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L-Ascorbate biosynthesis in strawberries: L-Galactono-1,4-lactone dehydrogenase expression during fruit development and ripening

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Abstract

L-Galactono-1,4-lactone dehydrogenase (GLDH; E.C. 1.3.2.3), the enzyme responsible for the last step of L-ascorbate biosynthesis in plants, was cloned from strawberries (*Fragaria* × *ananassa* Duch, cv. Campineiro) and its activity and expression followed during fruit development and ripening. Properties of strawberry GLDH were similar to those of other plants, what can be explained by the high identity at the amino acid level. Enzymatic and molecular analysis of fruits at different developmental stages indicated that the main changes in activity and expression occurred during the enlargement phase, when the L-ascorbate surpassed the L-dehydroascorbate content. The infiltration of the substrate L-galactono-1,4-lactone through the fruit petiole at the small green and turning stage resulted in a 50% increase in total L-ascorbate content, but no differences in enzyme activity and expression.
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1. Introduction

L-Ascorbate has a pivotal role in plant cells as an antioxidant molecule that prevents oxidative stress caused by photosynthesis, oxidative metabolism or exposure to pollutants. This compound is also an important enzyme cofactor and it is involved in cell division, expansion and elongation (Smirnoff, 1996; Arrigoni et al., 1997; Loewus, 1999). Besides its role in plant metabolism, L-ascorbate and its oxidation product L-dehydroascorbate act as Vitamin C, an essential nutrient for humans, mostly provided by fruits and vegetables.

The importance of L-ascorbate in plant biosynthetic pathways was not clearly understood until recently. Wheeler et al. (1998) proposed a biosynthetic pathway via GDP-D-mannose, GDP-L-galactose, L-

Abbreviations: Cyt C, cytochrome C; GalUR, D-galacturonic acid reductase; GL, galactono-1,4-lactone; GLDH, L-galactono-1,4-lactone dehydrogenase

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galactose and L-galactono-1,4-lactone, which was supported by biochemical and molecular genetic evidence (Conklin et al., 1999, Loewus, 1999). It was demonstrated that GDP-D-mannose-3,5-epimerase converts GDP-D-mannose into GDP-L-galactose, which in turn provides the L-galactose used as substrate by the enzyme L-galactose dehydrogenase to produce the L-ascorbate precursor L-galactono-1,4-lactone (Wheeler et al., 1998).

The last step in L-ascorbate biosynthesis is the reduction of L-galactono-1,4-lactone by the enzyme L-galactono-1,4-lactone dehydrogenase (GLDH, E.C. 1.3.2.3), that was first studied by Mapson and Breslow (1958). The conversion of L-galactono-1,4-lactone into L-ascorbate seems to be the most well characterized step in L-ascorbate biosynthesis in plants, and the studies on the GLDH revealed that the mature enzyme from several plants is a monomer around 56–57 kDa, which is highly specific for L-galactono-1,4-lactone and cyt C as substrates (Ôba et al., 1994, 1995; Imai et al., 1998). The enzyme is located at the inner mitochondrial membrane (Bartoli et al., 2000; Mutsuda et al., 1995; Siendones et al., 1999), as a component of the mitochondrial complex I (Millar et al., 2003), and it has been cloned from several plant species. Based on the comparison of the amino acid sequences deduced from the cDNA clones, GLDH seems to be a highly conserved protein.

As mentioned above, the main route for L-ascorbate biosynthesis in plants seems to involve the L-galactose production via GDP-D-mannose epimerization to GDP-L-galactose. However, other minor biosynthetic routes seem to be also operative in L-ascorbate biosynthesis, as demonstrated by the efficient conversion of methyl D-galacturonic acid into L-ascorbate by *Arabidopsis* cell suspension culture (Davey et al., 1999), and the cloning of a D-galacturonic acid reductase that allows the conversion of D-galacturonic acid in L-galactonic acid, a precursor of L-galactono-1,4-lactone (Agius et al., 2003). Regardless the metabolic route, the majority of plant L-ascorbate seems to be produced by the conversion of L-galactono-1,4-lactone through GLDH activity. In this way, GLDH plays an essential role in L-ascorbate synthesis and, as a consequence, its activity is essential to the metabolism of plant cell.

Evidence of the importance of GLDH for the regulation of L-ascorbate level in plants was provided by Ôba et al. (1994), who detected a stimulation of GLDH activity followed by an increase of L-ascorbate level in sliced potato tubers exposed to air, and also by Tabata et al. (2001), who obtained L-ascorbate-deficient tobacco cells by antisense GLDH. The suppression of GLDH activity in these transgenic cells affected cell division, growth and structure. According to Tabata et al. (2002) the expression of GLDH can be suppressed by increased amounts of exogenous L-ascorbate and it can be induced by light, suggesting a relationship between enzyme activity, L-ascorbate availability and photoxidation. Oxidative stress in fresh-cut potato also promoted a significant increase in GLDH activity (Tudela et al., 2003) and the authors observed that its increase was related to a change in the isoelectric point, suggesting post-translational modification or induction of a different isoenzyme under these experimental conditions, as another control over GLDH activity. Another recent contribution to the understanding of the regulatory role of GLDH was brought by Millar et al. (2003), who found that respiration can control L-ascorbate biosynthesis in *Arabidopsis* and stimulation in synthesis can result from enhanced activity of GLDH. In spite of the importance of GLDH for L-ascorbate metabolism in plants, the information about this enzyme in fruits is limited to a recent work by Pateraki et al. (2004). Fruits are interesting models of study since they undergo many physiological and chemical changes during development and ripening, and the ascorbate biosynthesis regulation studies are important because high levels of Vitamin C increase the nutritional value of these edible plant organs. This can be illustrated by the recent paper by Agius et al. (2003), showing an alternative route for L-ascorbate biosynthesis through D-galacturonic acid, that is argued to be operative during strawberry ripening. This work resulted not only in the identification of another possible route for L-ascorbate biosynthesis but also in the identification of an interesting point for the engineered increase of Vitamin C level in plants.

Although the differences in L-ascorbate content seem to be highly dependent on the cultivar (Cordenunsi et al., 2002) and can also be affected by the postharvest conditions (Cordenunsi et al., 2003), strawberry fruit is a good source of L-ascorbate and also a useful model to study L-ascorbate biosynthesis. In this way, we present the cloning of GLDH from strawberries, along with the changes in its activ-

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