The Role of Ascorbate in Plant Growth and Development

Ana Ortiz-Espín, Antonio Sánchez-Guerrero, Francisca Sevilla, and Ana Jiménez

Abstract The participation of redox changes and antioxidants in the control of plant growth and development is important for the normal course of these physiological processes. Ascorbate (AsA) is a multifunctional molecule that is highly necessary for both plants and animals. It performs a variety of functions such as being a major redox buffer and cofactor for enzymes involved in multiple cell processes, including hormone biosynthesis, photosynthesis, and respiration as well as in the regeneration of other antioxidants. In addition to its well-known antioxidant properties, AsA is able to influence normal cell cycle progression and plant growth, and it takes part in signal transduction events. Reactive oxygen and nitrogen species and other antioxidants are also involved in these processes. This chapter focuses on the involvement of AsA and its interaction with redox regulatory signaling networks during growth and development at cellular, tissue, and organ levels, including its participation in cell cycle and proliferation, seed germination and during embryogenesis and meristem development in root and shoots, as well as during flowering. The book chapter seeks to supply valuable information of the current state of research on this multifaceted molecule in these processes at various organization levels.

Keywords Cell cycle · Cell elongation · Embryogenesis · Seed development · Root development · Shoot development · Flowering

1 Introduction

Ascorbate (AsA) is one of the main antioxidants in the cell, but its functions go beyond this in the cell metabolism, acting as a key regulator in plant growth and development. The presence of AsA in plants seems to be indispensable for survival and indeed, plants without ascorbate are inviable (Dowdle et al. 2007). The

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M. A. Hossain et al. (eds.), *Ascorbic Acid in Plant Growth, Development and Stress Tolerance*, https://doi.org/10.1007/978-3-319-74057-7_2

isolation of mutants in the biosynthetic AsA pathway containing less AsA or the application of a biosynthesis inhibitor like lycorine are useful tools for the study of plant development in a low AsA content environment. Some of the clearest evidence of the role of AsA in plant growth has been reported in *Arabidopsis thaliana* vitamin C (*vtc*) mutants. These plants have altered the AsA biosynthetic pathway, and hence levels of this antioxidant are lower than in wild-type (WT) plants. Interestingly, these mutants showed a slow-growth phenotype and accelerated senescence (Conklin et al. 1999; Veljovic-Jovanovic et al. 2001a; Pastori et al. 2003; Dowdle et al. 2007). On the other hand, the inhibition of the synthesis of AsA by lycorine reduces the rate of cell division which is complemented with the addition of AsA (Arrigoni et al. 1997b; Stasolla et al. 2008). The correlation between AsA and growth capacity has been reported in different plant tissues (Liso et al. 1988; Córdoba-Pedregosa et al. 2003). AsA pool and redox status together with GSH and other metabolites regulate cell cycle progression and the maturation of several organs, including seeds, roots, shoots, or flowers (Fig. 1).

Plant growth and development are strongly guided by phytohormones. AsA acts as a cofactor of dioxygenases in the biosynthesis of several phytohormones such as ethylene, gibberellins (GA), auxins (IAA), and abscisic acid (ABA). The endoge-

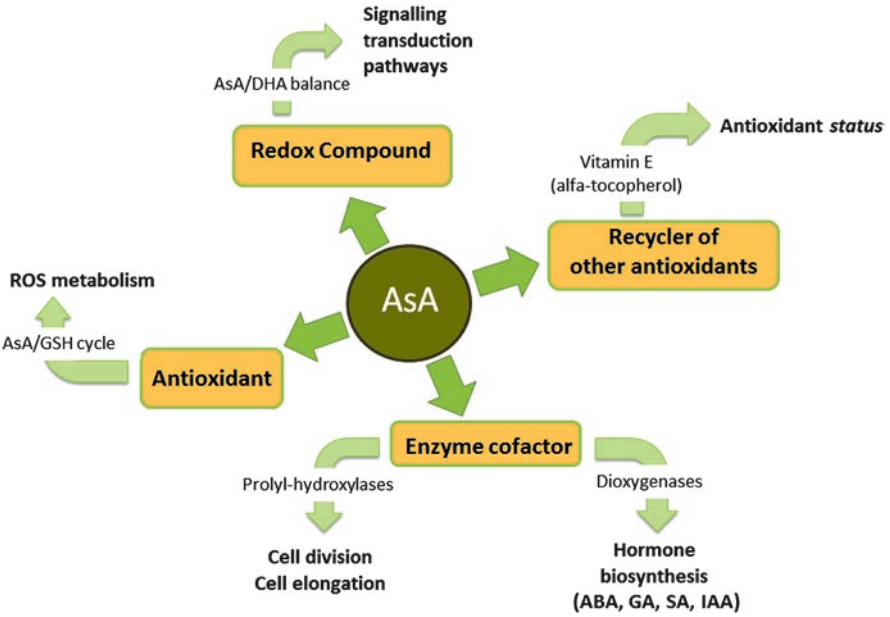

Fig. 1 Main roles of AsA in plant growth and development. AsA participates in different pathways leading to plant growth and development. AsA acts as an antioxidant, removing ROS through AsA-GSH cycle and it also stimulates the antioxidant capacity of the cell by recycling other antioxidants, such as vitamin E. The relative pool of reduced versus oxidized AsA forms perturbs the cell redox status, leading to different signaling transduction pathways. AsA also acts as enzyme cofactor of enzymes involved in cell growth and hormone biosynthesis

nous AsA influences the biosynthesis of phytohormones, as well as their signal transduction pathway. For example, some findings support the view that ABA and ABA signaling participate in the ascorbate-dependent control of growth. Arabidopsis mutants that show low levels of AsA induce high levels of ABA and hence, a restrained growth (Pastori 2003; Kerchev et al. 2011). IAA is also largely involved in the stimulation of plant growth, and it indirectly modifies the AsA redox status and consequently the growth state. These findings suggest that AsA is involved in the metabolic cross talk among redox-regulated pathways and hormones (Fig. 1).

All these aspects encourage us to deepen our knowledge of the role of AsA in the growth and plant development to unravel not only the biological significance of this key antioxidant in plants but also to increase the yields of our harvests (Paciolla et al. 2001).

2 Cell Cycle and Cell Elongation

There is a lot of evidence pointing to the level of AsA being a modulator of progression during the cell cycle. One of the earliest reports dealing with this topic is by Liso et al. (1984, 1988) in onion root cells. Roots are divided into different zones according to cell division and differentiation (see Sect. 5). Particularly studied are the cells from the quiescent center (QC) located in the root tip. These cells suffer a low rate of division and a larger G1 phase. The addition of AsA to the root tips leads to a shortening of the G1 phase and a prompted entry into the S-phase, stimulating the activity of the QC cells but also cell proliferation in the entire root meristem and in the pericycle (Liso et al. 1988; Arrigoni et al. 1989; Innocenti et al. 1990). In contrast, the inhibition of the AsA synthesis by lycoporine blocked the cell cycle in G1 and G2 phases, indicating a role for AsA in the cell cycle progression through the G1/S and G2/M transitions. A similar correlation was also observed during the growth of *Zea mays* (Kerk and Feldman 1995), *Lupinus albus* seedlings (Arrigoni et al. 1997a), pea roots (Citterio et al. 1994), and tobacco BY2 cells (de Pinto et al. 1999).

The state of competence in cells is an important issue when evaluating the effects of AsA in cell division. This state makes cells able to respond to antioxidant and hormones stimuli, which lead to cell cycle progression. Only in competent cells of embryo roots is the quick transition through the G1/S-phase checkpoint possible due to high levels of AsA. In some species, AsA does not promote cell cycle progression if cells are not competent (Arrigoni et al. 1989; Innocenti et al. 1990), suggesting that competence is a state which is necessary but not sufficient for cell cycling; the progression has also to be stimulated.

The influence of AsA redox status and AsA oxidized forms are also important in cell cycle progression. Ascorbate acts as an electron donor and is converted to a free radical intermediate monodehydroascorbate (MDHA) which presents a relatively short lifetime and can be spontaneously deprotonated to the final oxidized form, dehydroascorbate (DHA). The regeneration of these compounds to the reduced state is carried out by two reductases: MDHA reductase (MDHAR) using NADPH as

electron donor and DHA reductase (DHAR) which uses GSH as reductant. This last reaction generates glutathione disulfide (GSSG), which is in turn re-reduced to GSH by NADPH, a reaction catalyzed by glutathione reductase (GR). All these reactions are part of the so-called Foyer-Halliwell-Asada cycle (Foyer and Halliwell 1976), which together with ascorbate peroxidase (APX), is in charge of H_2O_2 scavenging. This cycle constitutes the main redox buffer in the cell (Jiménez et al. 1997; Foyer and Noctor 2011) and also a mechanism for the regeneration of the oxidized forms of ascorbate and glutathione, the main antioxidants in the plant cell (Noctor and Foyer 1998; Sevilla et al. 2015).

Changes in the AsA redox state (ratio AsA/DHA) have been observed during cell division (with a ratio of 6–10) and elongation (ratio of 1–3), indicating that the reduced state of AsA is predominant during cell division (Arrigoni et al. 1992). This correlation between AsA redox status and cell proliferation was later corroborated by (Kerk and Feldman 1995) in QC of maize cells, and by de Pinto et al. (1999) in tobacco BY2 cells. In this sense, sometimes the influence exerted by the oxidized forms of AsA on the cell cycle is not attributable to a rise in ascorbate concentration. As an example, De Cabo et al. (1993) demonstrated the action of MDHA and not AsA, in the shortening of the G1 phase in onion root meristem cells, so stimulating growth. Moreover, a specific inhibitory effect of DHA on the cell cycle have been proved (Cordoba and Gonzalez-Reyes 1994). In this context, Arrigoni et al. (1992) found that levels of DHA were higher in non-meristematic than meristematic zones and also that its contents in different tissues were rather related to its mitotic activity (Foyer and Mullineaux, 1998). A relationship exists between the levels of DHA and expression and activity of the enzymes DHAR (Arrigoni et al. 1997a; de Pinto et al. 1999; Potters et al. 2004) and ascorbate oxidase (AO) (Pignocchi et al. 2003). Levels of DHAR are higher in meristematic zones than expanding cells, allowing lower levels of DHA in these areas and stimulating an elevated rate of cell division (Potters et al. 2000). The DHAR enzyme uses GSH as cofactor, so levels of this antioxidant can also contribute to regulate DHA levels and hence, cell cycle. However, the importance of GSH in the reduction of DHA by Halliwell-Asada mechanism in the cell cycle is under debate. Similarly to AsA, GSH has a positive effect on the progression to cell cycle. Whereas high levels of GSH induce cell proliferation, high levels of GSSG block the cell cycle (Potters et al. 2004). Additionally, high ascorbate and glutathione levels are required for normal progression of the cell cycle in meristematic tissues (Potters et al. 2000; Vernoux et al. 2000; Jiang 2003).

Despite the fact that both AsA and GSH act in the cell cycle, they seem to exert their functions independently. It has been reported that GSH did not affect AsA pools and did not revert the effects of DHA over cell division (de Pinto et al. 1999; Potters et al. 2004). All this suggests that DHA reduction may be occurring in a glutathione-independent pathway and that both antioxidants act independently in the control of plant growth (Potters et al. 2004). In this sense, the enzymes thioredoxin reductase and glutaredoxin have been described as alternative reductive enzymes of DHA (Potters et al. 2002; Meyer et al. 2012).

The redox state in each cellular compartment is highly important in the cell cycle progression (Pignocchi et al. 2003; Martí et al. 2009; Diaz Vivancos et al.

2010; Calderón et al. 2017). It has been demonstrated that the redox state of the nuclei is an important factor, and GSH content is low at early G1 whereas it increases during the G1- and S-phases of the cell cycle (Diaz Vivancos et al. 2010). These redox fluctuations in the nucleus create an oxidative status early in the G1 phase followed by a reductive environment determined by an increase in GSH, necessary for cell cycle progression and to overcome the G1/S checkpoint (De Simone et al. 2017). The redox state of the cell at the G1-S is determined by *de novo* synthesis of GSH (Diaz Vivancos et al. 2010), and the level of GSH may directly modulate the synthesis of DNA after mitogenic stimulation (Suthanthiran et al. 1990; Poot et al. 1995). On the other hand, *vtc* mutants with lower ascorbate showed a higher growth due to a major oxidative stress and a slow growth under the addition of ascorbate (De Simone et al. 2017). Also, the importance of AsA during the logarithmic growth phase in tobacco cultured cells has been emphasized (Kato and Esaka 1999; De Pinto et al. 2000). Transient oxidations have been described in the current model of cell cycle regulation, in which ROS bursts serve to regulate key proteins thiol–disulfide exchange reactions at critical cysteine residues and, in this way, regulate cell cycle progression (Menon and Goswami 2007). In this redox control, GSH and thiol reductases such as thioredoxins have redundant roles in cell proliferation. As an example, TBY-2-overexpressing PsTrxo1 mutants in culture cells increased proliferation rate, particularly in the basal and stationary phase coinciding with an upregulation of proteins involved in DNA synthesis and an increase in nuclear GSH levels, but a decrease in total GSH pool (Ortiz-Espín et al. 2015; Calderón et al. 2017).

Several reports indicate that symplastic and apoplastic AsA play a relevant role in the control of cell division and proliferation (Arrigoni 1994; de Pinto et al. 1999; Horemans et al. 2000). The apoplast constitutes the space between the plasma membrane and the cell wall, and it plays an important role in the transition of cells from division to elongation (Cordoba and Gonzalez-Reyes 1994; Kato and Esaka 1999). Besides, being in contact with external environment, the apoplast constitutes the first line of defense against stressor and environmental clues (Hernández et al. 2001; Pignocchi and Foyer 2003). In the apoplast, AsA is oxidized to MDHA by AO. As we commented above, MDHA is an unstable radical and rapidly deprotonate to yield DHA and AsA. DHA is then transported into the cytosol by a specific carrier that preferentially translocates the oxidized form in exchange for the reduced form. In this way, a continuous flux of reducing power to the cell wall is ensured (Horemans et al. 2000), balancing AsA/MDHA levels in apoplast and symplast according to the needs of the cell. Changes in expression and activity of the apoplastic enzyme AO have been related with changes in DHA along the progression of the cell cycle (de Pinto et al. 1999; Pignocchi and Foyer 2003; De Pinto and De Gara 2004; Fotopoulos et al. 2006). The stimulation of AO activity provokes an increase in ascorbate oxidized forms which stimulates cell elongation without affecting cell division (de Pinto et al. 1999; Pignocchi et al. 2003; Li et al. 2017). The regulation of the ascorbate redox state in the apoplast is one of the mechanisms proposed to explain how ascorbate promotes cell expansion and elongation. An increase of AO is detected in rapidly elongating tissues and higher contents of MDHA and DHA are found in

these tissues. MDHA acts as an electron acceptor in transmembrane electron transport, where cytochrome B, transports electrons from NADH across the plasma membrane onto MDHA, which is reduced to AsA. The intensification of this process results in plasma membrane hyperpolarization which is followed by activation of plasma membrane H^+-ATPase. The activity of this enzyme provokes an acidification of apoplast that promotes cell wall loosening (González-Reyes et al. 1994). Simultaneously, the NADH oxidation enhances acidification in cytoplasm, activating vacuolar H^+-ATPase, which increases cell vacuolization and hence, cell expansion (González-Reyes et al. 1994; Horemans et al. 2000). Moreover, AsA can transform O_2 to H_2O_2 in the presence of Cu^{2+} and by means of Fenton reaction generates $OH^{\cdot}$ radicals, which intensify the oxidative status in the apoplast. The acidification of apoplast triggers the oxidative degradation of polysaccharides (Fry 1998; Schopfer 2001) and delayed cell wall lignification, reducing precursors of peroxidase-dependent lignin synthesis (Takahama 1992; Padu 1999; Fig. 2).

Another mechanism points to the hydroxylation of proteins as a key factor of cell division and elongation. Ascorbate is a cofactor of the enzyme prolyl hydroxylase. This enzyme provokes the posttranslational hydroxylation of proline residues of cell wall-associated glycoproteins like extensins and arabinogalactan proteins, which are involved in cell wall stiffening, cell proliferation, and signaling (Cooper et al. 1994; De Tullio et al. 1999). The lack of AsA will prevent the hydroxylation of these proteins and hence, may block the cell cycle and in fact, it has been reported that a lower rate of hydroxylation of proline residues induced a cell cycle block during metaphase (De Tullio et al. 1999).

All the above evidence suggests the regulatory role of AsA pool and AsA redox status in the cell growth. In this sense, AsA redox status in the apoplast seems to be critical in the fate of the cell towards division or elongation.

3 Embryogenesis

The process of zygotic embryogenesis (ZE) in higher plants comprises the part of the development which leads to the formation of dry mature seeds and it is divided into three phases. The first stage is a process of morphogenesis, where the polar axis of the plant body is defined with the generation of the shoot and root poles, embryonic axis and cotyledons (West and Harada 1993). The second phase comprises the embryo maturation where storage reserves are accumulated and finally, the embryo becomes desiccated and the formation of the seed comes to an end. During the morphogenesis phase, AsA has been described as regulator of polarity. This process starts with the transverse division of a zygote into an apical, proembryo, and basal cells that gives rise to the suspensor. During this division, a reduced state of AsA promotes the transverse cell division, whereas an increase of DHA provokes a longitudinal division (Chen and Gallie 2012). The elongation of newly formed cells is favored by higher contents of DHA, which reduce AsA redox status (Belmonte and Yeung 2004). A declined in the reduced AsA carries on up to the end of seed

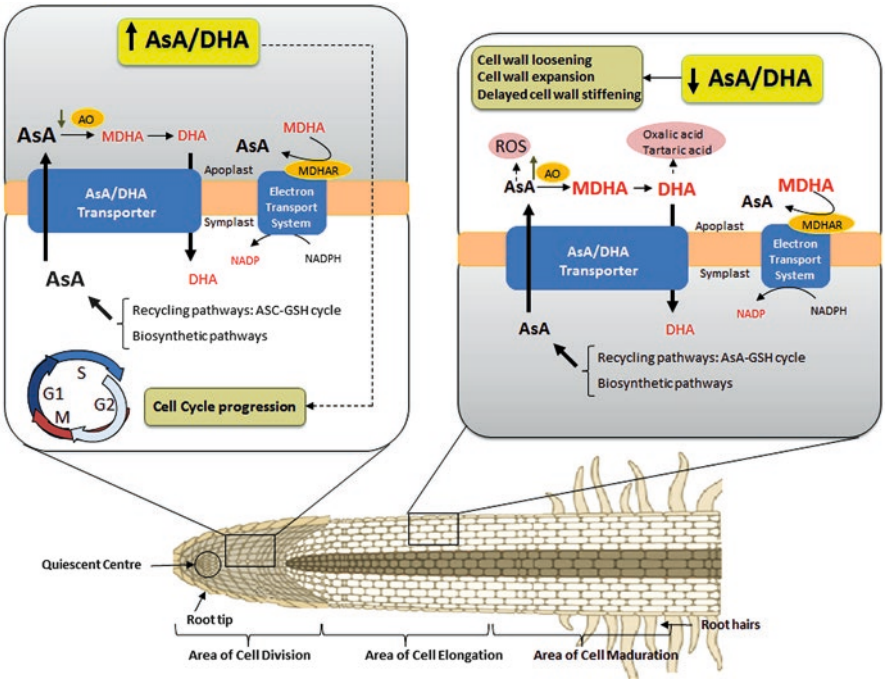

Fig. 2 AsA redox status in apoplast regulates cell cycle progression and cell wall elongation by antagonic mechanism in meristematic versus mature cells in root onion. According to acidic growth hypotheses, pH between apoplast and symplast regulates cell division and elongation. (1) In meristematic cells (area of cell division), high levels of AsA are correlated with low levels and activity of ascorbate oxidase (AO), which leads to a higher AsA/DHA ratio. This AsA redox status in apoplast enhances cell cycle, stimulating G1-S transition and consequently, cell division. (2) In differentiated cells (area of cell elongation), higher levels of ascorbate oxidase lead to an increase in AsA oxidized forms (MDHA) and products of secondary oxidations (DHA, oxalic acid, tartaric acid, ROS). Besides, the hyperpolarization of the plasma membrane induced by the oxidation of NADH triggers the activity of H^+ATPase, which further decreases the pH in apoplast. These processes decrease the AsA redox status and enhance oxidative degradation of wall polysaccharides (cell wall loosening), increasing vacuolar volume (cell wall expansion), and decreasing precursors of lignification (delayed cell wall stiffening). Consequently, cell wall elongation occurs

maturation, where almost all AsA is oxidized. Throughout this process, the role of DHAR is prevalent in controlling the AsA redox status. These results are in agreement with the general oxidative status observed in dry seeds. In this condition, it is easy to maintain a lower metabolic state and long-term storage (Tommasi et al. 2001).

Somatic embryogenesis is characterized by the growth of the plant from somatic cells or indirectly through an intermediate callus phase. The development of the new somatic embryos is reminiscent of that of ZE. This process is used in the production of genetically identical plants with desirable traits for agricultural and commercial applications. The role of AsA in the formation of somatic embryos has been

investigated using white spruce (*Picea glauca*) embryogenesis as a model system. Important changes in AsA metabolism have been observed in the maturation and germination processes in this species. During the first days of germination, embryos accumulate high levels of AsA with a high redox state and present a decrease in APX activity. Moreover, the inhibition of AsA synthesis by lycorine resulted in a lower DNA synthesis due to a decrease in the incorporation of purines and pyrimidines in nucleotides (Stasolla et al. 2008). Therefore, an increase in AsA pool and higher AsA/DHA ratio towards the reduced state is required for the progression of cell divisions within developing embryos (Stasolla and Yeung 2001). During the maturation of somatic embryos, AsA pool diminishes followed by a decrease in the ratio of AsA/DHA. The change from a reduced to an oxidized redox environment is favorable for the final development in the embryo. In fact, the application of glutathione biosynthesis inhibitor, buthionine sulfoximine, which turns GSH to GSSG, increased the quality of somatic embryos and their regeneration ability (Stasolla et al. 2008), inducing the expression of meristem formation genes such as *SHOOTMERISTEM/ZWILLE* and embryo maturation genes involved in ABA responsive and storage (*LEA* genes). Consequently, somatic embryos reach an appropriate morphology and physiology maturity, improving their post-embryonic growth (Stasolla and Yeung 2001; Stasolla et al. 2008; Stasolla 2010). On the other hand, a strict control of AsA pool must be exerted during the meristem formation, because an antagonic effect on development may be achieved above a threshold. The stimulating role of AsA during meristem formation in apical shoots involves inactivation of the peroxidase-dependent cell wall stiffening. An increase in AsA content decreases the activities of ferulic acid and guaiacol peroxidases allowing cell division and cell growth, while their activities increase in lycorine-treated embryos resulting in meristem abortion (Stasolla and Yeung 2007; Stasolla 2010). As another example, no apical dominance and early formation of auxiliary buds have been reported in tomato with suppressed AsA synthesis (Zhang 2012).

All these experiments demonstrate that the antioxidant pool and redox state of AsA and GSH are very important in the transition from cell division to maturity state during both zygotic and somatic embryogenesis. The knowledge of the mechanisms underlining the changes in these antioxidants will help us to unravel their specific role in the embryo development.

4 Seed Germination

A cell cycle of a seed involves three phases: development, maturation (see Sect. 3), and germination. Mature seeds are able to germinate under advantageous environmental conditions. The water imbibition of seed reactivates metabolism and the growth of seminal tissues. This process involves an increase of ROS and antioxidants including AsA, as well as redox proteins such as thioredoxin (Trx) or peroxiredoxin (Prx) (Simontacchi et al. 1993; De Gara et al. 1997; De Tullio and Arrigoni 2003; Martí et al. 2011; Ortiz-Espín et al. 2017). Oxidative imbalances can reduce

the potential for proper seed germination (Chen et al. 2014b) and so a tight regulation is required to balance ROS production and scavenging. AsA contents increase in the early stages of germination and this is correlated with an increase in the enzyme L-galactono-1,4-lactone dehydrogenase (GLDH), the last enzyme in AsA synthesis. In agreement with this, the DHA reduction capacity and APX activity decreases gradually (De Gara et al. 1991, 2000, Tommasi et al. 1999, 2001). Therefore, the higher AsA levels seem to be due to an *ex novo* synthesis rather than to AsA recycling (Tommasi et al. 1995, 2001). On the other hand, whereas in dry seed levels of GSSG and GSH are balanced, during germination the increase of GSH is accompanied by a strong decrease in GSSG and an increase in GR, indicating an increased recycling ability in the cell. Therefore, the opposite changes in DHAR and GR during germination indicate that GSH may have other functions not related with AsA-GSH cycle. In this sense, it has been demonstrated that the change in GSH redox status has a role in allowing protein synthesis by changes in the redox state of cysteine residues (Kranner and Grill 1993). In this regard, other thiol-redox proteins such as thioredoxins have been studied during germination (Ortiz-Espín et al. 2017). In this case, expression of promoter Trx::GUS in embryo axes and cotyledons coincided with AsA but not GSH, suggesting its role in redox regulation in these tissues rather than GSH and the existence of a feasible link between AsA and Trx (Ortiz-Espín et al. 2015). In fact, it has been reported in tobacco that DHA inhibits the activity of the enzymes regulated by the thioredoxin-peroxiredoxin system such as ribonuclease, involved in nucleotides synthesis (Morell et al. 1997), suggesting that AsA could stimulate cell proliferation (indirectly) through the Trx activity. Other evidences point to seeds of KO mutants of ascorbate oxidase and Trx under salt stress behaving in a similar way, causing early germination in salt imbibed seeds (Yamamoto et al. 2005; Ortiz-Espín et al. 2015). All these changes suggest that these components of the antioxidant and redox systems participate in the regulation of ROS levels and redox status during germination under stress conditions.

Seed germination is governed by an adequate ratio ABA/GA and during this process levels of ABA decreased in favor of GA which enhanced germination up to the seedling stage (Kucera et al. 2005; Finkelstein et al. 2008). The mechanism that directs this process seems to be related with ROS and AsA. The application of ABA to imbibed seeds provokes a decrease of ROS which leads to an inhibition of AsA production and a repression of GA biosynthesis. Moreover, GA biosynthesis was inhibited by lycorine, an inhibitor of AsA biosynthesis, and by reduced ROS levels, suggesting the role of AsA in this process. Additionally, the application of exogenous AsA can partially rescue seed germination from an ABA treatment (Ye et al. 2012). Therefore, AsA can control ABA/GA biosynthesis during seed germination.

Another interesting point is the influence of AsA in the germinating process by modulating the sensitivity to ABA and IAAs through APX6, which replaces APX1 as the dominant ascorbate peroxidase in dry seeds. The *apx6* defective mutant seeds showed an altered homeostasis of primary metabolites including accumulation of TCA cycle metabolites, ABA and IAAs and a repression of seed germination. These seeds respond to ABA or IAA in germination assays and show a repression of the ABA expression positive regulators, ABI3 and ABI5, but an induction of ABI4.

Therefore, the patterns of expression of these genes could be the reason for the inhibition of germination in *apx6* seeds in an ABI3-independent way (Chen et al. 2014a). These results imply the importance of APX6 activity for the seeds maturation-drying phase since it modulates the ROS signal cross talk with hormone signals to execute the germination program properly.

In short, all this evidence supports the role of AsA as an inductor of seed germination. AsA participates in a complex metabolic network integrated by ROS, hormones, and other antioxidants and redox proteins which regulate the development of the process.

5 Root and Shoot Development

AsA level is linked to root growth, development of root architecture, and root response to gravity (Olmos et al. 2006). The main evidence relating AsA with plant growth and development was obtained in onion root models (*Allium cepa* L.), (Liso et al. 1988). Roots can be divided into three parts according to the stage of differentiation: tip, medium zone, and base. Tip contains meristematic (apical meristem) and non-differentiated cells which include the quiescent center (QC), a group of cells where cell division proceeds very slowly or not at all, but the cells are capable of resuming meristematic activity if surrounding tissue is damaged. In the medium zone, the newly formed cells grow and elongate. Finally, at the base of the root, tissues are fully differentiated and hair roots and lateral roots emerge from this area (Fig. 2).

An analysis of AsA and AsA-GSH cycle partners inside the root provides deeper information about the requirements of cells according to their degree of differentiation. Studies of Córdoba-Pedregosa et al. (2003) showed a close relationship with the age of the tissue, which is related to the degree of differentiation and AsA contents. Whereas older tissues (base of root) have low AsA, younger tissues (tip root) have higher AsA levels, confirming the well-described relationship between AsA and cell proliferation (see Sect. 2). As stated above, AsA promotes cell division by inducing G1 to S progression of cells, and high AsA concentrations are required for normal progression of the cell cycle in meristematic tissues, including elongation of root tips in which AsA pool increases (De Pinto et al. 2000; Potters et al. 2000). In the QC of the root, the AsA content is substantially lower and AO mRNA and activity are found to be higher relative to the adjacent meristematic cells (Kerk and Feldman 1995). This condition enhances the oxidative status in apoplast, which blocks the cell cycle (Pignocchi 2006). As AO activity is auxin regulated (see Sect. 2), an involvement of this enzyme in the maintenance of QC cell identity has been suggested (Pignocchi 2006). AO-overexpressing tobacco plants showed high DHA in apoplast, which leads to an oxidation of the redox state in the apoplast and an insensitive response to IAA. The stimulation of ROS by auxin mediates plant growth reactions (Schopfer and Bechtold, 2002; Liszkay et al. 2004). Due to the high oxi-

dative status of the apoplast, auxin has minimal effect on oxidative signaling in these plants. On the other hand, AO has been shown to participate in the degradation of IAA by oxidative decarboxylation (Kerk et al. 2000), which suggests a role for AO in the regulation of auxin levels.

In addition, it is known that AsA and MDHA stimulate the elongation of onion roots and, simultaneously, high vacuolization of meristematic cells (Hidalgo et al. 1989; Cordoba and Gonzalez-Reyes 1994; González-Reyes et al. 1994, 1998). During root elongation, there is an increase in the cell volume by an intake of water by the protoplast and relaxation of the crossed bonds that link several components of the cell wall (McCann and Roberts 1994). In this process, AsA might facilitate elongation by inhibiting apoplastic peroxidases and activating hydroxylation of cell wall proteins as we commented on detail in Sect. 2 (Takahama 1992, 1993).

Knock out (KO) *vtc* Arabidopsis mutants deficient in AsA are very useful to explore the effects of low AsA content in the root and shoot development. Among the five *vtc* mutant lines (*vtc1-5*), only *vtc1* and *vtc2* showed changes in root growth, particularly in the length of primary roots and the presence of lateral roots (LR) (Conklin et al. 2000; Olmos et al. 2006; Dowdle et al. 2007; Tyburski et al. 2012). *Vtc2* mutant, which has 30% less AsA than *vtc1*, showed more and larger primary roots and a greater number of LRs than WT and *vtc1*. Besides, this mutant lost its gravitropism (Olmos et al. 2006) evidencing the importance of controlled oxidation in the tropic responses mediated by ROS and auxins (Joo et al. 2001). However, related to shoot development, both *vtc1* and *vtc2* showed shorter shoots than WT plants (Olmos et al. 2006). The main reasons for these differences seem to reside not only in the low levels of AsA, but also in its cellular location. *Vtc* mutants presented low levels of AsA in the apoplast (Veljovic-Jovanovic et al. 2001a) with the *vtc2* being the one with the lowest values. These experiments support the evidence that the redox state of the apoplast is essential in the modulation of plant growth responses (Olmos et al. 2006).

Another interesting aspect is the relationship between AsA and nutrients as regulators of root growth. It has been reported that MDHAR activity stimulates nutrient intake in Arabidopsis and is required for LR growth (González-Reyes et al. 1994; Foreman et al. 2003; Millar et al. 2003). Different studies using limiting sources of nitrogen and phosphorous have been carried out to analyze the effect on the shoot and root development and the participation of these nutrients in the signaling involved in the root architecture (Zhang and Forde 2000; Signora et al. 2001; Olmos et al. 2006). Low nitrate levels stimulate the formation of LRs in *A. thaliana*, whereas higher levels are inhibitory, and this effect is mitigated by increasing sugar, suggesting the antagonistic interaction between nitrate and sugar in the regulation of LR. However, levels of nitrate in *vtc1* and *vtc2* mutants did not alter the root architecture, suggesting that the enhanced LR growth in *vtc2* may depend entirely on an observed increase in ROS by the activation of the AtrbohC NADPH oxidase (Olmos et al. 2006). Nitrate could exercise its effect through ROS signaling and hence, the oxidative stress in *vtc2* mutant could attenuate the effect of nitrate. Interestingly, the short-root phenotype in *vtc1* has no relation with AsA deficiency

or the accumulation of H_2O_2 (Barth et al. 2010) and this mutant showed a disturbed protein N-glycosylation, which is associated with auxin and ethylene homeostasis and/or nitric oxide signaling (Barth et al. 2010).

Deficiencies in phosphorous (P) provoke a decreasing primary root elongation and development of abundant LRs (López-Bucio et al. 2002; Sánchez-Calderón et al. 2005, 2006; Nacry et al. 2005). Ascorbate can partially reverse the effects of P deficiency so increasing primary root elongation, although under high P levels, AsA inhibits the growth of primary roots. The differences in AsA content and redox state in the apical parts of primary roots of plants grown under various regimes of phosphate availability explain the observed differences. It has been suggested that the effects of AsA on root growth are possibly mediated by its effect on cell division activity in the apical root meristem. However, in these conditions, the behavior of *vtc1* mutant was found to be similar in the presence and absence of P (Tyburski et al. 2012), suggesting that other factors apart from AsA influence the root growth. A poorly adjusted auxin transport and signaling could lead to an auxin accumulation in the root apical meristem and provoke the arrest of root growth under low P availability (Sánchez-Calderón et al. 2005; Nacry et al. 2005).

The cross talk between hormones and redox status is emerging as a key regulatory point of control of plant growth and developmental processes. Emergence of LR is a highly coordinated process in which auxin plays a central role and, in fact, plants containing high levels of auxins produce more LRs than plants impaired in auxin signaling (Fukaki et al. 2007). The importance of auxins in AsA metabolism was also demonstrated by the observed increase in AO protein and transcripts in root tissues incubated with auxin (Pignocchi and Foyer 2003). In this context, it has been suggested that ROS, auxins and antioxidants like AsA and GSH form a redox signaling network are involved in plant development and response to adverse environments (De Tullio et al. 2010). ROS have been proposed as signaling actors during auxin-regulated LR formation although the underlined mechanisms are poorly understood. In *aux* mutants presenting a disruption in the auxin-mediated cell wall accommodation and remodeling in the sites of LR formation, ROS specifically are deposited in the apoplast during LR emergence (Orman-Ligeza et al. 2016). Also in tomato roots, auxin treatments increased the oxidized form DHA and induced root growth arrest but also provoked the production of LRs as an oxidative-mediated response (Tyburski et al. 2008). In Arabidopsis, auxin receptor (TIR1/AFB) mutants *tir1 afb2* presented an increased tolerance to salinity and displayed a reduced accumulation of H_2O_2 as well as enhanced antioxidant activity and showed higher levels of AsA than WT plants, indicating the involvement of auxin/redox interaction in the adaptation (Iglesias et al. 2010).

Therefore, these results indicate that the growth of primary and lateral roots is stimulated by antagonistic mechanisms. Whereas the growth of lateral roots seems to be stimulated by a high oxidative stress which is triggered by auxins and a low AsA availability, the growth of primary roots does not seem to depend on these factors.

6 Flowering

Timing of flowering is crucial for plant reproduction. The initiation of the process under unfavorable conditions can result in an aborted fruit development and hence, the plant will die without leaving offspring. Therefore, plants are able to activate or repress flowering in response to different environmental clues. One of the first indications of the relationship between flowering and environment was reported by J. Tournois (1914), who showed the strong influence of day/night length on flower induction. A century later, several papers have confirmed the influence of light (photoperiod, light quality, light intensity) in the transition from the vegetative to the reproductive state (Imaizumi et al. 2006; Johansson and Staiger 2014; Song and Shim 2015). Temperature is also an important factor and several plants need short exposure to low temperature (vernalization) to sense the end of the cold season (Lokhande et al. 2003; Thines et al. 2014; Airoldi et al. 2015; Capovilla et al. 2015). Other environmental factors such as drought (Kenney et al. 2014), levels of nutrients (Levy and Dean 1998), or CO2 concentration (Jagadish et al. 2016) are equally important. In addition, plants contain a set of internal factors such as hormones (ABA, GA) and antioxidants (AsA, GSH), which participate in the regulation of flowering (Ogawa et al. 2001; Barth et al. 2006; Chai et al. 2012; Weng et al. 2016). Therefore, both internal and external clues are sensed and integrated in the plant in order to establish properly the moment to flourish. In this context, several reports indicate that AsA can act as a repressor or activator of flowering depending on culture conditions such as photoperiod or growth media. Suppressed expression of the apoplastic AO gene delays flowering (Yamamoto et al. 2005) while exogenous addition of AsA or the precursor L-galactono-γ-lactone (GalL) in WT plants delayed growth and flowering in long-day-grown Arabidopsis plants (Attolico and De Tullio 2006) but induced growth and early flowering in short-day-grown plants (Barth et al. 2006). In the opposite case, Arabidopsis AsA-deficient *vtc* mutants showed an earlier flowering compared to WT plants and the addition of exogenous AsA delayed flowering (Conklin and Barth 2004; Kotchoni et al. 2009) irrespective of photoperiod. In this case, although AsA acts as a repressor of flowering, the pathway involved must differ from that suggested by Barth et al. (2006). Interestingly, these results contrast with data obtained by other authors, whose report a delayed instead of early flowering in *vtc1* mutants (Veljovic-Jovanovic et al. 2001b; Pastori 2003). However, in this case, the growth conditions differed, justifying the different behavior. Another set of evidence points to a lack of AsA oxidizing enzymes (APX or AO) as important elements in the regulation of flowering and mutants in genes that showed a delayed flowering with respect to WT (Pnueli et al. 2003). Consequently, all these experiments show that AsA (as repressor or activator) is related to time of flowering and that this is strongly governed by culture conditions. Besides, synthesis and recycling AsA pathways seem equally important in flowering. Nevertheless, the reason for the reported contrasting flowering phenotypes remains unresolved.

The transition of a plant from vegetative to reproductive stage increase the levels of antioxidants and ROS suggesting that plants must undergo an oxidative stress during the flowering process (Hirai et al. 1995; Lokhande et al. 2003; Shen et al. 2009). This oxidative stress has been hypothesized as being the cause of the early flowering in plants from lower latitudes (Lokhande et al. 2003). However, in *vtc* mutants, levels of H_2O_2 are similar to or slightly higher than in WT plants. In addition, the AsA redox state in the low AsA pool of these mutants remained unchanged, suggesting that oxidative stress and AsA redox state are not directly related with the early-flowering phenotype observed in *vtc* mutants. Curiously, other AsA mutants, which showed a delayed-flowering phenotype, such as the double mutant deficient in cytosolic and thylakoid AsA peroxidase (APX) exhibited early flowering under oxidative stress (Pnueli et al. 2003). Moreover, mutants lacking AO displayed both, higher levels of AsA and AsA redox status than WT plants in control conditions (Yamamoto et al. 2005). Therefore, flowering time can be explained by differences in AsA redox state and ROS levels, but only in some mutants. The relationship between the oxidative metabolism, AsA, and flowering has gone further with the studies of (Senthil Kumar et al. 2016) in orchids. The application of AsA to orchids significantly elevated the NO content and enzyme activities associated (nitrate and nitrite reductase). On the other hand, the addition of an NO donor on Arabidopsis *vtc1* mutant delayed flowering and decreased the expression level of some flowering-associated genes suggesting that NO signaling is vital for flowering repression (Senthil Kumar et al. 2016). In contrast, treatment with L-galacturonate of an NO-deficient *noa1* mutant did not alter its flowering time, suggesting that AsA is necessary for NO-biosynthesis involved flowering-repression pathway mediated by NO (Van Ree et al. 2011; Senthil Kumar et al. 2016). Therefore, although the role of AsA in the oxidative metabolism during flowering remains open, the direct implication in nitrosative metabolism is an interesting point of future research into the role of AsA in flowering pathways.

The relationship of AsA with flowering is reinforced when we analyze genetic data. Gene expression studies show that AsA affects the expression of flowering-related genes such as LEAFY, which encode a key transcription factor in flowering-inductive pathways (Attolico and De Tullio 2006). The addition of L-galactono-1,4-lactone to plants under long-day delayed expression of the LEAFY gene. In contrast, the application of GAs, an inductor of flowering (see below), engendered both, an early LEAFY expression and flowering. Later, a more integral study was performed by (Kotchoni et al. 2009). The gene expression analysis of *vtc* mutants showed an upregulation of genes related to flowering, circadian clock, and photoperiod pathway. In contrast, these genes were downregulated in the presence of AsA (Kotchoni et al. 2009), independently of photoperiod. Most important evidence points to AsAs possibly participating downstream in flowering pathways through expression of LEAFY genes, or upstream through circadian clock and photoperiod pathways, although its specific role remains inconclusive.

Flowering is a process intrinsically governed by hormones. It has been demonstrated that the application of GA or salicylic acid (SA) induces early flowering, whereas the application of ABA represses it. As commented in previous sections,

AsA participates as a key cofactor in the biosynthesis of hormones (Arrigoni and De Tullio 2000, 2002), so one would expect the influence of this antioxidant in flowering. Indeed, *vtc* mutants show high levels of ABA (Pastori 2003) and SA (Conklin and Barth 2004) and low contents of GA (Kiddle 2004; Foyer 2007) which is congruent with their delayed-flowering phenotype. The limiting activity of different GA-oxidases, which participates in the biosynthesis of GA (Kiddle 2004; Barth et al. 2006; Foyer 2007) could be responsible for low GA levels. Ascorbate acts as a cofactor of 2-oxoglutarate-dependent dioxygenases (2-ODDs) preventing their inactivation by over-oxidation (De Tullio et al. 2004; Clifton et al. 2006). On the other hand, an increase in NCED dioxygenase activity (Pastori 2003), enzyme involved in ABA biosynthesis pathways, may contribute to the late-flowering phenotype in short days. Surprisingly, these results seem to be contradictory because ABA needs AsA for their biosynthesis. However, Pastori speculates this behavior as a compensation mechanism for the decreased cofactor availability. Abscisic acid may (indirectly) contribute to the downregulation of LEAFY transcription factor, and hence to the delayed flowering.

All in all, a lot of evidence indicates the participation of AsA in flowering; the early or delayed phenotype observed in overexpression and AsA-deficient mutants respectively, the up/downregulation of genes related to flowering in the absence/presence of AsA and the changes in ABA/GA contents with AsA. Thus, the differences observed in the oxidative metabolism in the different mutants point to AsA being a relevant enzymatic cofactor, rather than antioxidant, in the flowering process.

7 Conclusions

The studies reported in this chapter indicate that AsA plays important roles in growth, differentiation, and metabolism. Ascorbate participates in plant growth-promoting cell division in meristems and cell elongation in differentiated tissues. Ascorbate participates in hormone biosynthesis and the AsA redox state is governed by recycling and oxidizing enzymes which respond to hormone stimulus. These elements are integrated in a complex metabolic network which leads to plant growth and development. The role of AsA is supplemented by other antioxidants and redox proteins such as GSH or thioredoxins which participate as signaling molecules and contribute to the redox state of the cell. In this regard, the manipulation of AsA/GSH redox state opens up vast possibilities for their use in the improvement of tissue culture and plant regeneration methods.

Acknowledgments This work was supported by Séneca Foundation, Murcia, Spain (Project 19876/GERM/15 and contract of A O-E), and MINECO/FEDER (project BFU2014-52452-P and FPI grant of A S-G). The authors apologize to the scientists that are not cited because of space limitation and thank Steve Hasler for correction of the written English of the manuscript.

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