Dharmendra K. Gupta
José M. Palma
Francisco J. Corpas *Editors*

Redox State as a Central Regulator of Plant-Cell Stress Responses

Springer

Chapter 4
Physiological Processes Contributing to the Synthesis of Ascorbic Acid in Plants

C.G. Bartoli, M.E. Senn and G.E. Gergoff Grozeff

Abstract Ascorbic acid (AA) is present in high concentration in plant tissues and participates of many vital processes. Its biosynthetic pathway in plants has been recently established. Nowadays, research is focused on understanding the regulation of this pathway. One of the aspects is to unravel the importance of each enzyme through the establishment of limiting reactions and specific controlling processes. Interestingly, the synthesis of AA presents a high interaction with several physiological processes. The photosynthetic activity directly affects AA synthesis, and the last reaction of its formation takes place in mitochondria depending on the mitochondrial electron transport chain. In addition, it is now known how some plant hormones regulate this pathway. The incident light constitutes a key environmental factor controlling the synthesis of AA in plants. Light regulates the levels of several enzymes, but its effect on AA biosynthesis is also mediated through modifications in the previously mentioned physiological processes. The knowledge about the regulation of AA synthesis will allow the development of manipulative strategies leading to effectively increase the concentration of AA in edible plant organs.

Keywords Ascorbic acid · Leaves · Fruit · Photosynthesis · Plant hormones · Respiration

4.1 Introduction

Ascorbic acid (AA) plays several functions in plant biology. It has a central participation in the antioxidant defense, it is crucial for the optimization of photosynthesis, and it is needed for the division and elongation of cells.

C.G. Bartoli (✉) · M.E. Senn · G.E. Gergoff Grozeff
Instituto de Fisiología Vegetal (INFIVE), Facultades de Ciencias Agrarias y Forestales y de Ciencias Naturales y Museo, Universidad Nacional de La Plata, CCT-CONICET La Plata, cc 327 (1900) La Plata, Argentina
e-mail: carlos.bartoli@agro.unlp.edu.ar

Despite the importance of AA for plant cell growth and development, its biosynthetic pathway was recently established. The reactions of AA biosynthesis were clearly demonstrated in green tissues, but the intermediates of its formation may be different in other organs such as fruit which is actively investigated by different research groups. In addition, progresses on our understanding about how environmental factors and physiological processes regulate the accumulation of this antioxidant have been achieved during the last years. These aspects of AA formation will be analyzed here, but first, a brief description of its synthesis in plants is following described.

4.2   AA Synthesis in Plant Tissues

Early studies demonstrated the occurrence of AA formation in plant mitochondria (Mapson et al. 1954), but the complete sequence of reactions conducing to the synthesis of this antioxidant has been recently established (Ishikawa et al. 2006; Linster et al. 2007). Glucose is considered the primary compound of this pathway consisting of a set of ten reactions detailed in Fig. 4.1.

Initial reactions of AA synthesis are shared with those related with the formation of sugars composing the cell wall (Valpuesta and Botella 2004). The last common metabolite of these pathways is GDP-L-galactose (GDP-L-Gal) formed through the epimerization of GDP-D-mannose a reaction catalyzed by GDP-D-mannose-3,5-epimerase (GME). Deficient tomato plants for this enzyme present low AA content (Gilbert et al. 2009), but they also have a disorder in the composition of cell walls since a 60 % deficiency in L-Gal content was observed in the side chain A of rhamnogalacturonan II (Voxeur et al. 2011). After this common reaction, the following steps are considered to be exclusively dedicated to AA synthesis (Linster et al. 2007). Among them, GDP-L-galactose phosphorylase (GGP, VTC2/VTC5 from Vi-t-amin C mutants obtained in Arabidopsis) catalyzes the synthesis of L-galactose-1-phosphate (L-Gal-1-P) which is dephosphorylated by the participation of L-Gal-1-P phosphatase (GPP, VTC4). Next reaction requires the activity of L-Gal dehydrogenase (L-GalDH) yielding L-Gal-1,4-lactone (L-GalL) that is finally oxidized for the production of AA. This last reaction takes place in mitochondria and is catalyzed by L-GalL dehydrogenase (L-GalLDH).

The selection of ethyl methanesulfonate-mutagenized Arabidopsis plants (*vtc* mutants) with low concentration of AA and high susceptibility against oxidative stress conditions was very useful to identify some of the enzymes shown in Fig. 4.1 (Conklin et al. 2000). For example, the studies of one of these mutants lead to the identification of the enzyme GDP-D-mannose pyrophosphorylase (GMP, VTC1). A modification on the gene coding for this enzyme is responsible of only 25 % AA content in *vtc1* compared with wt plants (Conklin et al. 1999). Then, the generation of plants with down-regulation or over-expression for different proteins was also utilized for the demonstration of their role in AA synthesis (and in some cases in alternative functions) in different organs and plant species. Table 4.1 summarizes

Fig. 4.1 AA biosynthetic and regeneration pathways in plants. The metabolite bobbled in a circle gives the name of each pathway. The enzymes in each pathway are as follows: *1* hexokinase; *2* glucose-6-phosphate isomerase; *3* mannose-6-phosphate isomerase (PMI); *4* phosphomanno-mutase (PMM); *5* GDP-D-mannose pyrophosphorylase (mannose-1-phosphate guanylyltrans-ferase) (VTC1 or GMP); *6* GDP-D-mannose 3′,5′-epimerase (GME); *7* GDP-L-galactose phosphorylase (VTC2 or GGP); *8* L-galactose-1-phosphate phosphatase (VTC4 or GPP); *9* L-galactose dehydrogenase (GalDH); *10* L-galactono-1,4-lactone dehydrogenase (L-GalLDH); *11* nucleotide pyrophosphatase or sugar-1-phosphate guanyltransferase; *12* sugar phosphatase; *13* L-gulose dehydrogenase; *14* L-gulonolactonase; *15* L-gulono-1,4-lactone oxidase; *16* polygalacturonate 4-alpha-galacturonosyltransferase; *17* D-galacturonate-1-phosphate uridyl-transferase; *18* D-galacturonate reductase (GalUR); *19* aldonolactonase; *20* phosphoglucomutase; *21* UDP-glucose-pyrophosphorylase; *22* UDP-glucose dehydrogenase; *23* glucuronate-1-phosphate uridylyltransferase; *24* D-glucurono-1-phosphate phosphatase; *25* *myo*-inositol oxygenase (MIOX); *26* D-glucuronate reductase; *27* monodehydroascorbate reductase; and *28* dehydroascorbate reductase

several of these studies involving altered plants where progresses in these subjects were achieved in different organs. Particularly, genetically modified plants lacking GGP or L-GalLDH are not viable (Dowdle et al. 2007; Alhagdow et al. 2007). These studies gave concluding evidences for the participation of these enzymes in AA biosynthesis and, more importantly, proved the vital role of AA for plant survival.

GGP/VTC2 is considered a specific key regulatory enzyme of this pathway (Linster and Clarke 2008). In Arabidopsis, a homologue gene, *VTC5*, is present but with a very low expression, and consequently, the corresponding enzyme activity

Table 4.1 Human manipulation of enzymes affecting the synthesis and the concentration of AA in different organs and plant species. Decrease concentration and increase concentration of AA are indicated as − and +, respectively

Enzyme modification	AA content	Plant species	Organs studied	References
i. Phosphomanose isomerase (PMI1) down-regulation	−	Arabidopsis	Leaves	Maruta et al. (2008)
ii. GDP-mannose pyrophosphorylase (GMP/VTC1) mutant/complementation	∓	Arabidopsis	Leaves	Conklin et al. (1999)
GMP over-expression	+	Tomato	Fruit	Cronje et al. (2012)
GMP down-regulation/over-expression	∓	Potato	Leaves and tubers	Keller et al. (1999)
	∓	Tomato	Leaves and fruits	Zhang et al. (2013)
iii. GDP-D-mannose 3,5-epimerase(GME) down-regulation	−	Tomato	Leaves and fruits	Gilbert et al. (2009)
iv. GDP-L-Galactose phosphorylase(GGP/VTC2) overexpression	+	Arabidopsis	Leaves	Bulley et al. (2009)
		Tomato, strawberry, and potato	Fruit, tubers	Bulley et al. (2012)
GGP down-regulation	−	Arabidopsis	Leaves	Dowdle et al. (2007)
	−	Tomato	Leaves	Baldet et al. (2013)
v. L-galactose dehydrogenase (L-GalDH) over-expression	No effect	Tobacco	Leaves	Gatzek et al. (2002)
L-GalDH down-regulation	−	Arabidopsis	Leaves	Gatzek et al. (2002)
vi. L-Galactono-1,4-lactone deshidrogenase (L-GalLDH) over-expression	No effect	Tobacco	Leaves and roots	Imai et al. (2009)
L-GalLDH down-regulation	−	Tobacco	Tissue culture	Tokunaga et al. (2005)
	−	Tomato	Fruit and leaves	Alhagdow et al. (2007)

Table 4.1 (continued)

Enzyme modification	AA content	Plant species	Organs studied	References
vii. D-galacturonic acid reductase (FaGalUR) over-expression	+	Tomato and arabidopsis	Fruit and leaves	Agius et al. (2003)
	+	Tomato	Fruit	Amaya et al. (2014)
viii. Dehydroascorbate reductase over-expression	+	Tobacco and maize	Leaves	Chen et al. (2003)
	+	Potato	Leaves and tubers	Qin et al. (2011)
ix. *myo*-inositol oxigenase (MIOX4) over-expression	+	Arabidopsis	Leaves	Lorence et al. (2004)
(MIOX2) over-expression	No effect	Arabidopsis	Leaves	Endres and Tenhaken (2009)
	∓	Tomato	Leaves/fruit	Cronje et al. (2012)

makes a small contribution to the pool of AA. The over-expression and down-regulation of GGP/VTC2 produce a large effect on the AA pool of plant tissues (Dowdle et al. 2007; Bulley et al. 2009; Baldet et al. 2013). Since GGP/VTC2 catalyzes a reaction committed to AA synthesis, and deficient plants for this enzyme present very low AA content (Dowdle et al. 2007; Baldet et al. 2013), they constitute very useful tools to unravel specific roles of AA in physiological processes occurring in leaves and fruit. Although many aspects remain to be studied, it was established that VTC2 is localized in two compartments, the cytosol and the nucleus, suggesting enzymatic and regulatory functions for this protein (Müller-Moule 2008).

The above-mentioned synthesis pathway is observed in different organs and named as Smirnoff–Wheeler considering the substantial contribution to its discovery made by these researchers. It is also known as L-Gal pathway considering the first precursor committed to AA formation. However, other precursors may contribute to the AA synthesis. D-galacturonic acid (D-GalUA) derived from wall pectin is transformed after a few reactions in AA (Agius et al. 2003). The participation of this alternative route was particularly observed during last stages of fruit ripening (Badejo et al. 2012). However, D-GalUA and L-Gal pathways converge in the last reaction catalyzed by L-GalLDH (Fig. 4.1).

In addition, the oxidation of *myo*-inositol may provide metabolites to the pool of precursors for AA synthesis in plants; over-expression of *myo*-inositol oxigenase (MIOX) increases AA content in leaves and fruit, but this effect is not observed in all studies (Lorence et al. 2004; Endres and Tenhaken 2009; Cronje et al. 2012); consequently, its real function in AA formation has not been demonstrated in plants, but it may potentially be used as a biotechnological tool for increase AA in plant tissues.

GDP-L-gulose (GDP-L-Gul) was also proposed as a metabolite directly committed to AA synthesis through a non-dependent L-Gal pathway (Wolucka and Van Montagu 2003). Although the reaction would take place in plant cells, its participation does not seem to make a considerable contribution to AA content. This conclusion is based on the one hand in the absence (or exceptionally low) of increase in AA content after feeding tissues with L-Gul (Mellidou et al. 2012) and, on the other hand, the loss of viability of plants lacking L-GalLDH or simultaneously VTC2 and VTC5 proteins (Alhagdow et al. 2007; Dowdle et al. 2007).

Considering the redox function of AA in plant metabolism, its recovery from oxidized forms plays a crucial function keeping its pool largely as its reduced form. The recovery is also important because the oxidized forms, dehydro- and mono-dehydroascorbate (DHA and MDHA), are susceptible to be degraded through its conversion to L-tartaric or oxalic acids (Loewus 1999). Detailed analysis of AA degradation is analyzed in Chap. 5 of this book. Reduction of the oxidized forms is catalyzed by DHA and MDHA reductases (DHAR and MDHAR) using redox power provided by NAD(P)H and glutathione (Foyer and Noctor 2011). Additionally, these reactions are needed to keep working the water-water cycle proposed by Asada (1999) which is critical for the optimization of photosynthesis.

The AA biosynthesis experimented different evolutionary routes leading to the animal and plant pathways characterized by the participation of different terminal enzymes, L-gulonolactone oxidase (L-GulO) and L-GalLDH, respectively (Wheeler et al. 2015). According to the interesting analysis made by Wheeler et al. (2015), a common eukaryote ancestor possessed L-GulO as an ancestral gene that was kept by non-photosynthetic eukaryotes. Then, plants acquired L-GalLDH through endosymbiotic gene transfer from ancestral Archaeplastida that previously replaced L-GulO with L-GalLDH. Alternative evolutionary models and the advantages of using L-GalLDH in photosynthetic cells are beautifully analyzed in Wheeler et al. (2015).

4.3 Environmental Regulation of AA Synthesis in Plant Tissues

4.3.1 The Light Control of AA Synthesis

The regulation of the synthesis of AA is exerted by different factors either endogenous or exogenous. Among environmental conditions, light is clearly the main external factor influencing processes involved in AA formation in leaves and fruit and, of course, the most studied (Fig. 4.2). This is consistent with the central roles played by AA in the antioxidant defences and in the optimization of

Fig. 4.2 Relationship between AA content with irradiance and photosynthetic electron transport rate in rice leaves. *Green values* and *arrows* indicate the rates of photosynthetic electron transport (μmol electrons m^{-2} s^{-1}) measured in leaves at the different levels of photosynthetically active radiation (PAR). The correlation between AA content and ETR was estimated as $R^2 = 0.74$

photosynthesis. The amount (i.e., irradiance), the quality (i.e., red/far-red ratio), and the length of the day are important characteristics of light regulating its concentration under the continuously changing ambient.

The irradiance modulates the expression of some enzymes of the Smirnoff–Wheeler pathways such as GMP, GGP, GPP, and L-GalLDH (Yabuta et al. 2007). The promoter region of the genes coding GPP and L-GalLDH includes *cis*-elements responsive to changes in irradiance (Fukunaga et al. 2010). Furthermore, the expression and activity of L-GalLDH increase during the day and then decrease in the night getting its lower activity at dawn (Tamaoki et al. 2003). These modifications observed in the gene expression or enzyme activity are associated with the changes in the concentration of AA.

The red/far-red ratio of incident light controls the accumulation of both AA and glutathione in leaves under similar irradiance (Bartoli et al. 2009). Sunny or shade environments are characterized by high or low red/far-red ratio, respectively. This means that the red/far-red ratio is a critical signal for plants indicating the light ambient and consequently preparing their metabolism to cope with the risk of photooxidative damage. The increased AA content induced by high red/far-red ratio was associated with modifications in the activities of enzymes involved in both synthesis (such as L-GalLDH) and recovery from oxidized forms (such as DHAR and glutathione reductase) and with increased concentration of glutathione and NAD(P)H that contribute to the AA accumulation (Bartoli et al. 2009).

Although not experimental evidences could be finding, the length of the day seems to be important setting the concentration of AA in plant tissues. Shortening of the days induces the establishment of plant organ dormancy (Rohde and Bhalerao 2007), even under relatively high ambient temperature (i.e., end of summer) and concomitantly a decrease in the concentration of AA. Furthermore, when photoperiod increases, even under low temperature, dormant poplar buds start to grow with the previous increase in AA content (Gergoff Grozeff and Bartoli 2014). These correlative/indirect data suggest a regulation of antioxidant concentration by changes in the photoperiod during alternate seasons. While light regulates AA biosynthesis through gene expression, it also affects this pathway by modifications in plant metabolism and development. In the following sections, physiological processes affecting AA synthesis will be analyzed, and finally, an especial section will be dedicated to peculiarities of this pathway in fruit. Table 4.2 presents a summary of physiological processes involved in the regulation of AA synthesis, and Fig. 4.3 shows how these processes interact with this pathway in plant tissues.

Table 4.2 Physiological processes affecting the synthesis or the content of AA in different organs and plant species. Decrease and increase in the synthesis or concentration of AA are indicated as − and +, respectively

Physiological processes	AA synthesis/content	Plant species	Plant organ involved	References
i. Photosynthesis (Electron transport rate)	+	Arabidopsis	Leaves	Yabuta et al. (2007, 2008)
ii. Respiration				
• Cyt c pathway (Availability of oxidized cyt c)	+	Potato	Tubers	Bartoli et al. (2000)
		Arabidopsis	Leaves	Millar et al. (2003)
• AOX pathway	+	Arabidopsis	Leaves	Bartoli et al. (2006)
iii. Active growth	+	Arabidopsis	Young leaves	Bartoli et al. (2000)
		Peach	Immature fruit	Imai et al. (2009)
iv. Dormancy	−	Poplar	Branch buds	Gergoff Grozeff and Bartoli (2014)
	−	Wheat	Seeds	De Gara et al. (1997)
v. Senescence	−	Arabidopsis	Leaves	Barth et al. (2006)
	−	Chrysanthemum	Flowers	Bartoli et al. (1997)
vi. Ripening	−	Kiwifruit	Fruit	Bulley et al. (2009)
	+	Tomato	Fruit	Badejo et al. (2012)
vii. Hormones				
• Ethylene	−	Spinach and arabidopsis	Leaves	Gergoff Grozeff et al. (2010)
• Gibberellins	+	Arabidopsis	Leaves	Millar et al. (2003)
• Brassinosteroids	+	Tomato	Leaves	Mazorra et al. (2014)
• Jasmonates	+	Arabidopsis and tobacco	Cell suspensions	Wolucka et al. (2005)

Fig. 4.3 Physiological processes and light signaling involved in positive or negative regulation of AA synthesis in plants (ETC, electron transport chain; cyt *c*, cytochrome *c*; AOX, alternative oxidase)

4.4 Physiological Processes Affecting AA Synthesis

4.4.1 Feedback Regulation and Other Specific Regulators

Although AA reaches high levels in plant tissues, its synthesis is negatively controlled by its own concentration. The AA synthesis from glucose decreases (and turnover increases) after feeding tissues with ^{14}C-labeled glucose under increased concentration of AA (Pallanca and Smirnoff 2000). A specific control of AA on its own pathway was recently established identifying a posttranscriptional regulation of GGP synthesis (Laing et al. 2015). This work shows a GGP synthesis inhibition through ribosome stalling by a translated *cis*-acting upstream open reading frame taking place under high AA concentration.

While AA concentration inhibits its own formation rate high level of its primary precursor, glucose stimulates this pathway. The over-expression of a pyrophosphatase increases inorganic phosphate and AA contents (Osorio et al. 2013). However, the effect of the concentration of AA was attributed to an increased level of soluble sugar (i.e., glucose and sucrose) providing more substrate for AA synthesis but also to a higher expression of GGP, L-GalLDH and MDHAR. In addition, the reaction catalyzed by GMP produces both GDP-man and pyrophosphate; the

hydrolysis of pyrophosphate by increased pyrophosphatase activity may also stimulate the pathway on the direction of AA formation.

Plants with disruptions in the gene called AA mannose regulator 1 (*AMR1*) present higher concentration of AA (Zhang et al. 2009). The results found in this work indicate that *AMR1* negatively regulates AA accumulation. Moreover, *AMR1* levels inversely correlate with the expression of GMP, GME, GGP, GPP, L-Gal dehydrogenase (L-GalDH), and L-GalLDH.

An interesting protein involved in AA synthesis regulation has been proposed recently (Conklin et al. 2013). This protein named VTC3 has the unusual presence of both kinase and phosphatase domains which are needed to regulate AA formation. Plants defective in the VTC3 protein have lower AA content (Conklin et al. 2000) and loose the capacity to increase the pool of this antioxidant when challenged to modifications in light and temperature (Conklin et al. 2013). The particular biochemical mechanisms involved are still unknown.

4.4.1.1   Relationship of AA Synthesis with Plant Metabolism

Interaction with Photosynthesis

In spite of light-dependent regulation of the biosynthetic pathway, the increment of AA is connected with the chloroplast metabolism. Some evidences demonstrate the stimulation of AA synthesis by the photosynthetic activity. Figure 4.2 shows the relationship between AA content with irradiance and photosynthetic electron transport rate. The increment of both photosynthesis and amount of light is accompanied by the content of AA in leaves showing a strong correlation. The application of photosynthetic electron transport chain (ETC) inhibitors such as atrazine and DCMU drastically decreases the concentration of AA in leaves (Yabuta et al. 2007; Bartoli et al. 2009). These results demonstrate the participation of the redox state of plastoquinone in the formation of AA, but the signal involved in this interaction is not known. However, light-induced AA accumulation was observed to require of both mitochondrial and nuclear protein synthesis (Mastropasqua et al. 2012). It has been observed that the photosynthetic ETC blockage produces a decrease in the expression of *GMP/VTC1, GGP/VTC2, GPP/VTC4,* and *L-GalLDH,* giving a further evidence of the protagonist role of the L-Gal pathway in the AA synthesis in leaves. In addition, it must be kept in mind that ETC inhibition also has an impact in the recovery of oxidized forms (Bartoli et al. 2009) that uses NADPH for DHA and MDHA reduction.

Considering that AA is synthesized from glucose, changes in CO_2 uptake by photosynthesis activity might impact on the provision of precursors for its synthesis. The effect of ETC inhibitors may also be mediated through a decreased provision of monosaccharides that contribute to the AA pool.

Interaction with Respiration

Early studies demonstrated that ʟ-GalL conversion to AA takes place in plant mitochondria and suggested the involvement of cytochrome c in this reaction (Mapson et al. 1954). More recently, the interaction of the last reaction in AA formation with respiratory activity of plants was demonstrated (Bartoli et al. 2000). ʟ-GalL oxidation feeds electrons into mitochondrial ETC visualized by the increase in oxygen consumption after ʟ-GalL feeding to isolated mitochondria. The addition of cyanide does not affect the in vitro activity of ʟ-GalLDH (Mapson and Breslow 1958) but inhibits the formation of AA by mitochondria (Bartoli et al. 2000). The activation of the alternative oxidase under stress conditions stimulates AA synthesis capacity further suggesting the requirement of oxidized cytochrome c as the electron acceptor (Bartoli et al. 2006). Interestingly, this reaction is stimulated by light and depends on an active photosynthetic ETC and protein synthesis (Yabuta et al. 2008). In addition, active Complex I is needed to get functional the ʟ-GalLDH protein; the supplement with malate, pyruvate, and ADP stimulates AA synthesis by isolated mitochondria, but the addition of rotenone (an inhibitor of Complex I) almost stops this reaction (Millar et al. 2003).

ʟ-GalLDH constitutes a distinct protein of plant mitochondria localized in the inner mitochondrial membrane (Siendones et al. 1999). Furthermore, this protein is not only functionally associated; it is also a component of Complex I particularly in the small type Complex I of 850 kDa (Millar et al. 2003). ʟ-GalLDH is integrated into two different protein subcomplexes of about 470 and 420 kDa (Schertl et al. 2012). Those plants with very low ʟ-GalLDH amounts have normal AA content but present some growth disorders (Alhagdow et al. 2007). These observations suggest that the amount of ʟ-GalLDH does not impose a restriction for the synthesis of AA and its function in plants is not limited to this pathway. It was observed that the capacity to synthesize Complex I is lost in mutants lacking ʟ-GalLDH (Pineau et al. 2008; Schimmeyer et al. 2016). These works demonstrate the crucial participation of this enzyme as an assembly factor for the mitochondrial Complex I independently of its function in AA synthesis.

Another interrelationship of mitochondrial activity with AA accumulation consists in the recovery from oxidized forms mediated by mitochondrial ETC. Szarka et al. (2007) showed a malonate inhibition on DHA reduction by isolated mitochondria, suggesting the involvement of succinate dehydrogenase activity in this reaction.

In spite of the relationship with mitochondrial ETC, alteration of Krebs cycle also affects AA accumulation. Down-regulation of mitochondrial malate dehydrogenase increases AA content, and this was associated with a decreased respiration with no changes in ʟ-GalLDH activity (Nunes-Nesi et al. 2005). Unfortunately, the precise physiological mechanisms involved in this increase in AA content induced by the inhibition of Krebs cycle remain to be elucidated.

Regulation of AA Synthesis by Plant Hormones

Regular patterns are observed in the fluctuations of AA content during plant growth and development or during plant acclimation to different environmental conditions, processes that are tightly controlled by hormones. High AA concentration is present in active growing tissues of young plants, and then, it decreases during the senescence of different organs. As previously mentioned, dormant tissues have very low AA content but increase when active growth is initiated. These kinds of associations suggest the participation of hormones in the regulation of its synthesis and level according to the physiological status of the plant. In spite of the existence of the large and active research about the participation of hormones on different plant processes, only a few works dealt with their effect on AA metabolism, evidences that are described below; unfortunately, the role of other hormones such as cytokinin, a vital plant growth promoter, on AA synthesis remains to be studied.

Ethylene is a plant hormone produced during the late stages of plant development stimulating some senescence-related processes, such as chlorophyll or protein degradation. While production and sensitivity to ethylene increases, AA concentration deeply decreases in aged plant organs such as flowers and leaves (Bartoli et al. 1996, 1997; Barth et al. 2006). Similarly, *never-ripe* tomato mutant plants with low sensitivity to ethylene present fruit with higher AA content (Alba et al. 2005). Arabidopsis mutants with reduced ethylene signaling present higher leaf AA content, synthesis (L-GalLDH activity), and recovery (DHAR and MDHR activities) than wild-type plants; in addition, these physiological parameters are kept high in leaves of these mutants induced to senesce under darkness (Grozeff et al. 2010). Interestingly, *AMR1* is expressed at the final developmental stages. The expression of this gene might be regulated by ethylene setting AA level during the development of plant organs.

Brassinosteroids constitute an important group of steroid hormones regulating the growth and development of plants (Hao et al. 2013). Brassinosteroids-deficient mutants present low concentration and synthesis of AA in their leaves, but it is recovered to wild-type levels when these hormones are increased (Mazorra et al. 2014). Interestingly, mutants are dwarf and produce higher amounts of ethylene than wild-type plants. The specific inhibition of ethylene signaling with 1-methyl cyclopropene treatment also restores AA content and synthesis, demonstrating an antagonistic effect of these hormones controlling the antioxidant metabolism (Mazorra et al. 2014).

Gibberellins stimulate plant growth and also AA synthesis. This was particularly observed in mitochondria isolated from gibberellin-treated leaves (Millar et al. 2003). These isolated mitochondria showed an increased capacity to form AA from L-GalL compared with those obtained from untreated plants. The leaves of Arabidopsis plants treated with gibberellins presented higher content of AA, but the expression of the L-GalLDH was not changed (Kiddle 2004). These results suggest that gibberellins control the synthesis of AA through a modification in the activity but not in the amount of the L-GalLDH protein. Interestingly, *vtc1* plants that have

low AA content and growth rate present similar gibberellins content (Kerchev et al. 2011) but increased abscisic acid compared with wild type (Pastori et al. 2003). The balance between these two hormones determines many physiological processes such as seed germination or bud dormancy and might be also important to regulate the AA accumulation and synthesis in plant tissues.

Other hormones regulating the concentration of antioxidants are jasmonantes (jasmonic acid and the derivative methyl ester). Treatment with these hormones induces the increment in the concentration of AA in suspension cells. The effect was only observed in actively growing cells (Wolucka et al. 2005). The synthesis was detected by the increment of AA accumulation from ^{14}C-labeled mannose and gene expression of enzymes of the AA biosynthetic pathways (Wolucka et al. 2005). Besides the increment in the expression of biosynthetic genes, Sasaki-Sekimoto et al. (2005) demonstrated that jasmonates induce the increases in the expression and the activities of enzymes of AA recovery from oxidized forms. Other plant-stimulating hormones are auxins; treatments with them increase AA content (Piotrowska-Niczyporuk and Bajguz 2014), but the effect on its synthesis remains to be studied.

4.5 The Synthesis of AA in Fruits

Fruit constitute a great source of Vitamin C for human consumption, and the agents affecting the content of this vitamin are of special interest for research (Lee and Kader 2000; Giovannoni 2004). The concentration of AA is highly variable in fruit ranging from less than 8 mg g^{-1} FW in watermelon (Vanderslice et al. 1990) to 5000 mg g^{-1} FW in tropical fruit such as camu-camu (Rodrigues et al. 2001). Apart from different species, this variability is observed within the same species such as tomato (Stevens et al. 2007) or kiwifruit (Nishiyama et al. 2004) or within the cultivars in some berries (Wang and Line 2000).

Besides this variability, the concentration of AA changes during fruit ripening stage; some species such as pear (Franck et al. 2003) and kiwi (Bulley et al. 2009) present a high concentration in young fruit, during the cell division stage, and a drop in the content approximately at harvest time in both cases. In other species, such as tomato, the content of AA increases during ripening (Badejo et al. 2012; Ioannidi et al. 2010). In addition, environmental conditions also affect AA synthesis and concentration in fruit. AA can be synthesized inside the fruit (Ioannidi et al. 2010; Bulley et al. 2009; Hancock et al. 2007) or transported from long distances from source to sink tissues (Tedone et al. 2004; Li et al. 2008). Interestingly, the translocation from leaves is high in green immature fruit and decreases during ripening (Mellidou et al. 2012).

The synthesis of AA in fruit consists of a complex net of precursors and pathways changing their contributions depending on the species and fruit development. Both L-Gal and L-GalUA pathways are active sources of AA in fruit (Ishikawa et al. 2006). As mentioned before, *myo*-inositol offers another enter to the AA synthetic

pathway (Wolucka and Van Montagu 2003; Lorence et al. 2004), but there are not concluding evidences showing any relevance for AA accumulation in fruit.

A large body of evidences indicates that L-GalL pathway makes a substantial contribution to the AA synthesis in fruit. Silencing of GME leads to a half drop in the AA content in fruit (Gilbert et al. 2009). Similar result was observed with down-regulated expression of GMP, but the effect on the content of AA was more pronounced in leaves than in fruit (Zhang et al. 2013). An elevated gene expression of L-Gal pathway enzymes such as GMP, GME, GGP, and GPP was found during initial peach fruit growth coinciding with the highest content of AA (Imai et al. 2009).

In agreement with the previous results, the over-expression of GGP from *Actinidia chinensis* (kiwifruit), under the control of the 35S promoter, gives a three- to sixfold increase in AA content in tomato (Bulley et al. 2012). In the same way, the simultaneous over-expression of GGP and GME produces an even higher accumulation of AA in fruit (Bulley et al. 2009). It is important to keep in mind that over-accumulation of AA in tomato produces seedless fruit (i.e., parthenocarpic fruit) with no mucilage in the interior of the cavity (Bulley et al. 2012). This observation suggests that production and concentration of AA must be finely regulated during fruit growth (Mellidou et al. 2012).

Above-mentioned results clearly demonstrate the participation of the L-Gal route in AA synthesis in fruit. However, it is really important to remark that the expression of genes of the AA pathway not always correlates with the final concentration of this metabolite; in tomato, the concentration of AA increases with the progress of ripening reaching the highest at red stage when L-Gal pathway gene expression is low (Badejo et al. 2012). Alternative precursors increasing AA pool may come from the degradation of cell wall during fruit growth and maturing. It was observed an association between increased expressions of genes involved in the degradation of pectin and high AA accumulation in tomato fruit (Di Matteo et al. 2010). Red tomato fruit present higher capacity to synthesize AA from exogenous added D-GalUA and higher expression of D-GalUA reductase (D-GalUAR) compared with green immature fruit (Badejo et al. 2012). In the same way, ethylene would have an important role stimulating the degradation of cell wall providing pectin-derived precursors for AA formation (Di Matteo et al. 2010). Depending on the ripening stage, tomato fruit use different AA biosynthetic pathways; this is the L-Gal pathway during green stage and the D-GalUA pathway during the red stage (Badejo et al. 2012; Amaya et al. 2014). Similarly, an increase in the D-GalUAR enzyme concomitant with an increase in the content of AA was observed at the ripening stage of strawberry fruit (Cruz-Rus et al. 2011). In spite of these results, no conclusive evidence demonstrates the actual participation of D-GalUA pathway; the effect of knockdown regulation of D-GalUAR will give a direct proof unequivocally confirming the role of this pathway in AA synthesis in fruit or other plant tissues. Besides synthesis, the increased activity of recovery from oxidized forms help to keep high AA content in tomato at red stages (Mellidou et al. 2012). In pepper fruit, concentration of AA also increases during maturation; this is associated with the increment in NADPH levels that may contribute to a higher capacity to recovery from oxidized forms (Mateos et al. 2009). Furthermore, ripening of pepper fruit at low temperature produces a reduction in the activities of NADPH

generating enzymes and a concomitantly shift to a more oxidized AA/DHA rate (Mateos et al. 2013). Globally, these works suggest that depending on the species and development stage different pathways contribute to the synthesis of AA in fruit.

4.5.1 The Effect of Light on the Synthesis of AA in Fruit

AA is a small and mobile molecule that can move over the plant from source to sink tissues. Although fruit show the capacity to synthesize AA, this metabolite is gained in fruit by translocation from source tissues, such as leaves (Franceschi and Tarlyn 2002). Considering that AA primary precursors are produced from photosynthesis, light affects AA accumulation in leaves and other organs. However, some works give evidences of a direct effect of light in fruit antioxidant accumulation (Gautier et al. 2009; Massot et al. 2012). It was observed a drastically reduction in the content of AA in shaded tomato fruit without modification in the content of sugars, indicating a non-limiting substrate availability (Gautier et al. 2009). Furthermore, Gautier et al. (2009) also show that shading fruit was more effective to decrease the concentration of AA in fruit than shading leaves.

Apple fruit developed in shade zones of the canopy present less AA content, but they still have the capacity of producing this antioxidant via L-Gal and D-GalUA pathways (Li et al. 2008). These authors also found that the effect of light is localized in the peel zone associated with a drop in the activity of L-GalLDH as well as in the recycling enzymes in the shaded fruit (Li et al. 2009). In contrast with the previous results, AA decreases in kiwi fruit when the complete plant was shaded but not when only the fruit was kept in the dark (Li et al. 2010). The drop in AA was associated with the decrease in the expression of genes of L-Gal and L-GalUA biosynthetic pathways and recycling enzymes.

Taken together, these works suggest that the effect of light on the synthesis and accumulation of AA in young fruit depends on both leaves and fruit, but the relative contribution of these organs depends on the plant species. Unraveling how different factors affect plant functions may be important to improve the quality of edible plant organs. In that sense, the application of blue and white LED was successfully used to increase the glucose and AA in fruit (Xu et al. 2012).

4.6 The Changes of AA During Plant Domestication

The rises in the fruit yield of different species have decreased the natural content of the AA pool in fruit (Charmet 2011). Decreasing concentration of AA has been observed throughout domestication (Gest et al. 2013) as a consequence of an increasing focus of the human manipulation on fruit yield instead of nutraceutical/nutritional properties. This has been reported several times in commercial fruit of low AA content such as grape (DeBolt et al. 2006;

Melino et al. 2011) and apple (Davey and Keulemans 2004) and high AA content fruit such as kiwifruit (Huang et al. 2004) or tomato (Stevens et al. 2007).

The colocalization of QTL with antagonistic allele effects for fruit fresh weight and sugar concentration was observed in tomato (Prudent et al. 2009). Plant thinning reduces the number of fruit while increase their size. Kim et al. (2006) found that the amount of AA per fruit can be reduced due to an effect of plant thinning; this means that the number of fruit per plant may affect the AA content. Similarly, a nocturnal light treatment increases the fresh weight of tomato fruit but simultaneously reduced the concentration of soluble sugars and AA (Gergoff Grozeff et al. 2016).

4.7 Concluding Remark

The experimental evidences obtained confirm the L-Gal pathway as the crucial route leading to the synthesis of AA. However, in special cases such as ripen fruit, D-GalUA pathway makes a protagonist contribution.

High AA content correlates with active growth status, while lower AA content correlates with non-growing physiological conditions. This correlation may be mediated by hormones that regulate the accumulation of AA in plant tissues. The data suggest, in general terms, that those hormones associated with active growth stimulate, while those associated with senescence or dormancy inhibit the AA synthesis. In addition, the association of growth and AA concentration suggests a cooperative rather a competitive interaction between both cell wall sugar and AA synthesis. In agreement with these observations, active photosynthetic and respiratory metabolisms are needed to get maximal AA biosynthetic rates. Considering that the synthesis of AA is the result of the interaction with both photosynthesis and respiration, AA may play a role coordinating the activities of chloroplasts and mitochondria.

Understanding the processes involved in this pathway will allow the development of an efficient manipulation of plants to get increased concentration of AA, improving the nutritional quality of edible plant organs.

References

Agius F, Gonzalez-Lamothe R, Caballero JL, Munoz-Blanco J, Botella MA, Valpuesta V (2003) Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. Nat Biotechnol 21:177–181

Alba R, Payton P, Fei Z, McQuinn R, Debbie P, Martin GB, Tanksley SD, Giovannoni JJ (2005) Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. Plant Cell 17:2654–2965

Alhagdow M, Mounet F, Gilbert L, Nunes-Nesi A, Garcia V, Just D, Petit J, Beauvoit B, Fernie AR, Rothan C, Baldet P (2007) Silencing of the mitochondrial ascorbate synthesizing enzyme L-galactono-1,4-lactone dehydrogenase affects plant and fruit development in tomato. Plant Physiol 145:1408–1422

Amaya I, Osorio S, Martinez-Ferri E, Lima-Silva V, Doblas VG, Fernández-Muñoz R, Fernie AR, Botella MA, Valpuesta V (2014) Increased antioxidant capacity in tomato by ectopic expression of the strawberry D-galacturonate reductase gene. Plant Biotech J 10:490–500

Asada K (1999) The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annl Rev Plant Physiol Plant Mol Biol 50:601–639

Badejo AA, Wada K, Gao Y, Maruta T, Sawa Y, Shigeoka S, Ishikawa T (2012) Translocation and the alternative D-galacturonate pathway contribute to increasing the ascorbate level in ripening tomato fruits together with the D-mannose/L-galactose pathway. J Exp Bot 63:229–239

Baldet P, Bres C, Okabe Y, Mauxion JP, Just D, Bournonville C, Ferrand C, Mori K, Ezura H, Rothan C (2013) Investigating the role of vitamin C in tomato through TILLING identification of ascorbate-deficient tomato mutants. Plant Biotechnol 30:309–314

Barth C, De Tullio M, Conklin PL (2006) The role of ascorbic acid in the control of flowering time and the onset of senescence. J Exp Bot 57:1657–1665

Bartoli CG, Pastori G, Foyer C (2000) Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. Plant Physiol 123:335–343

Bartoli CG, Simontacchi M, Montaldi E, Puntarulo S (1996) Oxidative stress, antioxidant capacity and ethylene production during ageing of cut carnation (Dianthus caryophyllus) petals. J Exp Bot 47:595–601

Bartoli CG, Simontacchi M, Montaldi E, Puntarulo S (1997) Oxidants and antioxidants during aging of chrysanthemum petals. Plant Sci 129:157–165

Bartoli CG, Tambussi EA, Fanello DD, Foyer CH (2009) Control of ascorbic acid synthesis and accumulation by the incident light red/far red ratio in Phaseolus vulgaris leaves. FEBS Lett 583:118–122

Bartoli CG, Yu J, Gómez F, Fernández L, McIntosh L, Foyer CH (2006) Inter-relationships between light and respiration in the control of ascorbic acid synthesis and accumulation in Arabidopsis thaliana leaves. J Exp Bot 57:1621–1631

Bulley SM, Rassam M, Hoser D, Otto W, Schünemann N, Wright M, MacRae E, Gleave A, Laing W (2009) 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

Bulley SM, Wright M, Rommens C, Yan H, Rassam M, Lin-Wang K, Andre C, Brewster D, Karunairetnam S, Allan AC, Laing WA (2012) Enhancing ascorbate in fruits and tubers through over-expression of the L-galactose pathway gene GDP-L-galactose phosphorylase. Plant Biotech J 10:390–397

Charmet G (2011) Wheat domestication: lessons for the future. CR Biol 334:212–220

Chen Z, Young TE, Ling J, Chang SC, Gallie DR (2003) Increasing vitamin C content of plants through enhanced ascorbate recycling. Proc Nat Acad Sci U S A 100:3525–3530

Conklin PL, Norris SR, Wheeler GL, Williams EH, Smirnoff N, Last RL (1999) Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. Proc Nat Acad Sci U S A 96:4198–4203

Conklin PL, Saracco SA, Norris SR, Last RL (2000) Identification of ascorbic acid-deficient Arabidopsis thaliana mutants. Genetics 154:847–856

Conklin PL, DePaolo D, Wintle B, Schatz C, Buckenmeyer G (2013) 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

Cronje C, George GM, Fernie AR, Bekker J, Kossmann J, Bauer R (2012) Manipulation of L-ascorbic acid biosynthesis pathways in Solanum lycopersicum: Elevated GDP-mannose pyrophosphorylase activity enhances L-ascorbate levels in red fruit. Planta 235:553–564

Cruz-Rus E, Amaya I, Sánchez-Sevilla JF, Botella MA, Valpuesta V (2011) Regulation of L-ascorbic acid content in strawberry fruits. J Exp Bot 62:4191–4201

Davey MW, Keulemans J (2004) Determining the potential to breed for enhanced antioxidant status in Malus: mean inter- and intravarietal fruit vitamin C and glutathione contents at harvest and their evolution during storage. J Agric Food Chem 52:8031–8038

DeBolt S, Cook DR, Ford CM (2006) L-tartaric acid synthesis from vitamin C in higher plants. Proc Natl Acad Sci U S A 103:5608–5613

De Gara L, De Pinto MC, Arrigoni O (1997) Ascorbate biosynthesis and ascorbate peroxidase activity during the early stage of wheat germination. Physiol Planta 100:894–900

Di Matteo A, Sacco A, Anacleria M, Pezzotti M, Delledonne M, Ferrarini A, Frusciante L, Barone A (2010) The ascorbic acid content of tomato fruits is associated with the expression of genes involved in pectin degradation. BMC Plant Biol 10:163–173

Dowdle J, Ishikawa T, Gatzek S, Rolinski S, Smirnoff N (2007) Two genes in *Arabidopsis thaliana* encoding GDP-L-galactose phosphorylase are required for ascorbate biosynthesis and seedling viability. Plant J 52:673–689

Endres S, Tenhaken R (2009) Myoinositol oxygenase controls the level of myoinositol in Arabidopsis, but does not increase ascorbic acid. Plant Physiol 149:1042–1049

Foyer CH, Noctor CH (2011) Ascorbate and glutathione: the heart of the redox hub. Plant Physiol 155:2–18

Franceschi VR, Tarlyn NM (2002) L-Ascorbic acid is accumulated in source leaf phloem and transported to sink tissues in plants. Plant Physiol 130:649–656

Franck F, Baetens M, Lammertyn J, Verboven P, Davey MW, Nicolaï BM (2003) Ascorbic acid concentration in cv. Conference pears during fruit development and postharvest storage. J Agric Food Chem 51:4757–4763

Fukunaga K, Fujikawa Y, Esaka M (2010) Light regulation of ascorbic acid biosynthesis in rice *via* light responsive *cis*-elements in genes encoding ascorbic acid biosynthetic enzymes. Bios Bio Biochem 74:888–891

Gautier H, Massot C, Stevens R, Sérino S, Génard M (2009) Regulation of tomato fruit ascorbate content is more highly dependent on fruit irradiance than leaf irradiance. Annl Bot 103:495–504

Gatzek S, Wheeler GL, Smirnoff N (2002) 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

Gergoff Grozeff GE, Bartoli CG (2014) Participation of ascorbic acid in the dormancy establishment of poplar lateral branch buds. J For Res 19:301–304

Gergoff Grozeff GE, Chaves A, Bartoli CG (2010) Ethylene regulates ascorbic acid content during dark-induced leaf senescence. Plant Sci 178:207–212

Gergoff Grozeff GE, Senn ME, Alegre M, Chaves AR, Bartoli CG (2016) Nocturnal low irradiance pulses improve fruit yield and lycopene concentration in tomato. Sci Hort 203:47–52

Gest N, Gautier H, Stevens R (2013) Ascorbate as seen through plant evolution: the rise of a successful molecule? J Exp Bot 64:33–53

Gilbert L, Alhagdow M, Nunes-Nesi A, Quemener B, Guillon F, Bouchet B, Faurobert M, Gouble B, Page D, Garcia V (2009) The GDP-D-mannose epimerase (GME) plays a key role at the intersection of ascorbate and non-cellulosic cell wall biosynthesis in tomato. Plant J 60:499–508

Giovannoni JJ (2004) Genetic regulation of fruit development and ripening. Plant Cell 16:S170–S180

Hancock RD, Walker PG, Pont SDA, Marquis N, Vivera S, Gordon SL (2007) L-Ascorbic acid accumulation in fruit of Ribes nigrum occurs by in situ biosynthesis via the L-galactose pathway. Funct Plant Biol 34:1080–1091

Hao J, Yin Y, Fei SZ (2013) Brassinosteroid signaling network: implications on yield and stress tolerance. Plant Cell Rep 32:1017–1030

Huang HW, Wang Y, Zhang ZH, Jiang ZW, Wang SM (2004) Actinidia germplasm resources and kiwifruit industry in China. Hort Sci 39:1165–1172

Imai T, Ban Y, Terakami S, Yamamoto T, Moriguchi T (2009) L-Ascorbate biosynthesis in peach: cloning of six L-galactose pathway-related genes and their expression during peach fruit development. Physiol Plant 136:139–149

Ioannidi E, Kalamaki MS, Engineer C, Pateraki I, Alexandrou D, Mellidou I (2010) Expression profiling of ascorbic acid-related genes during tomato fruit development and ripening and in response to stress conditions. J Exp Bot 60:663–678

Ishikawa T, Dowdle J, Smirnoff N (2006) Progress in manipulating ascorbic acid biosynthesis and accumulation in plants. Physiol Plant 126:343–355

Kerchev PI, Pellny TK, Vivancos PD, Kiddle G, Hedden P, Driscoll S, Vanacker H, Verrier PJ, Hancock RD, Foyer CH (2011) The transcription factor ABI-4 is required for the ascorbic acid-dependent regulation of growth and regulation of jasmonate-dependent defense signaling pathways in Arabidopsis. Plant Cell 23:3319–3334

Keller R, Springer F, Renz A, Kossmann J (1999) Antisense inhibition of the GDP-mannose pyrophosphorylase reduces the ascorbate content in transgenic plants leading to developmental changes during senescence. Plant J 19:131–141

Kiddle G (2004) The role of ascorbate in plant defence and development. Ph. D. thesis. University of Bristol, UK

Kim JG, Takami Y, Mizugami T, Beppu K, Fukuda T, Kataoka I (2006) CPPU application on size and quality of hardy kiwifruit. Sci Hort 110:219–222

Laing WA, Martínez-Sánchez M, Wright MA, Bulley SM, Brewster D, Dare AP, Rassam M, Wang D, Storey R, Macknight RC, Hellens RP (2015) An upstream open reading frame is essential for feedback regulation of ascorbate biosynthesis in Arabidopsis. Plant Cell 27:772–786

Lee SK, Kader AA (2000) Pre and postharvest factors influencing vitamin C content in horticultural crops. Post Biol Technol 20:207–220

Li M, Ma F, Liu J, Li J (2010) Shading the whole vines during young fruit development decreases ascorbate accumulation in kiwi. Physiol Planta 140:225–237

Li M, Ma F, Shang P, Zhang M, Hou C, Liang D (2009) Influence of light on ascorbate formation and metabolism in apple fruits. Planta 230:39–51

Li M, Ma F, Zhang M, Pu F (2008) Distribution and metabolism of ascorbic acid in apple fruits (*Malus domestica* Borkh cv. Gala). Plant Sci 174:606–612

Linster CL, Clarke SG (2008) L-Ascorbate biosynthesis in higher plants: the role of VTC2. Trend Plant Sci 13:567–573

Linster CL, Gomez TA, Christensen KC, Adler LN, Young BD, Brenner C, Steven G, Clarke SG (2007) Arabidopsis VTC2 encodes a GDP-L-Galactose Phosphorylase, the last unknown enzyme in the Smirnoff-Wheeler pathway to ascorbic acid in plants. J Biol Chem 282:18879–18885

Loewus FA (1999) Biosynthesis and metabolism of ascorbic acid and of analogs of ascorbic acid in fungi. Phytochemistry 52:193–210

Lorence A, Chevone BI, Mendes P, Nessler CL (2004) Myo-inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. Plant Physiol 134:1200–1205

Mapson W, Breslow E (1958) Biological synthesis of ascorbic acid: L-Galactono-γ-lactone Dehydrogenase. Biochem J 68:395–406

Mapson LW, Isherwood FA, Chen YT (1954) Biological synthesis of L-ascorbic acid: the conversion of L-galactono-γ-lactone into L-ascorbic acid by plant mitochondria. Biochem J 56:21–28

Maruta T, Yonemitsu M, Yabuta Y, Tamoi M, Ishikawa T, Shigeoka S (2008) Arabidopsis phosphomannosei somerase 1, but not phosphomannose isomerase 2, is essential for ascorbic acid biosynthesis. J Biol Chem 283:28842–28851

Massot C, Stevens R, Génard M, Longuenesse JJ, Gautier H (2012) Light affects ascorbate content and ascorbate-related gene expression in tomato leaves more than in fruits. Planta 235:153–163

Mastropasqua L, Borraccino G, Bianco L, Paciolla C (2012) Light qualities and dose influence ascorbate pool size in detached oat leaves. Plant Sci 183:57–64

Mateos RM, Bonilla-Valverde D, del Río LA, Palma JM, Corpas FJ (2009) NADP-dehydrogenases from pepper fruits: effect of maturation. Physiol Plant 135:130–139

Mateos RM, Jiménez A, Román P, Romojaro F, Bacarizo S, Leterrier M, Gómez M, Sevilla F, del Río LA, Corpas FJ, Palma JM (2013) Antioxidant systems from Pepper (*Capsicum annuum* L.): involvement in the response to temperature changes in ripe fruits. Int J Mol Sci 14:9556–9580

Mazorra LM, Senn ME, Gergoff Grozeff GA, Fanello DD, Carrión CA, Núñez M, Bishop GJ, Bartoli CG (2014) Impact of brassinosteroids and ethylene on ascorbic acid accumulation in tomato leaves. Plant Physiol Biochem 74:315–322

Melino VJ, Hayes MA, Soole KL, Ford CM (2011) The role of light in the regulation of ascorbate metabolism during berry development in the cultivated grapevine *Vitis vinifera* L. J Sci Food Agric 91:1712–1721

Mellidou I, Keulemans J, Kanellis AK, Davey MW (2012) Regulation of fruit ascorbic acid concentrations during ripening in high and low vitamin C tomato cultivars. BMC Plant Biol 12:239

Millar AH, Mittova V, Kiddle G, Heazlewood JL, Bartoli CG, Theodoulou FL, Foyer CH (2003) Control of ascorbate synthesis by respiration and its implications for stress responses. Plant Physiol 133:443–447

Müller-Moule P (2008) An expression analysis of the ascorbate biosynthesis enzyme VTC2. Plant Mol Biol 68:31–41

Nishiyama I, Yamashita Y, Yamanaka M, Shimohashi A, Fukuda T, Oota T (2004) Varietal difference in vitamin C content in the fruit of kiwifruit and other Actinidia species. J Agric Food Chem 52:5472–5475

Nunes-Nesi A, Carrari A, Lytovchenko A, Smith AMO, Loureiro ME, Ratcliffe RG, Sweetlove LJ, Fernie A (2005) Enhanced photosynthetic performance and growth as a consequence of decreasing mitochondrial malate dehydrogenase activity in transgenic tomato plants. Plant Physiol 137:611–622

Osorio S, Nunes-Nesi A, Stratman M, Fernie AR (2013) Pyrophosphate levels strongly influence ascorbate and starch content in tomato fruit. Front Plant Sci 4:308

Pallanca JE, Smirnoff N (2000) The control of ascorbic acid synthesis and turnover in pea seedlings. J Exp Bot 345:669–674

Pastori GM, Kiddle G, Antoniw J, Bernard S, Veljovic-Jovanovic S, Verrier PJ, Noctor G, Foyer CH (2003) Leaf vitamin C contents modulate plant defense transcripts and regulate genes controlling development through hormone signaling. Plant Cell 15:939–951

Pineau B, Layoune O, Danon A, De Paepe R (2008) L-Galactono-1,4-lactone dehydrogenase is required for the accumulation of plant respiratory complex I. J Biol Chem 283:32500–32505

Piotrowska-Niczyporuk A, Bajguz A (2014) The effect of natural and synthetic auxins on the growth, metabolite content and antioxidant response of green alga *Chlorella vulgaris* (Trebouxiophyceae). Plant Growth Reg 73:57–66

Prudent M, Causse M, Génard M, Tripodi P, Grandillo S, Bertin N (2009) Genetic and physiological analysis of tomato fruit weight and composition: Influence of carbon availability on QTL detection. J Exp Bot 60:923–937

Qin A, Shi Q, Yu X (2011) Ascorbic acid contents in transgenic potato plant overexpressing two dehydroascorbate reductase genes. Mol Biol Rep 38:1557–1566

Rodrigues RB, De Menezes HC, Cabral LMC, Dornier M, Reynes M (2001) An amazonian fruit with a high potential as a source of vitamin C: camu-camu (*Myrciaria dubia*). Fruits 56:345–354

Rohde A, Bhalerao R (2007) Plant dormancy in the perennial context. Trend Plant Sci 12:217–223

Sasaki-Sekimoto Y, Taki N, Obayashi T, Aono M, Matsumoto F, Sakurai N, Suzuki H, Hirai MY, Noji M, Saito K, Masuda T, Takamiya K, Shibata D, Ohta H (2005) Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. Plant J 44:653–668

Schertl P, Sunderhaus S, Klodmann J, Gergoff Grozeff GE, Bartoli CG, Braun HP (2012) L-galactono-1,4-lactone dehydrogenase (GLDH) forms part of three subcomplexes of mitochondrial complex I in *Arabidopsis thaliana*. J Biol Chem 287:14412–14419

Schimmeyer J, Bock R, Meyer EH (2016) L-Galactono-1,4-lactone dehydrogenase is an assembly factor of the membrane arm of mitochondrial complex I in Arabidopsis. Plant Mol Biol 90:117–126

Siendones E, Gonzalez-Reyes JA, Santos-Ocaña C, Navas P, Córdoba F (1999) Biosynthesis of ascorbic acid in kidney bean. L-galactono-γ-lactone dehydrogenase is an intrinsic protein located at the mitochondrial inner membrane. Plant Physiol 120:907–912

Stevens R, Buret M, Duffe P, Garchery C, Baldet P, Rothan C, Causse (2007) Candidate genes and quantitative trait loci affecting fruit ascorbic acid content in three tomato populations. Plant Physiol 143:1943–1953

Szarka A, Horemans N, Kovács Z, Gróf P, Mayer M, Bánhegyi G (2007) Dehydroascorbate reduction in plant mitochondria is coupled to the respiratory electron transfer chain. Physiol Plant 129:225–232

Tamaoki M, Mukai F, Asai N, Nakajima N, Kubo A, Aono M, Saji H (2003) Light-controlled expression of a gene encoding L-galactono-g-lactone dehydrogenase which affects ascorbate pool size in Arabidopsis thaliana. Plant Sci 164:1111–1117

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

Tokunaga T, Miyahara K, Tabata K, Esaka M (2005) Generation and properties of ascorbic acid-overproducing transgenic tobacco cells expressing sense RNA for L-galactono-1,4-lactone dehydrogenase. Planta 220:854–863

Valpuesta V, Botella MA (2004) Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant. Trends Plant Sci 9:573–577

Vanderslice JT, Higgs DJ, Hayes JM, Block G (1990) Ascorbic acid and dehydroascorbic acid content of food-as-eaten. J Food Comp Anal 3:105–118

Voxeur A, Gilbert L, Rihouey C, Driouich A, Rothan C, Baldet P, Lerouge P (2011) Silencing of the GDP-D-mannose-3,5-epimerase affects the structure and cross-linking of the pectic polysaccharide rhamnogalacturonan II and plant growth in tomato. J Biol Chem 286:8014–8020

Wang SY, Line HS (2000) Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. J Agric Food Chem 48:140–146

Wheeler G, Ishikawa T, Pornsaksit V, Smirnoff N (2015) Evolution of alternative biosynthetic pathways for vitamin C following plastid acquisition in photosynthetic eukaryotes. Elife 4: e06369

Wolucka BA, Goossens A, Inzé D (2005) Methyl jasmonate stimulates the *de novo* biosynthesis of vitamin C in plant cell suspensions. J Exp Bot 56:2527–2538

Wolucka BA, Van Montagu M (2003) 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 28:47483–47490

Xu HI, Xu Q, Li F, Feng Y, Qin F, Fang W (2012) Applications of xerophytophysiology in plant production-LED blue light as a stimulus improved tomato crop. Sci Hortic 148:190–196

Yabuta Y, Mieda T, Rapolu M, Nakamura A, Motoki T, Maruta T, Yoshimura K, Ishikawa T, Shigeoka S (2007) Light regulation of ascorbate biosynthesis is dependent on the photosynthetic electron transport chain but independent of sugars in Arabidopsis. J Exp Bot 58:2661–2671

Yabuta Y, Maruta T, Nakamura A, Mieda T, Yoshimura K, Ishikawa T, Shigeoka S (2008) Conversion of L-Galactono-1,4-lactone to L-ascorbate is regulated by the photosynthetic electron transport chain in Arabidopsis. Biosci Biotechnol Biochem 72:2598–2607

Zhang C, Ouyang B, Yang C, Zhang X, Liu H, Zhang Y, Zhang J, Li H, Ye Z (2013) Reducing AsA leads to leaf lesion and defence response in knock-down of the AsA Biosynthetic enzyme GDP-D-Mannose Pyrophosphorylase gene in tomato plant. PLoS ONE 8:e61987

Zhang W, Lorence A, Gruszewski HA, Chevone BI, Nessler CL (2009) *AMR1*, an Arabidopsis gene that coordinately and negatively regulates the mannose/L-galactose ascorbic acid biosynthetic pathway. Plant Physiol 150:942–950