DOI: 10.1007/s10535-015-0540-z BIOLOGIA PLANTARUM 59 (4): 783-787, 2015

BRIEF COMMUNICATION

Gene expression and enzyme activities of the D-mannose/L-galactose pathway influence L-ascorbic acid content in *Myrciaria dubia*

J.C. CASTRO[1]*, M. COBOS[2], J.D. MADDOX[3], S.A. IMÁN[4], A. EGOAVIL[1], J. TORRES[1], and F. GUTIERREZ[1]

Unidad Especializada de Biotecnología, Centro de Investigaciones de Recursos Naturales de la Amazonía, Universidad Nacional de la Amazonía Peruana, 16006, Iquitos, Perú[1]
Laboratorio de Biotecnología y Bioenergética,Universidad Científica del Perú, 16006, Iquitos, Perú[2]
The Field Museum of Natural History, 60605, Chicago, IL, USA[3]
Instituto Nacional de Innovación Agraria, 16006, Iquitos, Perú[4]

Abstract

The aim of this work was to elucidate the molecular and biochemical mechanisms that control L-ascorbic acid (AsA) content variation in *Myrciaria dubia.* The AsA was quantified by high-performance liquid chromatography, gene expression by real-time quantitative PCR, and enzyme activities by spectrophotometric methods from leaves and immature fruits of two genotypes (*Md*-60,06 and *Md*-02,04) with pronounced (about 2 times) differences in the AsA content. In either genotype, the fruit peel had ~ 1.5 times more AsA than the fruit pulp and ~ 15.0 times more than the leaf. All tissues examined demonstrated the capability for AsA biosynthesis through the D-mannose/L-galactose pathway because mRNAs of the six key genes [GDP-D-mannose pyrophosphorylase (*GMP*), GDP-D-mannose-3',5'-epimerase (*GME*), GDP-L-galactose phosphorylase (*GGP*), L-galactose-1-phosphate phosphatase (*GPP*), L-galactose dehydrogenase (*GDH*), and L-galactono-1-4-lactone dehydrogenase (*GLDH*)] and catalytic activities of the corresponding enzymes (GMP, GDH, and GLDH) were detected. The differential expressions of genes and enzyme activities mostly correlated with the respective AsA content. Thus, the expression of several genes of the D-mannose/ L-galactose pathway determined the AsA content variation in tissues of *M. dubia.*

Additional key words: GDP-D-mannose-3',5'-epimerase; GDP-D-mannose pyrophosphorylase; GDP-L-galactose phosphorylase; L-galactono-1-4-lactone dehydrogenase; L-galactose dehydrogenase; L-galactose-1-phosphate phosphatase.

Myrciaria dubia (Kunth) McVaugh (common name camu-camu) is an Amazonian fruit shrub that produces several bioactive phytochemicals, such as anthocyanins (Zanatta *et al*. 2005), ellagic acid derivatives, and other phenolics (Fracassetti *et al*. 2013). However, the most valuable is its high L-ascorbic acid (AsA; *i.e*., vitamin C) content in fruits which can be as much as 2 g of AsA per 100 g of pulp (Imán *et al*. 2011). It is also interesting the large variation in AsA pool size both among different tissue types of the same individual and among individuals (Castro *et al*. 2013a).

Several studies have shown that a variety of genetic and environmental factors influence variation in AsA content in plant tissues (Davey *et al*. 2006, Roselló *et al*. 2011). These factors, directly or indirectly, influence the metabolic pathways of AsA biosynthesis (Conklin *et al*. 2013). Although a combination of radio-labelling, mutant analysis, and transgenic manipulation provides evidence for multiple pathways of AsA biosynthesis in plants, the D-mannose/L-galactose (Smirnoff-Wheeler) pathway is generally considered the most important (Valpuesta and Botella 2004, Wheeler

Submitted 4 March 2015, *last revision* 27 April 2015, *accepted* 29 April 2015.
Abbreviations: AsA - L-ascorbic acid; DTT - dithiothreitol; EDTA - ethylenediaminetetraacetic acid; f.m. - fresh mass; GDH - L-galactose dehydrogenase; GLDH - L-galactono-1,4-lactone dehydrogenase; GME – GDP-D-mannose-3',5'-epimerase; GMP - GDP-D-mannose pyrophosphorylase; GPP - L-galactose-1-phosphate phosphatase; PMSF - phenylmethylsulfonyl fluoride; PP2A - serine/threonine protein phosphatase; PVP - polyvinylpyrrolidone.
Acknowledgements: This research was financially supported by the Consejo Nacional de Ciencia, Tecnología e Innovación Tecnológica (CONCYTEC) from Perú (Contract N° 280-2010-CONCYTEC-OAJ), and the UNAP (R.R. 2012-UNAP).
* Corresponding autor; fax: (+51) 065 263569, e-mail: juanccgomez@yahoo.es

et al. 1998).

The factors that affect the variation in AsA content among tissues and genotypes of *M. dubia* are largely unknown. Therefore, an objective in our study was to understand the molecular and biochemical mechanisms that control AsA pool size variation by testing the hypothesis that differential gene expression and enzyme activities of the D-mannose/L-galactose pathway influence AsA pool size in *M. dubia*. To test this hypothesis, we measured and compared the AsA content, gene expression, and activities of key enzymes of the D-mannose/L-galactose metabolic pathway in different tissues (*i.e*., leaves, fruit pulp, and peel) of two plant genotypes characterized by their low and high AsA content in fruit pulp.

Leaves and unripe fruits (65 d after anthesis) were collected from *Myrciaria dubia* (Kunth) McVaugh genotypes *Md*-02,04 and *Md*-60,06 belonging to the *M. dubia* germplasm bank at the Instituto Nacional de Innovación Agraria of Peru. These plants were previously characterized by a low [53 ± 4 μmol(AsA) g^{-1}(f.m.)] and high [122 ± 10 μmol(AsA) g^{-1}(f.m.)] AsA content, respectively (Castro *et al*. 2013b). The AsA content was measured using high-performance liquid chromatography (*HP 1100*, *Elite La Chrome*, Waldbronn, Germany) according to Ledezma-Gairaud (1993).

For determination of GDP-mannose pyrophosphorylase (GMP), L-galactose dehydrogenase (GDH), and L-galactono-1,4-lactone dehydrogenase (GLDH) activities, tissue extracts were prepared by grinding each sample (1 g) in a mortar and pestle in 5 cm^3 of ice cold 100 mM Tris-HCl (pH 7.5) containing 1 mM $MgCl_2$, 2 mM Na_2EDTA, 3 mM dithiothreitol (DTT), 1 mM benzamidine hydrochloride, 1 mM aminocaproic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF), 3 % (m/v) polyvinylpyrrolidone, 0.2 % (m/v) *Triton X-100*, and 20 % (v/v) glycerol (Gatzek *et al*. 2002, and Hancock *et al*. 2003, Conklin *et al*. 2006). Cell debris was removed by centrifugation at 20 000 *g* and 4 °C for 15 min, and the supernatant was desalted by dialysis (membrane *MWCO 6000-8000*, *FisherBrand*, PA, USA) in 1 dm^3 of a dialysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM benzamidine hydrochloride, 0.1 mM aminocaproic acid, and 0.1 mM PMSF) at 4 °C for 6 h. Denatured proteins were removed by centrifugation at 20 000 *g* and 4 °C for 10 min. The supernatant was used for the assays to determine the activities of GMP, GDH, and GLDH according to Davis *et al*. (2004), Gatzek *et al*. (2002), and Hancock *et al*. (2003), respectively.

Total RNA was isolated from leaves, fruits pulp, and peel using the cetyltrimethylammonium bromide (CTAB) method, solvent extractions, and DNase treatment as described previously (Castro *et al*. 2013b). The RNA quality and quantity were assessed by standard measurement of absorbance at 230, 260, and 280 nm (Fig. 1 Suppl.) and by formaldehyde denaturing gel electrophoresis (Sambrook *et al*. 1989). Single-stranded cDNA was obtained from 1.5 μg of total RNA using *MuLV* reverse transcriptase and oligo$(dT)_{16}$ following the manufacturer's instructions (*Applied Biosystems*, Foster City, CA, USA).

The expression profiles of the D-mannose/L-galactose pathway genes in leaves, fruit pulp, and fruit peel were determined by real-time quantitative PCR using *SYBR Green I* technology on a *Mastercycler ep Gradient realplex*^{S} (*Eppendorf*, NY, USA) thermal cycler. The expression stability of candidate reference genes was evaluated in our sample set with *actin*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and serine/threonine protein phosphatase (*PP2A*) standards using the *NormFinder* software in R (Andersen *et al*. 2004). All PCR reactions were performed following the manufacturer's instructions of a *SYBR Green* PCR core kit (*Applied Biosystems*) and using specific primers (Table 1 Suppl.). A melting curve analysis ranging from 55 °C to 95 °C with incremental steps of +0.5 °C s^{-1} was used to confirm specificity of amplifications. Reaction efficiency and C_T were determined in our sample set using the *LinRegPCR* software (Ruijter *et al*. 2009). In each run, three technical replications were performed for each of the biological samples, and the relative quantification of expression was performed using the comparative C_T method (Simon 2003). All expression data were calculated as expression ratio relative to *PP2A* which was the most stable reference gene. Means, standard deviations, and statistical significance of differences were performed with one-way *ANOVA*, the Tukey's HSD-test, and Student's *t*-test. Differences at $P < 0.05$ were considered significant.

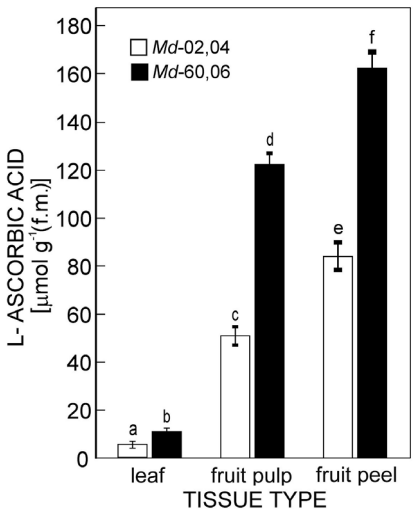

Fig. 1. The AsA content in leaves, fruit pulp, and fruit peel of *M. dubia*. Means ± SD, $n = 3$. Different letters above columns indicate statistically significant differences ($P < 0.001$).

All possible comparisons of AsA pool size both among tissues types of the same individual and among individuals of the same tissue types showed a significantly different AsA content (Fig. 1). The fruit peel had ~ 1.5× more AsA than the fruit pulp and ~ 15.0× than

the leaves. Similarly, the fruit pulp AsA content was ~ 10× higher that the leaf AsA content. Finally, *Md*-60,06 had on average a 2× higher AsA content than *Md*-02,04.

The experimental data indicate that the leaves, fruit pulp, and fruit peel of *M. dubia* possessed the capability for AsA biosynthesis through the D-mannose/L-galactose pathway because the six genes and the corresponding enzymes (GMP, GDH, and GLDH) of this metabolic pathway were detected in all three tissues analyzed. However, in all the tissues, these genes and enzymes showed differential expression and activities, respectively.

The expressions of all six genes were higher in the fruit pulp and peel in comparison with the leaves (Fig. 2). Also, the expressions of these genes were higher in genotype *Md*-60,06 in comparison with genotype *Md*-02,04 in the leaves (*GME*, *GLDH*), fruit pulp (*GMP, GME, GGP, GPP*, and *GLDH*) and peel (*GMP, GME, GGP*, and *GLDH*). Additionally, in both the genotypes, the genes *GME* and *GLDH* showed a strong positive correlation with the AsA content in the respective tissues. The other genes, such as *GMP*, *GGP*, and *GPP*, exhibited a weak positive correlation among the expressions and AsA content. In contrast, the expression of *GDH* did not correspond to the AsA content in all the three tissue types.

With regard to the enzyme activities, it is evident that the three enzyme activities evaluated here were higher in the fruit pulp and peel in comparison with the leaves (Fig. 2). The enzyme activities of GMP and GLDH were higher in genotype *Md*-60,06 than *Md*-02,04 for all the three tissue types. Furthermore, there was a strong positive correlation between the GLDH activity and AsA content. The other enzymes evaluated showed a weak but positive correlation (GMP) or no correlation (GDH) with the AsA content. Finally, it is evident that GLDH showed positive correlations among the gene expression, enzyme activities, and AsA content (Fig. 1).

Our results indicate that the leaves, fruit pulp, and peel of *M. dubia* had the capability for AsA biosynthesis,

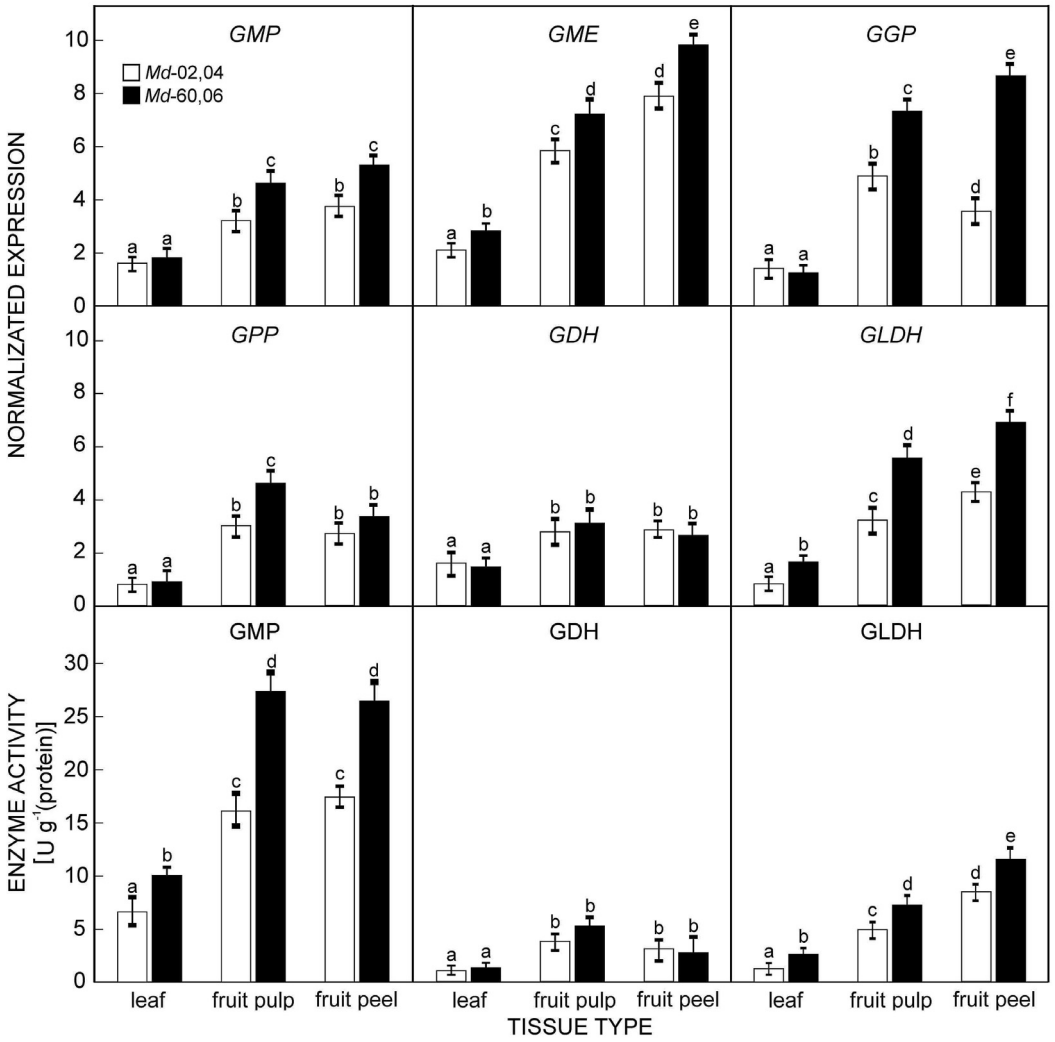

Fig. 2. The relative gene expressions and enzyme activities of the D-mannose/L-galactose pathway in leaves, fruit pulp, and fruit peel of *M. dubia*. Means ± SD, $n = 3$. Different letters above columns indicate statistically significant differences ($P < 0.05$).

because all the genes and the corresponding enzymes (GMP, GDH, and GLDH) of the D-mannose/L-galactose pathway were detected (Fig. 2). These findings are similar to previous reports in other plant species, such as *Malpighia glabra* (Badejo *et al*. 2009), *Actinidia* spp. (Li *et al*. 2010), and *Vitis vinifera* (Melino *et al*. 2009), confirming that fruits have capability for *in situ* biosynthesis of AsA *via* the D-mannose/L-galactose pathway.

These results also demonstrate that the AsA content variation in the leaves, fruit pulp, and fruit peel in *M. dubia* was due, in part, to differential gene expressions and enzyme activities of the D-mannose/L-galactose pathway. Although this biosynthetic pathway is considered most important for AsA biosynthesis in plants (Wheeler *et al*. 1998), several studies have shown that plants possess other biosynthetic pathways, such as myo-inositol pathway (Lorence *et al*. 2004), L-gulose pathway (Wolucka and Van Montagu 2003), galacturonic acid pathway, and animal-like pathway (Valpuesta and Botella 2004). It is thus likely that the AsA pool size variation among these tissues was caused by differential contributions of these metabolic pathways.

An AsA content in fruit pulp can be increased by three processes: *1*) *ex situ* biosynthesis (leaf, fruit peel, phloem, *etc*.) and import by long distance transport to fruit pulp (Franceschi and Tarlyn 2002, Tedone *et al*. 2004), *2*) *in situ* biosynthesis (Li *et al*. 2010), and *3*) recycling through the ascorbate-glutathione cycle (Gest *et al*. 2013). Conversely, an AsA pool size can be decreased by the following processes: *1*) ROS elimination (Foyer and Halliwell 1976), particularly H_2O_2 elimination by ascorbate peroxidase (Dunajska-Ordak *et al*. 2014), *2*) utilization as enzyme cofactor in anabolic pathways for phytohormones, anthocyanins, and hydroxyprolin rich proteins biosynthesis (Valpuesta and Botella 2004, Gallie 2013), and *3*) catabolism to threonate, oxalate, and tartrate (Smirnoff and Wheeler 2000). The relative contributions of these biochemical processes, therefore, were likely to influence the AsA content in tissues of *M. dubia* more than any single process, though this needs be tested empirically.

The experimental data show that the differential gene expressions and enzyme activities of the D-mannose/ L-galactose pathway were responsible for the AsA pool size variation in the tissues of *M. dubia*. These findings are consistent with reports from Bulley *et al*. (2009) who showed that fruits of *Actinidia eriantha* with a high expression of genes *GME* and *GGP* have a 3 to 16× higher AsA content than genotypes with their low expression. Likewise, Yang *et al*. (2011) showed that a higher AsA accumulation in fruit pulp of *Citrus sinensis* may be related to a higher expression of *GME*, *GPP*, *GDH*, and *GLDH*. However, Mellidou *et al*. (2012) showed that only the expression of one ortologue of *GGP* correlates with a high AsA content found in a tomato cultivar characterized by fruits with a high AsA content.

In conclusion, our data clearly show that the expression of several genes of the D-mannose/ L-galactose pathway correlated with the AsA pool size variation in the tissues of *M. dubia*, and that AsA biosynthesis was thus controlled primarily through the transcriptional activation and corresponding translation of the enzymes of this metabolic pathway.

References

Andersen, C.L., Jensen, J.L., Orntoft, T.F.: Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. - Cancer Res. **64**: 5245-5250, 2004.

Badejo, A.A., Fujikawa, Y., Esaka, M.: Gene expression of ascorbic acid biosynthesis related enzymes of the Smirnoff-Wheeler pathway in acerola (*Malpighia glabra*). - J. Plant Physiol. **166**: 652-660, 2009.

Bulley, S.M., Rassam, M., Hoser, D., Otto, W., Schünemann, N., Wright, M., MacRae, E., Gleave, A., Laing, W.: Gene expression studies in kiwifruit and gene over-expression in *Arabidopsis* indicates that GDP-L-galactose guanyltransferase is a major control point of vitamin C biosynthesis. - J. exp. Bot. **60**: 765-778, 2009.

Castro, J.C., Gutierrez, F., Acuña, C., Cerdeira, L.A., Tapullima, A., Cobos, M., Imán, S.A.: Variation of vitamin C and anthocyanins in *Myrciaria dubia* "camu camu". - Rev. Soc. Quím. Perú **79**: 319-330, 2013.

Castro, J.C., Egoávil A.C., Torres, J., Ramírez, R., Cobos, M., Imán, S.A.: Isolation of high-quality total RNA from leaves of *Myrciaria dubia* "camu camu". - Prep. Biochem. Biotechnol. **43**: 527-538, 2013.

Conklin, P.L., DePaolo, D., Wintle, B., Schatz, C., Buckenmeyer, G.: Identification of *Arabidopsis* VTC3 as a putative and unique dual function protein kinase::protein phosphatase involved in the regulation of the ascorbic acid pool in plants. - J. exp. Bot. **64**: 2793-2804, 2013.

Conklin, P.L., Gatzek, S., Wheeler, G.L., Dowdle, J., Raymond, M.J., Rolinski, S., Isupov, M., Littlechild, J.A., Smirnoff, N.: *Arabidopsis thaliana* VTC4 encodes L-galactose-1-phosphatase, a plant ascorbic acid biosynthetic enzyme. - J. biol. Chem. **281**: 15662-15670, 2006.

Davey, M.W., Kenis, K., Keulemans, J.: Genetic control of fruit vitamin C contents. - Plant Physiol. **142**: 343-351, 2006.

Davis, A.J., Perugini, M.A., Smith, B.J., Stewart, J.D., Ilg, T., Hodder, A.N., Handman, E.: Properties of GDP-mannose pyrophosphorylase, a critical enzyme and drug target in *Leishmania mexicana*. - J. biol. Chem. **279**: 12462-12468, 2004.

Dunajska-Ordak, K., Skorupa-Kłaput, M., Kurnik, K., Tretyn, A., Tyburski, J.: Cloning and expression analysis of a gene encoding for ascorbate peroxidase and responsive to salt stress in beet (*Beta vulgaris*). - Plant mol. Biol. Rep. **32**: 162-175, 2014.

Foyer, C.H., Halliwell, B. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. - Planta. **133**: 21-25, 1976.

Fracassetti, D., Costa, C., Moulay, L., Tomás-Barberán, F.A.: Ellagic acid derivatives, ellagitannins, proanthocyanidins

and other phenolics, vitamin C and antioxidant capacity of two powder products from camu-camu fruit (*Myrciaria dubia*). - Food Chem. **139**: 578-588, 2013.

Franceschi, V.R., Tarlyn, N.M.: L-ascorbic acid is accumulated in source leaf phloem and transported to sink tissues in plants. - Plant Physiol. **130**: 649-656, 2002.

Gallie, D.R.: L-ascorbic acid: a multifunctional molecule supporting plant growth and development. - Scientifica **2013**: 1-24, 2013.

Gatzek, S., Wheeler, G.L., Smirnoff, N.: Antisense suppression of L-galactose dehydrogenase in *Arabidopsis thaliana* provides evidence for its role in ascorbate synthesis and reveals light modulated L- galactose synthesis. - Plant J. **30**: 541-553, 2002.

Gest, N., Gautier, H., Stevens, R.: Ascorbate as seen through plant evolution: the rise of a successful molecule?. - J. exp. Bot. **64**: 33-53, 2013.

Hancock, R.D., McRae, D., Haupt, S., Viola, R.: Synthesis of L-ascorbic acid in the phloem. - BMC Plant Biol. **3**: 1-13, 2003.

Imán, S., Bravo, L., Sotero, V., Oliva, C.: Vitamin C content in fruits of camu camu *Myrciaria dubia* (H.B.K) Mc Vaugh, in four states of maturation, coming from the collection of germplasm of the INIA Loreto, Peru. - Sci. Agropecul. **2**: 123-130, 2011.

Ledezma-Gairaud, M.L.: Validation of the method: determination of total vitamin C by high resolution liquid chromatography "HPLC".” - Tecnología En Marcha **417**: 15-23, 1993.

Li, M., Ma, F., Liang, D., Li, J., Wang, Y.: Ascorbate biosynthesis during early fruit development is the main reason for its accumulation in kiwi. - PloS One **5**: e14281, 2010.

Lorence, A., Chevone, B.I., Mendes, P., Nessler, C.L.: Myo-inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. - Plant Physiol. **134**: 1200-1205, 2004.

Melino, V.J., Soole, K.L., Ford, C.M.: Ascorbate metabolism and the developmental demand for tartaric and oxalic acids in ripening grape berries. - BMC Plant Biol. **9**: 145, 2009.

Mellidou, I., Keulemans, J., Kanellis, A.K., Davey, M.W.: Regulation of fruit ascorbic acid concentrations during ripening in high and low vitamin C tomato cultivars. - BMC Plant Biol. **12**: 239, 2012.

Roselló, S., Adalid, A.M., Cebolla-Cornejo, J., Nuez, F.: Evaluation of the genotype, environment and their interaction on carotenoid and ascorbic acid accumulation in tomato germplasm. - J. Sci. Food. Agr. **91**: 1014-1021, 2011.

Ruijter, J.M., Ramakers, C., Hoogaars, W.M.H., Karlen, Y., Bakker, O., Van den Hoff, M.J.B., Moorman, A.F.M.: Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. - Nucl. Acids Res. **37**: e45, 2009.

Sambrook, J., Fritsch, E.F., Maniatis, T.: Molecular Cloning: A Laboratory Manual. 1st Ed. - Cold Spring Harbor Laboratory Press, Cold Spring Harbor - New York 1989.

Simon, P.: Q-Gene: processing quantitative real-time RT-PCR data. - Bioinformatics **19**: 1439-1440, 2003.

Smirnoff, N., Wheeler, G.L.: Ascorbic acid in plants: biosynthesis and function. - Crit. Rev. Biochem. mol. Biol. **35**: 291-314, 2000.

Tedone, L., Hancock, R.D., Alberino, S., Haupt, S., Viola, R.: Long-distance transport of L-ascorbic acid in potato. - BMC Plant Biol. **4**: 16, 2004.

Valpuesta, V., Botella, M.A.: Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant. - Trends Plant Sci. **9**: 573-577, 2004.

Wheeler, G.L., Jones, M.A., Smirnoff, N.: The biosynthetic pathway of vitamin C in higher plants. - Nature **393**: 365-369, 1998.

Wolucka, B.A., Van Montagu, M.: GDP-mannose-3',5'-epimerase forms GDP-L-gulose, a putative intermediate for the *de novo* biosynthesis of vitamin C in plants. - J. biol. Chem. **278**: 47483-47490, 2003.

Yang, X.Y., Xie, J.X., Wang, F.F., Zhong, J., Liu, Y.Z., Li, G.H., Peng, S.A.: Comparison of ascorbate metabolism in fruits of two citrus species with obvious difference in ascorbate content in pulp. - J. Plant Physiol. **168**: 2196-2205, 2011.

Zanatta, C.F., Cuevas, E., Bobbio, F.O., Winterhalter, P., Mercadante, A.Z.: Determination of anthocyanins from camu-camu (*Myrciaria dubia*) by HPLC-PDA, HPLC-MS, and NMR. - J. Agr. Food Chem. **53**: 9531-9535, 2005.