosier, among others, and the theoretical topology and structure have been accordingly proposed ([105,48,49,77,94,6]).

GalLDH has been depicted to contain membrane-spanning regions, so this peculiarity and its electron-transferring role indicate that this protein should be associated to the intermembrane space. Thus, GalLDH has been reported as an integral protein of the mitochondrial inner membrane [12,48,87], but some other authors have suggested that GalLDH is a peripheral protein [61,84]. More recently, this protein was described to form part of the mitochondrial complex I in *Arabidopsis* [84], where it seems that, besides donating electrons to the transfer chain, it also functions as an assembly factor [85]. This step of the GalLDH seems to be an important regulatory point in the ascorbate biosynthetic pathway and in the accumulation of ascorbic acid, as it has been thoroughly reported ([82,93,94,6,109,60,65,98]). Recently it has been reported that pepper fruits is one of the richest vitamin C sources of plant origin in our diet, and an important role of ascorbate in the fruit physiology was proposed [80]. Thus, the investigation of the modulation of the ascorbate synthesis in plant crops with agronomic interest such as pepper is an issue which deserves our attention.

In the latest years, it has been postulated that, in plants, nitric oxide (NO) and its derivatives called reactive nitrogen species (RNS), including *S*-nitrosoglutathione (GSNO) and peroxynitrite ($ONOO^-$), among others, are involved in important and vital physiological issues such as the defence response to both biotic and abiotic, as a regulators of growth, development, immunity and environmental interactions, in processes such as germination, flower setting and flower development, growth and development of roots, senescence, and very recently, in delaying ripening of fruits [18,59]. In many of those cases, it has been reported that these roles of RNS are mediated through post-translational modifications (PTMs) of proteins which, then, modulate the processes indicated above by mechanisms of cellular signaling. Most of these RNS-mediated PTMs include events such nitrosylation of thiol and amine groups, and nitration of tyrosine and other amino acids [18]. To our knowledge, no data are available on the modulation of the ascorbate biosynthesis in crop species by NO and other RNS.

In this work, due to the relevance of ascorbate in the metabolism of pepper fruits [80], where the GalLDH might then play an important role in the physiology throughout the plant life cycle, the full characterization of the GalLDH from fruits was achieved. The full length cDNA clone of the GalLDH was accomplished and its molecular and immunological properties were studied. The enzyme activity and gene expression of GalLDH in leaves, flowers, stems and fruits were also determined. Besides, the modulation of the ascorbate metabolism in pepper fruits through the regulation of the GalLDH by distinct RNS was investigated, and the obtained results on that issue provide evidence which may explain the increased ascorbate levels during fruit ripening.

2. Materials and methods

2.1. Plant material and growth conditions

Pepper (*Capsicum annuum* L.) fruits, leaves, flowers and stems were obtained from plants grown in experimental glass-covered greenhouse (Syngenta Seeds, Ltd., El Ejido, Spain), with optimal nutrients supplementation applied on rockwood as substrate. Fresh fruits from the same plants at distinct ripening stages (immature green and mature red phenotypes) were used for this study. When treatment of pepper fruits with NO was carried out, experiments were performed according to [18]. Briefly, pepper fruits at a breaking-point stage were subjected to an NO-enriched atmosphere (5 ppm) in a hermetic box for 1 h. This condition was set by the use of a Nitric Oxide Meter (Environmental Sensors Co., Boca Raton, FL, USA). Afterwards, fruits were maintained under room temperature for 10 days and, finally, they were processed for diverse analyses such as determination of ascorbate content, and enzyme activity and gene expression of the L-galactono-1,4-lactone dehydrogenase. Fruits at breaking point were used to investigate the modulation of GalLDH during the ripening process and not those at steady stages, either green or red, as indicated above. Fruits harvested at immature green stage do not ripe, so they never shift into red colour, and mature red fruits do not ripe any longer but senesce after several weeks. Thus, breaking point fruits are the only material where a dynamic process such as ripening (colour shift takes 3–8 days in pepper depending on the cultivar) can be monitored. In our experimental conditions, fruits were subjected to NO treatment at the breaking point stage, while sampling for further assays (ascorbate and GalLDH) of both treated and untreated fruits was done when they had already ripened and were red (10 days after treatment; Supplementary Fig. 1). In previous studies, it was proved that NO delayed ripening of pepper fruits with untreated fruits ripening after 3 days whereas NO-treated ones did several days later [18].