

Chapter 2

Biological Role of Ascorbate in Plants

Ascorbate, as a strong and active antioxidant, plays an important role in keeping human health. It helps promote iron absorption, increase antibody concentration and improve human immunity. Ascorbate is used to regenerate α -tocopherol (vitamin E), and remove radicals which could induce cancer and senescence. Ascorbate is beneficial to strengthen blood vessel and decrease cholesterol concentration. Ascorbate can help prevent arteriosclerosis related cardiovascular diseases as well as hypertension and apoplexy. Ascorbate prevents disease associated with connective tissue (e.g., scurvy), promotes the formation of collagen and development of cartilage, strengthens tooth, tightens skin and facilitates wound healing [1].

In contrast to most animals, humans lack the ability to synthesize ascorbate as a result of a mutation in the last enzyme required for ascorbate biosynthesis. Human must absorb ascorbate from dietary sources, especially plant products such as fresh fruit and vegetable [2].

Consistent with its multi-function in human and animals, ascorbate in plants has beneficial influences on various aspects in plants. Through modifying gene expression, ascorbate not only act to regulate defense and survival but also act via phytohormones to modulate plant growth [3]. Emerging research results indicate that ascorbate, existing widely in plants as the abundant micromolecule substance, fulfils its essential roles in series of physiological processes such as plant defense against oxidization, co-factor of key enzymes, plant cell division, cell expansion, growth and development, and senescence [4–6]. Ascorbate, at least in some plant species, is also the substrate for the biosynthesis of oxalate and tartrate [7, 8].

However, the multifunction of ascorbate makes it complicated to decipher its exact role under certain physiological process. *Arabidopsis thaliana* mutants lacking ascorbate cannot grow well, but it is not known which function is critical: control of reactive oxygen or the proposed roles in modulating cell expansion and division [9]. Thus, there is need to increase our understanding of this enigmatic molecule since it could be involved in a wide range of important functions from

antioxidant defence and photosynthesis to growth regulation. The collection of *vtc* mutants should contribute to a deeper understanding of the proposed functions of this multifaceted molecule.

2.1 Role in Plant Growth and Development

Ascorbate is an important growth regulator as well as antioxidant for plants. Both the ascorbate mutants in *Arabidopsis* and the various transgenic plant species with relation to ascorbate synthesis are frequently reported to show altered growth and development. Up to date, the plant mutants without ascorbate have not been identified, which may be a demonstration that the ascorbate is an indispensable molecule for plants to survive [10]. The much low amount of ascorbate (15 % of the wild-type control) in transgenic tomato with gene suppression of GDP-D-mannose-3,5-epimerase (GME) leads to severe growth retardation (unpublished data). These evidences together with the fact that ascorbate accumulation level varies with developmental stages in plants strongly indicate that the ascorbate is closely involved in plant growth and development.

Ascorbate as well as its metabolism related enzymes is involved in the control of plant growth processes. Ascorbate modulates plant growth probably by controlling several basic biological processes, such as (i) the biosynthesis of hydroxyproline-rich proteins required for the progression of G1 and G2 phases of the cell cycle, (ii) the crosslinking of cell wall glycoproteins and other polymers, and (iii) redox reactions at the plasmalemma involved in elongation mechanisms. The ascorbate free radical induces a high vacuolization responsible for elongation. This effect may be dependent on the activity of the redox system linked to the plasmalemma. The modulation of the plasmalemma energetic state derived from the ascorbate-induced hyperpolarization and the activity of an intrinsic transplasmalemma ascorbate-regenerating enzyme has been the basis of ascorbate-mediated plant growth regulation.

Recent research results propose that the mechanism for ascorbate regulation of plant growth and development may reside in its interaction with phytohormones. Ascorbate is cofactor for biosynthesis of several phytohormones such as ethylene, gibberellins (GA) and abscisic acid (ABA). The endogenous ascorbate influences the biosynthesis of phytohormones, as well as the signal transduction pathway mediated by phytohormones [3]. The ascorbate in leaves could regulate the plant growth through interaction with phytohormones [3]. Transcript changes indicate that growth and development are constrained in ascorbate defective mutant, *vtc1*, by the modulation of abscisic acid signaling. Abscisic acid contents are significantly higher in *vtc1* than that in the wild-type [3].

The onion root has been the model for investigating the role of ascorbate on plant growth [11]. Exploring how ascorbate participates in the plant growth and development regulation as an antioxidant will deepen insights into the biological function of the ascorbate.

2.1.1 Cell Division

Ascorbate is frequently reported to be related with cell division in plants. The ascorbate content in the meristem is usually higher than that in cell division-inactive tissue, such as quiescent center of the maize root tip. This is consistent with fact that the expression level of ascorbate oxidase gene (*AO*), which acts in ascorbate oxidation and metabolism, in the quiescent center was higher than that in the surrounding meristem cells [12]. Ascorbate has been implicated in regulation of cell division by influencing progression from G1 to S phase of the cell cycle [4]. The exogenous ascorbate promoted the G1 to S progression in root meristem and pericycle of onion resulting in decreasing cell number in the quiescent center.

The ascorbate and dehydroascorbate exerted significant impact on the process of cell division in the tobacco suspension cells [13]. Lycorine, the inhibitor of ascorbate, could prevent the cell division, while supplementary ascorbate could restore the cell division [14]. The decreasing of endogenous ascorbate resulted in retarded cell division, lowered growth rate of young branches, and slow plant growth, as demonstrated both *Arabidopsis* mutant [15] and transgenic tobacco [16].

The plasmalemma is a dynamic interface that perceives and transmits information concerning changes in the environment to the nucleus to modify gene expression. In plants, ascorbate is an essential part of this dialogue. The concentration and ratio of reduced to oxidized ascorbate in the apoplast possibly modulates cell division and growth [5]. Although ascorbate consumption is more or less the same during cell division and cell expansion, the ascorbate/monodehydroascorbate ratio is 6–10 during cell division and 1–3 during cell expansion, indicating the reduced state of ascorbate is more required for cell division [14].

Callus formation, the process in which cell division is vigorously activated, is also affected by ascorbate. Foliar application with ascorbate is shown to promote the callus formation from the cut surfaces of scion stems, and eventually improved the survival rate of scion after grafting [17].

2.1.2 Cell Wall Metabolism and Cell Expansion

The plant grows as the result of cell division, elongation and differentiation. The ascorbate, as well as several enzymes involved in ascorbate synthesis and metabolism is reported to affect the cell wall metabolism and cell growth directly or indirectly.

Ascorbate may act to regulate cellular process including cell wall metabolism by linking its biosynthesis with other cell metabolism. VTC4, the enzyme of L-galactose 1-phosphate phosphatase (GPP) for ascorbate synthesis, is shown to act as a bifunctional enzyme that affects myoinositol and ascorbate biosynthesis in plants. Myoinositol synthesis and catabolism are crucial for the production of

phosphatidylinositol signaling molecules, glycerophosphoinositide membrane anchors, and cell wall pectic noncellulosic polysaccharides [18].

The ascorbate helps to eliminate free radicals involved in xylogen synthesis, regulate the polymerization of xylogen monomers and the lignification of cell wall. The exogenous monodehydroascorbate will promote the cell growth and rooting of onion [19, 20]. The ascorbate peroxidase (APX) keeps the plasticity of cell wall by reducing hydrogen peroxide [19, 20]. The ascorbate reversibly inhibits the activity of apoplast APX, prevents the conversion and secretion of free radicals into the apoplast, and regulates the lignification of plant cell wall [20, 21]. The equilibrium between ascorbate and hydrogen peroxide regulates the polymerization of xylogen monomers, and thus modulates the cell wall lignification [22].

The monodehydroascorbate is formed by oxidizing the ascorbate with AO enzyme, and then reduced by cytochrome b on the plasmalemmas, during which transmembrane electron transport promotes the cell growth [4]. Addition of monodehydroascorbate to ascorbate-loaded plasmalemma vesicles caused a rapid oxidation of the cytochrome, followed by a slower re-reduction [23, 24]. Thus, the ascorbate free radical could act as an electron acceptor to the cytochrome-mediated electron transport reaction, and eventually promote cell growth, as the transmembrane electron transport has been shown to stimulate cell growth.

The ascorbate and dehydroascorbate in the cell wall can affect the crosslinking of cell wall protein and polysaccharide, leading to loosening of the cell wall [25]. The ascorbate-induced hydroxyl radicals was shown to promote oxidative scission of plant cell wall polysaccharides [26]. It is proposed that ascorbate non-enzymically reduces O_2 to H_2O_2 , and Cu^{2+} to Cu^+ , and that H_2O_2 and Cu^+ react to form $^{\bullet}OH$, which causes oxidative scission of polysaccharide chains. Although $^{\bullet}OH$ radicals are often regarded as detrimental, they are so short-lived that they could act as site-specific oxidants targeted to play a useful role in loosening the cell wall, e.g. during cell expansion, fruit ripening and organ abscission.

Ascorbate acts as substrate for oxalate biosynthesis, and apoplast oxalate can influence the crosslinking of pectin and elongation of cell wall by binding Ca^{2+} . The dehydroascorbate is converted to cell wall oxalate, which binds Ca^{2+} into crystallization and regulates the Ca^{2+} level in cell wall indirectly [22]. In addition, ascorbate is a cofactor for prolyl hydroxylase that posttranslationally hydroxylates proline residues in cell wall hydroxyproline-rich glycoproteins required for cell division and expansion [25].

The cell wall-localized AO enzyme, which is involved in ascorbate oxidation and metabolism, has been implicated in control of cell growth. AO enzyme are closely related to cell expansion and cell division [27, 28]. AO enzyme activity is found mainly in plant cell wall, especially in the fast growing cells [29, 30]. High AO activity in the cell wall is correlated with areas of rapid cell expansion.

The enzyme responsible for ascorbate synthesis is also reported to be involved in cell growth. In addition to ascorbate synthesis, L-galactose dehydrogenase (GalLDH), an enzyme for ascorbate synthesis, could play an important role in the regulation of cell growth-related processes in plants. In all *SIGalLDH*-RNAi plants with reduced *SIGalLDH* transcript and enzyme activity, plant growth rate was

decreased [31]. The most affected lines studied exhibited up to an 80 % reduction in SIGALDH activity and showed a strong reduction in leaf and fruit size, mainly as a consequence of reduced cell expansion.

All these evidence indicate that ascorbate and/or enzymes involved in its synthesis and metabolism are closely associated in the process of cell wall metabolism and cell expansion.

2.1.3 Shoot Apical Meristem Formation

Ascorbate level in plants can influence the plant shoot apical meristem formation [32]. Among the transgenic tomato plants expressing *GME* gene, which is responsible for ascorbate synthesis, several co-suppression transgenic plants were identified. Interestingly, the inhibition of apical buds formation and early initiation of auxiliary buds were observed in *SIGME2* co-suppression tomato seedlings (unpublished data).

The ascorbate content in the *SIGME2* co-suppression plants with abnormal shoots was only about 1.9–15.0 % of the wild-type control. This sharp decreasing in ascorbate in the *SIGME2* co-suppression lines was found to be associated with abnormal phenotype in seedlings. The apical bud growth was retarded, leading to the absence of apical dominance and early formation of auxiliary buds (Fig. 2.1) (unpublished data).

The *GME* co-suppression lines with significant decreasing of ascorbate content also exhibited brown spots on the apical buds and shortened internodes (Fig. 2.1; Table 2.1). In the *GME* co-suppression tomato plants, the brownish region appeared on the apical shoots of the seedlings of 4–5 true leaf stage (Fig. 2.1). The brown sector expanded 1 week later and the apical shoots were retarded 2 weeks later. At the same time, the axillary shoots below the apical shoots were generated and grew upward instead of the apical shoots. Some of the grown-up axillary shoots were also retarded in the same pattern with that of apical buds, and replaced by the surrounding clusters of axillary buds (Fig. 2.1). This phenotype changes were consistently observed in open filed and greenhouse condition in three generations, indicating that the phenotype alteration was most likely caused by gene suppression instead of environment or tissue culture (unpublished data).

This phenotype alteration was shown to be inheritable from T_0 to T_2 generations. However, in the T_2 generation of the *GME* co-suppression lines, not all the offspring plants exhibited the shoot retardation. The abnormal growth ratio of the co-suppression line of *GME2-1* and *GME2-3* was 17.8 and 13.9 %, respectively. To investigate the phenotype more accurately, the two-month old seedlings were utilized to analyze the average plant height, internode length, and axillary bud number. The *GME2-1* and *GME2-3* lines with growth retardation has the plant height of 6.93 cm (36 % of control) and 3.80 cm (20 % of control), respectively, while the untransformed control plant was 19.17 cm high (Table 2.1; Fig. 2.2). The co-suppression lines with growth retardation of *GME2-1* and *GME2-3* had the internode length of 0.86 cm (45 % of the control) and 0.89 cm (46 % of the

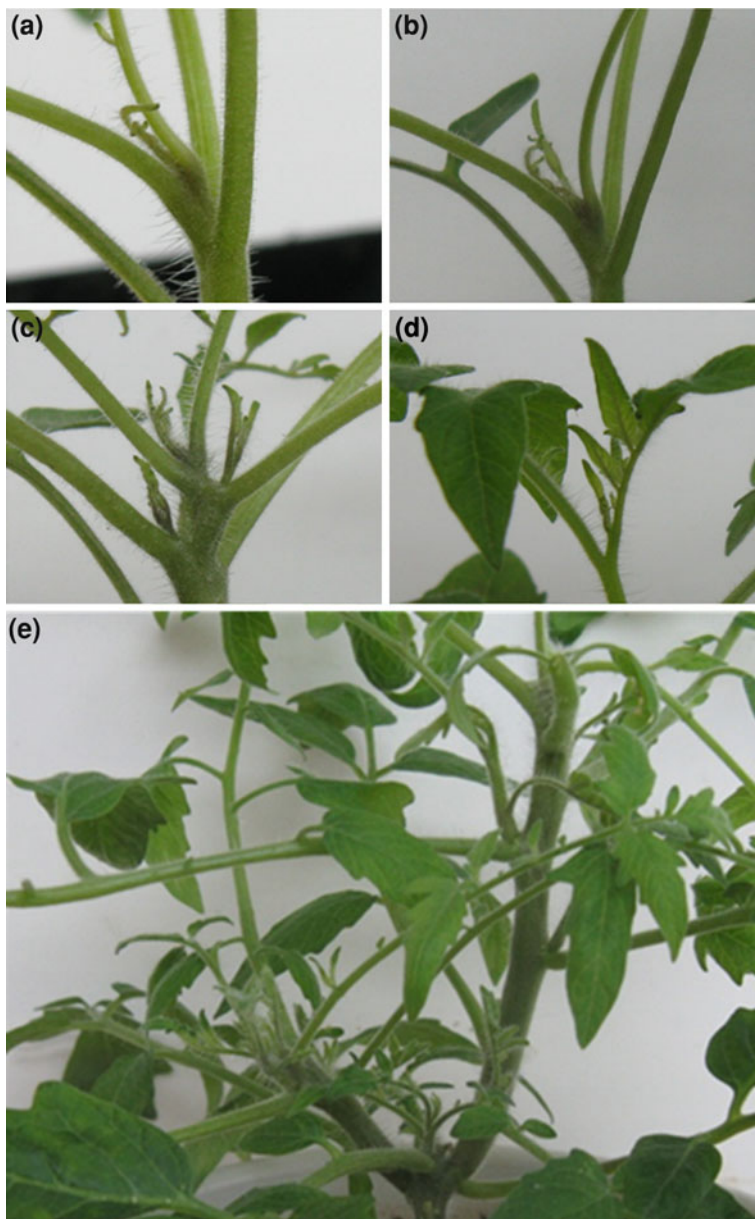


Fig. 2.1 Abnormal apical buds in seedlings of *GME* co-suppression tomato. **a** Apical buds with brown spots in *GME* co-suppression line; **b** Increase of brown areas on buds in *GME* co-suppression line; **c** Formation of lateral buds in *GME* co-suppression line; **d** Apical buds of wild-type seedlings; **e** Lateral shoot clusters grow over the apical shoots in *GME* co-suppression lines (courtesy of Professor Zhibiao Ye and Dr. Chanjuan Zhang)

Table 2.1 The average plant height, internode length and lateral bud number of *GME* transgenic plants and the wild-type control (WT)

Line	The average plant height (cm)	The average internode length (cm)	Number of lateral buds
WT	19.17 ± 1.47	1.92 ± 0.15	0
GME2-1(S)	6.93 ± 2.19 ^a	0.86 ± 0.30 ^a	1–2 ^a
GME2-3 (S)	3.8 ± 0.64 ^a	0.89 ± 0.23 ^a	2–3 ^a
GME2-1 (N)	20.9 ± 1.15	1.99 ± 0.14	0
GME2-3 (N)	17.05 ± 1.94	1.69 ± 0.03	0
GME1-40 (O)	19.13 ± 1.56	1.85 ± 0.18	0
GME2-6 (O)	21.05 ± 2.03	2.05 ± 0.25	0

S denotes the offspring plants with *GME* co-suppression and abnormal phenotype. *N* represents the transgenic offspring with mild co-suppression and normal phenotype. *O* represents the overexpressing lines with normal phenotype. ^a indicates values that are significantly different from those of wild-type

Fig. 2.2 *GME* co-suppression tomato (GME2-3) exhibited shortened internode length and lower plant height (courtesy of Professor Zhibiao Ye and Dr. Chanjuan Zhang)



control), respectively. However, the co-suppression lines of GME2-1 and GME2-3 without growth retardation and the *SIGME* overexpressing lines showed similar plant height and internode length with that of wild-type plants. Among the different lines tested, the axillary buds appeared only on the co-suppression lines of GME2-1 and GME2-3 with growth retardation (Table 2.1) (unpublished data).

It should be noted that ascorbate content in the co-suppression lines of GME2-1 and GME2-3 with growth retardation was much lower than that in other mild co-suppression lines without phenotype alterations. That means the restrained ascorbate level is supposed to be associated with the plant development retardation.

On the other hand, spraying ascorbate in advance could avert the *GME* co-suppression lines from abnormal growth. The co-suppression seedlings supplemented with exogenous ascorbate did not show brown spots or abnormal growth, while the control plants (sprayed with water) eventually exhibited the abnormal phenotype. For the four leaf stage seedlings, the co-suppression line of GME2-3 were hydroponically cultured for 2 days and then sprayed with 100 mmol/L ascorbate. The mock spraying (with water) lines developed more brown regions and the apical shoots were retarded with developing axillary buds.

The ascorbate supplement, however, resulted in a limited growth of axillary buds, and restored apical shoot growth. One week after the ascorbate spraying stopped, however, the resorted the apical shoots turned brownish again with abnormal growth, probably because the exogenously supplemented ascorbate was consumed, resulting in ascorbate shortage in the seedlings.

That the co-suppression of *SIGME2* resulted in retardation of the apical shoot growth in seedlings, which could be restored by spraying ascorbate solutions, suggested the abnormal phenotype was related to extreme deficiency of ascorbate. The abnormal apical shoots appeared again shortly after the exogenous ascorbate spraying stopped possibly because supplemented ascorbate was consumed and the plants were again confronted with ascorbate shortage. Up to now, in all the ascorbate defective mutants identified in *Arabidopsis*, the ascorbate content in leaves was no less than 30 % of the wild-type. The extremely low content (less than 15 % of wild-type) of ascorbate in the *SIGME2* co-suppression tomato plants was possibly the threshold for plants to survive.

That is reminiscent of the previous investigation that the RNA interference of the *SIGME1* and *SIGME2* simultaneously in tomato resulted in growth retardation [33]. However, the abnormal apical shoot development was not observed in this case. The exogenous supplement of ascorbate did not restore the phenotype in seedling [33]. The mild decreasing in *SIGME1-SIGME2 RNAi* plants might not activate plant sensitivity to the ascorbate supplement.

The retarded apical shoot development in the *GME* co-suppression tomato plants may result from cell apoptosis in apical meristem as revealed by cytological analysis (unpublished data). In the *SIGME2* co-suppression plants with abnormal shoot development, the paraffin section and transmission electron microscopy observation showed that most of the parenchymatous cells in the medulla adjacent to shoot apical meristem were collapsed or apoptotic (Fig. 2.3) (unpublished data).

The seedlings of five leaves stage were utilized for the paraffin section analysis. The vertical section of the *GME2-1* and *GME2-3* co-suppression lines showed that the brown spots did not affect the shoot apical meristem significantly. The normal leaf primordia could be observed. However, the parenchyma cells in the co-suppression lines were unusually dyed with abnormal structure compared to those regular well-organized cells in the wild-type control (WT). At the same time, more vascular bundles were observed in the co-suppression lines, indicating the abnormal apical shoot development probably came from cell death in the medulla (Fig. 2.4). And the more vascular bundles suggested that the apical shoot meristem of co-suppression lines activated early cell differentiation (unpublished data).

The plant aerobic metabolism such as respiration and photosynthesis will produce large amount of ROS, and the surplus ROS exert damages on the protein, unsaturated fatty acid and DNA, resulting oxidation injury and function disorder in cells. Both enzymatic and non-enzymatic antioxidants are utilized in plants to scavenge the excessive ROS. The decreasing amount of ascorbate, one of the most important antioxidants, in the *SIGME2* co-suppression plants, will certainly affect the plant's ability of scavenging ROS, resulting in the accumulation of ROS and oxidization damage.

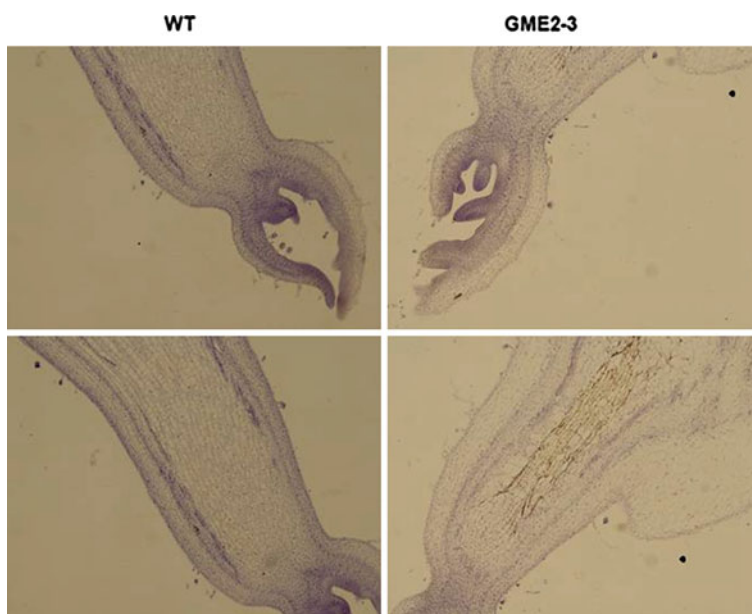


Fig. 2.3 Microsections of seedlings shoot apical meristem and stem from GME2-3 co-suppression line and control plants (WT) at five leaf stage (courtesy of Professor Zhibiao Ye and Dr. Chanjuan Zhang)

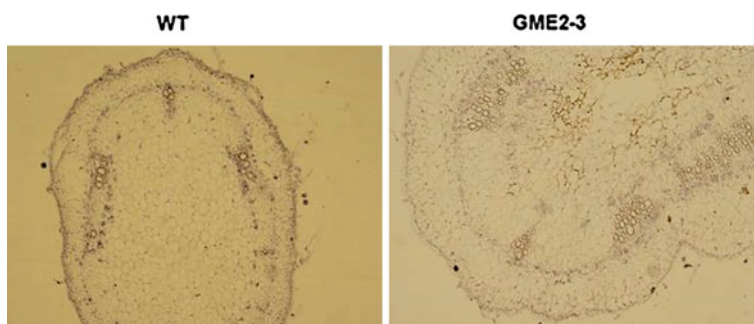


Fig. 2.4 Microsections of stem adjacent to shoot apical meristem from GME2-3 co-suppression line and control plants (WT) at five leaf stage (courtesy of Professor Zhibiao Ye and Dr. Chanjuan Zhang)

The *SIGME2* co-suppression lines showed increased sensitivity to oxidative stress. This is likely attributed to the sharply decreasing ascorbate in the co-suppression lines, since ascorbate metabolism affects the plant capacity to scavenge ROS and resistance to oxidative stress. The ROS act as cell death-inducing signal as well as xylem differentiation regulator. The increasing xylem number in the stem of tomato seedlings with *SIGME2* co-suppression was probably attributed to the accumulation of hydrogen peroxide (Fig. 2.4).

Thus the interesting co-suppression lines of *GME* presented a good demonstration that ascorbate is closely related with apical shoot development. The ascorbate promotion of shoot formation is also supported by physiological evidence. Application of ascorbate promotes shoot formation from the cut surface of tomato stems. Ascorbate levels and the activities of antioxidant enzymes in plants treated with ascorbate were higher than those in control plants. Conversely, hydrogen peroxide concentrations and malondialdehyde contents in plants treated with ascorbate were lower than those in control plants. It is supposed that cells in the calli formed at the cut surfaces re-differentiated earlier in ascorbate-treated plants than in those in control plants [34].

2.1.4 Root Development

Ascorbate level in plants is reported to be linked to development of root architecture and root response to gravity. And the ascorbate defective mutants in *Arabidopsis* provide efficient genetic tool to investigate ascorbate function in root development.

The root architecture is reported to be modulated in the *Arabidopsis* ascorbate defective mutants with moderately low (*vtc1*) or very low (*vtc2*) ascorbate content, as compared to wild-type [35]. Although the shoot development was comparable in all accessions over the first 14 d of growth, the production of primary roots was slightly different in *vtc1*, *vtc2*, and wild-type plants. The *vtc* mutants showed the antagonistic interaction between nitrate and sugar in the regulation of lateral root development that was observed in the wild-type. The *vtc2* mutant with much lower ascorbate level produced greater numbers of longer lateral roots than wild-type or *vtc1* plants at all levels of nitrate [35]. The most notable difference was that a high proportion of the primary roots of the *vtc2* plants grown on soil had lost the wild-type responses to gravity.

2.1.5 Photosynthesis

Ascorbate has proposed functions in photosynthesis as an enzyme cofactor (including synthesis of ethylene, gibberellins and anthocyanins). It has a major role in photosynthesis, acting in the Mehler peroxidase reaction with APX to regulate the redox state of photosynthetic electron carriers and as a cofactor for violaxanthin deepoxidase, an enzyme involved in xanthophyll cycle-mediated photo-protection [30].

Ascorbate accumulation in *Arabidopsis* leaves is increased by high light along with expression and activity of GDP-L-galactose phosphorylase (GGP, also VTC2), the enzyme responsible for ascorbate synthesis. That indicates the multiple roles of ascorbate during photosynthesis. These roles may include modulation of

hydrogen peroxide and singlet oxygen, enzyme cofactor in the xanthophyll cycle and, speculatively, a photosystem II electron donor during photoinhibition [9].

Role of ascorbate in photosynthesis is also supported by the transgenic plants regulating the enzyme for ascorbate synthesis. Suppressed expression of L-galactono-1,4-lactone dehydrogenase gene (*GLDH*), the gene encoding last step enzyme for ascorbate synthesis, in rice resulted in a loss of chlorophyll, a lower Ribulose 1,5-bisphosphate carboxylase/oxygenase protein content, and a lower rate of CO₂ assimilation. As a consequence, a slower rate of plant growth and lower seed set were observed. Conversely, increasing *GLDH* expression maintained high levels of chlorophyll, Rubisco protein, and a higher rate of net photosynthesis, resulting in higher seed set [36]. These data at least indicate that the ascorbate level and/or *GLDH* enzyme is closely associated with plant photosynthesis and growth.

2.1.6 Regulation of Florescence

The florescence of higher plants is jointly modulated by both endogenous and exogenous regulators. The external factors include day length, illumination and temperature, while the internal factors include gibberellins etc. Recent study indicated that ascorbate participates in the physiological process of florescence regulation.

The Arabidopsis ascorbate defective mutants, *vtc1-1*, *vtc2-1*, *vtc3-1* and *vtc4-1*, exhibited earlier florescence as compared to wild-type plants regardless of long or short days [37]. However, late flowering was observed in double mutant Arabidopsis lacking both thylakoid APX and cytosolic APX [38]. Antisense suppression of *AO* gene in transgenic tobacco resulted in delayed flowering time with respect to the wild-type control under normal growth, while alteration in other phenotypes was not observed [39]. Feeding the wild-type plants of Arabidopsis with L-galactono-1,4-lactone, the direct precursor for ascorbate synthesis, resulted in five-day delay of florescence. The L-galactono-1,4-lactone feeding on transgenic Arabidopsis expressing fusion gene *LEAFY::GUS* led to postponed expression of the *LEAFY* gene in apical parts of Arabidopsis [40]. All these evidences consistently suggest that ascorbate level is potentially correlated with the flowering time. Nevertheless, whether the flowering time is associated with the ROS accumulation or ascorbate level remain undetermined.

The mechanism underlying the ascorbate mediated regulation of florescence remains elusive. Break through came in 2009 about the ascorbate regulation of Arabidopsis florescence. Expression analysis of florescence related genes in Arabidopsis ascorbate defective mutant showed that gene expression alteration coincided with the phenotype of early florescence. The expression level of genes related to florescence in ascorbate defective mutant was significantly higher than that of wild-type. The feeding of L-galactose resulted in improved ascorbate content in the both wild-type plants and the defective mutant, and delayed

florescence as compared to the mock control (feeding with water). The L-galactose feeding also caused decreasing expression of genes related to flowering and photoperiodicity as compared to water feeding [37]. Double mutants combining ascorbate defective mutant *vtc* and photoperiodic or autonomous pathway mutants showed decreasing ascorbate content in Arabidopsis and delayed flowering time. Thus ascorbate might act upstream of photoperiodic and autonomous pathways to regulate the flowering time. Kotchoni et al. suggested that ascorbate act as an endogenous signal to influence the flowering time by modulating the related gene expression and metabolism process [37].

2.1.7 Regulation of Leaf Senescence

Senescence, a type of programmed cell death in plants, is initiated as the last stage during the leaf development. Both external and internal factors can trigger and aggravate leaf senescence. External factors include extreme temperature, drought, malnutrition, ozone, illumination deficiency and disease, while the internal factors affecting the leaf senescence are comprised of physiological age and developmental stages of reproductive organ.

Series of physiological and biochemical changes, such as cell structure, cell metabolism, and related gene expression, take place in plant cells during the process of senescence. The early stage of leaf senescence shows chlorophyll degradation and decreasing photosynthetic capacity due to the decreasing expression of genes related to Rubisco smaller subunit and chlorophyll a/b binding proteins, which are termed as senescence-down-regulated genes (*SDGs*). Some other genes are up-regulated in the early stage of leaf senescence, which are called senescence-associated genes (*SAGs*). The latter stage of leaf senescence shows cell peroxidation, DNA degradation and eventually disintegrated organelles.

Ascorbate is shown to influence the senescence of plants by modulating the expression of *SAGs*. Low accumulation of ascorbate accelerates senescence, while high content of ascorbate postpones the plant senescence. When detached leaves were treated in dark, leaves of ascorbate defective mutant, *vtc1*, lost chlorophyll more quickly and were induced to senesce earlier than wild-type plant leaves. Early expression of senescence-associated genes such as *SAG13*, *SAG15* were observed in ascorbate mutant of *vtc1* [41]. When supplemented with exogenous ascorbate, however, the expression levels of *SAG13* of the mutant were restored to the levels of wild-type. Additionally, the expression abundance of some atypical *SAGs* such as *PR-1*, *PR-2* and *PR-5*, in the mutants of *vtc1* and *vtc2* was higher than that of wild-type control.

The ROS are shown to promote the expression of senescence-associated genes, during which ascorbate is supposed to be involved in the transcriptional regulation of *SAGs*. In the leaves treated with silver nitrate, a ROS generating reagent, the *LSC54* and *LSC94* were significantly up-regulated until the subsequent treatment with ascorbate [42]. Expression of the *LSC54* and *LSC94* gene has been shown

previously to increase during leaf senescence and cell death. Supplement of ascorbate will reduce the ROS accumulation, alleviate oxidative damage to photosynthetic tissue, and consequently delay the process of senescence. During this process, the ascorbate may regulate leaf senescence by modulating the ROS level and/or the expression of *SAGs*.

The role of ascorbate in senescence regulation is also supported with physiological evidence. At later stages of plant development, the *vtc* rosettes were smaller than those of the wild-type and the leaves showed intracellular structural changes that are consistent with programmed cell death (PCD). PCD symptoms such as nuclear chromatin condensation, the presence of multivesicular bodies, and extensive degradation and disorganization of the grana stacks were observed in 8-week-old *vtc2* leaves and in 10-week-old *vtc1* leaves [35].

The *GMP* gene was reported to impose influences on the plant senescence. Decreasing ascorbate content resulted in early senescence in transgenic potato with antisense expression of GDP-D-mannose pyrophosphorylase gene (*GMP*), a gene in ascorbate synthesis pathway [43]. Brown spots appeared on the stem and leaves of *GMP* antisense transgenic plants and spread from bottom to top 10 weeks after transferring to soil. Those antisense transgenic lines with the most significant decrease of *GMP* enzyme activity and the most serious symptoms withered and died three months after transferring to soil, while the wild-type plants did not start to senesce [43]. On the contrary, *GMP* overexpression in potato resulted in improved ascorbate content and increasing ratio of ascorbate/dehydroascorbate in both leaves and tubers of transgenic potato plants. Both pigment content and photosynthetic rate were much higher in transgenic plants than that in wild-type plants. *GMP* overexpressing plants showed a distinguishable change in phenotype and delayed senescence [44].

Consistently, in the *GMP* RNAi-suppressed tomato plants, similar early senescence phenotype was observed. The senescence in the *GMP* suppressed tomato plants happened even earlier than that of transgenic potato plants [43]. In the *GMP* RNAi transgenic tomato seedlings, brown spots were formed on young leaves and stem, resulting in rapid etiolation and abscission of the bottom leaves. As the plants developed, the early senescence and yellowing of leaves expanded from bottom to the upper parts of the plants, and eventually most of the leaves withered (unpublished data).

Although the decreasing ascorbate accumulation is connected with early senescence in plants, the *SIGME2* suppressed transgenic plants did not generate brownish spots or early senescence on leaves. This suggests that the early senescence in the *SIGMP* RNAi transgenic plants is probably caused by the ascorbate deficiency as well as the insufficient product of *GMP* enzyme catalyzing, GDP-D-mannose, which is vital for various cellular process. GDP-D-mannose is required for the biosynthesis of the glucomannan and galactomannan of the hemicellulose polymers in plant cell wall, the GDP-L-trehalose and glycoprotein in the cell wall, and the O-linked glycoprotein and N-linked glycoprotein in the protein glycosylation. Microarray analysis showed that several genes related to

cell wall synthesis and protein glycosylation were up-regulated in the break and ripening stage fruits of transgenic tomato overexpressing *SIGMP*, indicating that the biological processes other than ascorbate biosynthesis were altered by *GMP* overexpression [32]. Thus the early senescence and brownish spots on the *GMP* suppressing transgenic tomato plants might be the consequence of down-regulated gene expression related to cell wall synthesis and/or protein glycosylation. The mechanism underlying the interaction between ascorbate synthesis and leaf senescence invites further investigation.

Ascorbate is the cofactor for enzymes involved in biosynthesis of GA, ABA and ethylene. Emerging evidences indicate that ascorbate together with various phytohormones regulates the process of senescence. The phytohormones of ABA and ethylene promote senescence while GA prevents senescence. Thus, ascorbate possibly regulate the plant senescence either by modulating the ROS accumulation or influencing the signal pathway of the phytohormones, such as GA, ABA and ethylene [40]. Thus, in addition to acting simply as an antioxidant in the apoplastic space, ascorbate appears to be involved in a complex phytohormone-mediated signalling network that links together ROS responses and the onset of senescence [45].

Taken together, the redox status as well as the concentration of ascorbate is involved in the regulation of plant senescence [44]. The decreasing ascorbate content usually results in early senescence, which is also consistent with the fact that the ascorbate is negatively involved in the regulation of flowering time.

2.2 The Cofactors for Enzyme Activity

The ascorbate acts as cofactors to regulate the series of enzyme activity and facilitate enzymatic reactions [46]. Ascorbate interacts with enzymes having either monooxygenase or dioxygenase activity. Ascorbate is an essential cofactor in reactions catalyzed by Cu^+ -dependent monooxygenases and Fe^{2+} -dependent dioxygenases [22, 25]. In the plants, ascorbate is utilized as coenzyme for the Fe dioxygenase to participate in the post-translational modification of the cell wall protein. The dehydroascorbate can interact with the side chain of lysine and arginine in the enzyme and prevent protein crosslinking [47]. Ascorbate is also reported to modulate the ferritin-mediated iron uptake and release in plants [48]. The ascorbate keeps the reduced state of the metal ions in the enzymatic reaction center, and promotes the enzymatic activity [22].

Ascorbate is also a cofactor for some hydroxylase enzymes (e.g. prolyl hydroxylase) and violaxanthin deepoxidase. Ascorbate acts as prothetic group for prolyl hydroxylase and lysyl hydroxylase, catalyzing the synthesis of hydroxylysine and hydroxyproline [25]. And the violaxanthin deepoxidase enzyme links ascorbate to the photoprotective xanthophyll cycle [4]. As the cofactor of violaxanthin deepoxidase, ascorbate is thus involved in the pigment biosynthesis for photoprotection [22].

Ascorbate is shown to be enzyme cofactors involving synthesis of ethylene, GA and anthocyanins. Ascorbate is the coenzyme for 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase) and GA2-oxidase and thus involved in the biosynthesis of phytohormones of ethylene and GA [4]. In addition, the ascorbate can strongly activate myrosinase, a family of enzymes involved in plant defense against herbivores.

2.3 Plant Antioxidation Capacity

In both plant and animal metabolism, the biological functions of ascorbate are centered on the antioxidant properties of this molecule. Considerable evidence has been accruing in the last three decades of the importance of ascorbate in protecting not only the plant from oxidative stress, but also mammals from various chronic diseases that have their origins in oxidative stress. In plants, ascorbate is the most important antioxidant and, in association with other components of the antioxidant system, protects plants against oxidative damage resulting from aerobic metabolism, photosynthesis and a range of pollutants.

The hypersensitivity of some of the *vtc* mutants to ozone and UV-B radiation, the rapid response of APX expression and ascorbate level to oxidative stress, and the properties of transgenic plants with altered APX activity all support an important role for ascorbate in protecting plants against oxidative stress [4, 30]. In the *vtc-1* mutant of *Arabidopsis*, which had an ascorbate deficiency in chloroplasts of ca. 60 %, although low ascorbate did not cause oxidative stress in optimal growth conditions, it increased malondialdehyde levels in chloroplasts by ca. 60 %, and reduced α -tocopherol (vitamin E) and β -carotene by ca. 85 and 40 %, respectively, in water-stressed mutants, showing that ascorbate contributes to the protection of thylakoid membrane lipids from oxidation in stressed plants [49].

The ascorbate level as well as activities of the biosynthesis related enzymes in plants responds rapidly to oxidative stress. The content of ascorbate increased in plants grown in polluted air together with the enzymatic activities of dehydro-ascorbate reductase (DHAR) involved in its recycling. However, the APX activity remained unchanged in this case. The data obtained support the hypothesis that the glutathione-ascorbate cycle is stimulated in removing the hydrogen peroxide produced under oxidative stress [50]. APX, the enzyme responsible for ascorbate oxidation, is an important enzymatic ROS scavenger. APX enzyme activities are shown to be correlated with oxidation tolerance [51]. APX activity increased after ozone treatment and this effect was stronger if the plants were pre-treated with anti-ozonant ethylenediurea prior to O₃-exposure. The ozone exposure of plants stimulates the APX, whose enzymatic activity enhancement at the apoplastic and cytosolic levels may act as a biochemical defence against ozone damage [52].

Scavenging the ROS in plants is one of the major tasks of ascorbate. The ROS is generated during photosynthesis and respiration, as well as in various stress such as drought, extreme temperature, salt, ultraviolet etc. Excessive ROS will do harm

to lipid, nuclear acid, and protein, resulting in oxidative damages, physiological disorder, and eventually premature senescence and cell death [53]. Ascorbate is an effective radical scavenger to eliminate the ROS in plants. Ascorbate reacts with ROS generating monodehydroascorbate and dehydroascorbate. The dehydroascorbate is decomposed into tartaric acid and ketosuccinic acid. The monodehydroascorbate and dehydroascorbate are reduced into ascorbate by MDHAR enzyme using NADPH or glutathione as reducer. ROS can be consumed through the resultant ascorbate–glutathione cycle. For activity, ascorbate must be in the fully reduced state. Therefore, both the rate of ascorbate synthesis and recycling via dehydroascorbate and monodehydroascorbate reductases are critical in the process of ROS scavenging [45].

Ascorbate is a well-known antioxidant and cellular reductant with an intimate and complex role in the response of plants to ozone. It is clear from a number of studies that sensitivity to ozone is correlated with total ascorbate levels, and that a first line of defence against the ROS generated in the apoplastic space by ozone is ascorbate. The malondialdehyde is the product of membranous peroxidation. The malondialdehyde content represents the damage extent of the plant cells or the membrane permeability. The ascorbate can inhibit the membranous peroxidation, protect the cell from damaging, and delay the cell senescence by decreasing the production of malondialdehyde and stabilizing the cytomembrane structure.

The ascorbate plays a pivotal role in scavenging the ROS generated in the process of photosynthesis. The leaf ascorbate content is usually higher than that in other tissues of plants. This, from an evolutionary point of view, will help plant to defense against the ROS generating from photosynthesis. The leaf apoplast contains millimolar amounts of ascorbate that protects the plasmalemma against oxidative damage due to the process of photosynthesis [5]. High concentration of ascorbate is accumulated in the chloroplast, while chloroplast is in lack of catalase (CAT). Thus the hydrogen peroxide produced in the photosystem I has to be scavenged by APX [54]. The ascorbate is demonstrated to act as an electron donor to photosystem I in light-induced electron transport. It was found that ascorbate, at physiological concentrations, rapidly reduced photooxidized reaction center chlorophyll of photosystem I [55]. The oxidized ascorbate of monodehydroascorbate is also the direct electron acceptor in the photosystem I [56]. The levels and redox state of ascorbate modify the pattern of modulation of photosynthesis by mitochondrial metabolism [57]. Ascorbate is also co-factor of violaxanthin deepoxidase, which is a key enzyme of the xanthophyll cycle converting violaxanthin to cryptoxanthin on the thylakoid membrane. Thus the ascorbate plays an important role in consuming the excessive light energy and protecting photosynthesis for plants.

At a physiological level, the best-studied phenomena involving ascorbate is its participation in an oxygen scavenging pathway in the chloroplast known as the ascorbate–glutathione cycle [6]. However, either cytosolic ascorbate–glutathione cycle or chloroplastic ascorbate–glutathione cycle is primarily utilized for the detoxification of hydrogen peroxide may vary with plant species [58].

The antioxidative role of ascorbate also resides in its capacity to maintain quality of plant products, e.g. fruit and vegetable. The ascorbate shows inhibitive effect on the polyphenol oxidase (PPO), which oxidizes diphenols to quinones resulting in browning reactions in many wounded horticultural fruits and vegetables. The ascorbate can inhibit the activity of PPO enzyme by reducing the quinones and its derivatives into phenolic compounds or prevent the spontaneous polymerization of quinones into pigment substances. The ascorbate helps to keep another important antioxidant, vitamin E, in the reduced state.

Ascorbate-dependent detoxification of hydrogen peroxide is also shown to be associated with guaiacol-type peroxidases as well as ascorbate peroxidase, suggesting that ascorbate is the natural substrate for many types of peroxidase in situ and not just the ascorbate-specific peroxidases [59, 60]. The ascorbate-dependent destruction of hydrogen peroxide in the more acidic cellular compartments such as the vacuole may be an important function of such non-specific peroxidases [61].

2.4 Heavy Metal Evacuation and Detoxification

Ascorbate is supposed to act in the plant defence against heavy metal stress. Ascorbate may help plants to eliminate the heavy metals or ROS generated by this stress.

Ascorbate is shown to promote the Hg^0 emission originated from Hg^{2+} uptake by the roots. Homogenates of barley leaves added to dissolved Hg^{2+} induced a powerful volatilization at alkaline but not at acidic pH. The same pH dependence and emission kinetic together with the highest reduction capacity was observed for ascorbate as compared to other phyto-reductants. The electrochemical potentials of the reactions involved suggest an electron transfer from NADPH via glutathione and ascorbate to Hg^{2+} . The results showed plants transfer reduction equivalents via ascorbate to reduce Hg^{2+} ions, thus counteracting mercury toxicity by volatilizing the metal. This effect appears to be assisted by other light-dependent processes such as transpiration and ascorbate synthesis [62].

In an initial exposure to heavy metals (Cd^{2+} , Pb^{2+} and Hg^{2+}), the ascorbate levels increases in plants, suggesting that the ascorbate respond actively to evacuate heavy metals or detoxify the ROS derived from heavy metal stress [63].

2.5 Role in Stress Defense

Environmental stresses include both biotic and abiotic stresses. Abiotic stress is one of the limiting factors for crop production. Biotic stress comes from insects and diseases, while abiotic stress is comprised of drought, salt, cold, high temperature, ultraviolet, and heavy metals etc. These environmental stress factors cause redox imbalance and oxidative damage in plants. The ROS, such as

superoxide anion ($O_2^{\cdot-}$), hydroxyl free radical (OH), hydrogen peroxide (H_2O_2) and singlet oxygen, are generated under various abiotic and biotic stresses. ROS can cause the peroxidation of plasmalemma, DNA mutation, protein denaturation, and eventually cell death [64]. Thus it is quite indispensable to scavenge the surplus ROS for plant growth and development.

In recent years, the role of the plant antioxidant system for the stress response has become a research focus of plant stress physiology. Generally it is regarded that plant injury caused by stress comes from antioxidant system decreasing and membrane lipid peroxidation. A variety of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), APX and glutathione reductase (GR), etc., as well as non-enzymatic antioxidants such as ascorbate, glutathione, vitamin E and carotenoids play important roles in plant responses to stresses [29, 65]. These enzymatic antioxidants and non-enzymatic antioxidant are utilized to eliminate excess ROS in plant cells.

Ascorbate has been shown as an efficient ROS scavenger in the process of plant defense to various stresses [3, 28]. Ascorbate participates directly in eliminating ROS as electron donor, and the enzymes involved in ascorbate metabolism also act positively in plant defense against various stress. The ascorbate has great affinity with anionic peroxidase, which detoxifies the hydrogen peroxide during the processes of peroxidation of ascorbate to defend against peroxidative stress [66]. As the most important antioxidant in plant cells, one of the key functions of ascorbate is to protect the chloroplast from oxidative damage.

Ascorbate can scavenge the ROS directly or indirectly, which is generated during various physiological process like photosynthesis, oxidization and metabolism, and stress response, and thus help plants to overcome the oxidative damage and survive [30]. Ascorbate in apoplast is considered to be involved in signal perceiving and transduction from external environment [67, 68]. The reduced state of ascorbate as well as the oxidized state of dehydroascorbate act as signals regulating the interaction between plant and stresses and confer resistance to stresses, such as ozone, drought and pathogen [3, 69, 70]. Apoplastic ascorbate levels and redox state has an important role in plant responses to environmental stress. Apoplastic ascorbate may be involved in the protection of plant plasmalemma against environmental stress caused by oxidative damage, especially when the plants subjected to ozone or other atmospheric pollution stress.

In living cells, ascorbate redox system is composed of reduced ascorbate, semi-reduced (monodehydroascorbate) and oxidized ascorbate (dehydroascorbate). In the intracellular environment, a number of enzymatic reactions allow the ascorbate pool to maintain at a considerable reduced state, while in the extracellular environment, redox state of ascorbate is more dependent on plant species and the physiological status. Studies have shown that the ascorbate level, the redox state (ascorbate/dehydroascorbate ratio) of ascorbate and enzyme activity involved in ascorbate biosynthesis and metabolism are related to series of environmental stress responses.

Improvement of ascorbate content in plants will increase plant stress tolerance, while decreasing ascorbate content will result in stress sensitivity of plants. Ascorbate-deficient (*vtc*) mutants tend to be smaller, more sensitive to abiotic stresses and more resistant to biotrophic pathogens. The ascorbate defective mutant in Arabidopsis, *vtc1*, showed more sensitivity to ozone, sulfur dioxide, and ultraviolet. These evidence shows that plant stress tolerance is associated with the ascorbate level in plants [71]. Grain soaking in ascorbate could also counteract the adverse effects of salinity on the seedlings [72]. That plants with higher leaf ascorbate concentrations had higher leaf NO_2 uptake rates, suggest that leaf capacity for the scavenging of NO_x ($\text{NO} + \text{NO}_2$) by ascorbate may explain the variation in the ability of plants to absorb atmospheric nitrogen oxides [73]. The ascorbate content in plants increased in response to excess level of Zn and high irradiance stress, indicating that ascorbate is one of the effective defense mechanism against stresses in plants [74]. Salt stress can lead to significantly reduced ascorbate content in wheat. However, the decreasing extent of ascorbate in salt tolerant varieties is less than that in the salt-sensitive varieties. Protein profiles under stress indicate a positive role of ascorbate in the alleviation of the damage effects induced by abiotic stress [75].

Evidence shows it is feasible to improve plant stress tolerance by regulating ascorbate synthesis and recycling to increase ascorbate accumulation. Overexpressing galacturonate reductase gene from strawberry and L-gulonolactone oxidase gene from rat in potato resulted in improved ascorbate synthesis and higher survival rate under oxidation and salt treatment [76, 77]. Overexpressing the ascorbate recycling gene, *DHAR*, led to enhanced resistance to various stresses in transgenic plants. Overexpressing the *DHAR* gene can increase plant defense capacity to ozone in transgenic plants [78]. Ectopic expression of human *DHAR* gene in tobacco resulted in improved resistance to cold and salt stress [79]. Overexpressing rice *DHAR* gene in Arabidopsis improved the plant resistance to salt stress [80]. Overexpressing the cytosolic *DHAR* gene from Arabidopsis in tobacco resulted in enhanced resistance to drought, ozone and aluminium [81, 82]. That overexpression of *DHAR*, but not of *MDHAR*, confers aluminium tolerance, indicates the maintenance of a high level of reduced state ascorbate is important to aluminium tolerance in plants [82].

The redox state of the ascorbate influences the abiotic resistance in plants. The ROS is formed in apoplastic regions, where ascorbate is considered to play an important role in plant defense against oxidation and injury. The AO enzyme is located in apoplast. Overexpressing the melon AO gene in tobacco resulted in oxidation of the ascorbate in apoplast. The reduced state of ascorbate accumulated at low level in apoplast, with altered redox status. The increasing dehydroascorbate/ascorbate ratio resulted in extreme sensitivity to ozone in AO transgenic plants [68]. That altered stomatal dynamics was observed in AO overexpressing tobacco plants suggests AO enzyme or dehydroascorbate may be closely linked to stress response, as control of stomatal aperture is of paramount importance for plant adaptation to the surrounding environment [69]. Because the ascorbate is also directly involved in cell wall elongation and lignification, maintaining high

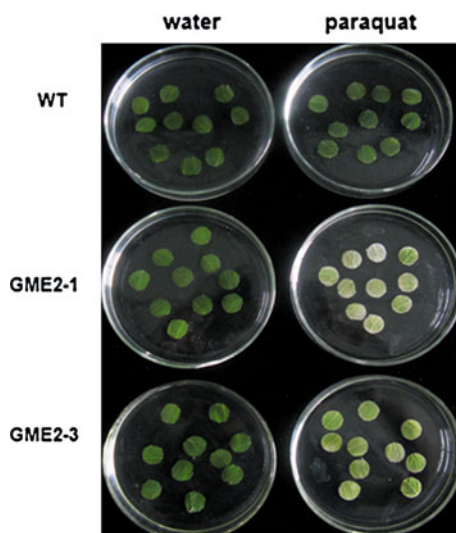
level and high redox state of apoplastic ascorbate is physiologically important for the plants during the environmental stress.

Under ozone stress conditions, the total ascorbate content of kidney bean leaves of ozone-tolerant varieties is higher than that of the sensitive varieties, and the tolerant species maintains relatively high levels of ascorbate pool. In addition, the content of apoplastic ascorbate of tolerance varieties is much higher than that of the sensitive cultivar. In the ozone tolerant varieties, the ascorbate/(ascorbate + dehydroascorbate) ratio is relatively high, suggesting that plant ozone tolerance is closely related to apoplastic ascorbate level and its redox state. Since the apoplast does not have most of the enzymes involved in the ascorbate–glutathione cycle, apoplastic ascorbate levels and its redox state further demonstrates unique ascorbate transport mechanism in plants.

The ascorbate–glutathione cycle constitutes one of the most important antioxidant systems in plants. In the ascorbate–glutathione cycle, the ascorbate and the glutathione are utilized as reducers and recycled through consuming the ATP and NAD(P)H [29], in which four enzymes, APX, MDHAR, DHAR and GR, are involved. The ascorbate–glutathione cycle enzymes are activated for the dissipation of excess excitation energy in the photosynthesis. Haem-containing enzymes include peroxidase and catalase (CAT) distributed among prokaryotes and eukaryotes and play a vital role in hydrogen peroxide detoxification [83]. The modulation of the ascorbate–glutathione cycle together with coordinated antioxidant activity involving increased activities of superoxide dismutase (SOD) and CAT, allowed plants to cope with oxidative stress [84].

As a player in the defence against ROS, the role of APX in plant stress response has been extensively studied at the biochemical and molecular level. APX, an enzyme scavenging hydrogen peroxide, is located in various organelles, such as cytoplasm, chloroplast, mitochondria and peroxisome [85]. The activity of APX, the ascorbate metabolism related enzyme, is triggered by abiotic stress. The cytoplasm-located APX enzyme, cAPX, is considered to play a crucial role in stress response of plants. The cAPX expression level increased significantly in leaves of pea and spinach after stress treatment of high light, drought, high temperature, and oxidation [86, 87]. The two APX genes in rice, *OsAPX1/2*, were up-regulated in response to injury, salicylic acid, abscisic acid and hydrogen peroxide [88]. Overexpressing cytosolic APX in tomato increased the plant resistance to abiotic stresses, such as ultraviolet, high temperature, cold and salt [89]. And the enzyme activity of APX was stimulated by ethylene, leading to increased resistance against ozone and other oxidative stress [90]. Interestingly, although the transcripts levels of APX were almost identical in control or salt-grown radish plants in both leaves and roots, the activity of APX enzyme was enhanced by the salt treatment in both leaves and roots [91], suggesting that the salt-induced APX activity is probably the consequence of post-transcriptional events. The drought acclimated leaves of wheat exhibited systematic increase in the activities of hydrogen peroxide scavenging enzymes particularly APX and CAT, and maintenance of ascorbate redox pool by efficient function of APX enzyme [92].

Fig. 2.5 Leaf discs of wild-type (WT) and transgenic tomato with *GME2* co-suppression (*GME2-1* and *GME2-3*) treated with 100 $\mu\text{mol/L}$ paraquat (courtesy of Professor Zhibiao Ye and Dr. Chanjuan Zhang)



The enzymes for ascorbate synthesis also affect the plant stress responses, possibly by regulating the ascorbate accumulation. *GME* co-suppression affects the tomato response to oxidative stress. Leaf discs of two month seedlings were treated with distilled water or 100 $\mu\text{mol/L}$ paraquat solution for 18 h and the chlorophyll content was investigated. Oxidation treatment of the *GME* co-suppression lines resulted in serious etiolation of leaf discs. The chlorophyll content of the transgenic plants in the water treatment was significantly lower than that of the wild-type control. The paraquat treatment of the leaf discs resulted in much more decreasing in the chlorophyll content in co-suppression lines than that in the wild-type control (Fig. 2.5). Experimental results showed that *SIGME2* co-suppression resulted in significantly decreasing ascorbate and chlorophyll content, as well as higher sensitivity to oxidative stress (unpublished data).

On the other hand, overexpression of *SIGME1* and *SIGME2* resulted in improved resistance to oxidation, chilling injury, and high salt in tomato [32]. Gene chip hybridization was utilized to analyze the gene expression profiles of *SIGME1* overexpressing tomato at the break stage fruit. The results showed that the genes related to stress resistance, lipid metabolism, flavonoid metabolism were significantly up-regulated. Several differentially expressed genes were both observed in the *SIGME1* and *SIGME2* overexpressing tomato with the similar expression profile [32].

The ascorbate is also involved in responses to biotic stress in plants. Activation of a defence gene-signalling network by both ozone and pathogens is influenced by the level of ascorbate. The ascorbate metabolism is shown to response dynamically to pathogenic bacteria infection [93]. And the ascorbate defective mutant, *vtc1*, activates numerous defense genes including those that encode pathogenesis-related proteins [3]. Interestingly, salicylic acid (SA)-deficient plants adapt to RNA virus infections better with a lighter symptom and less ROS accumulation. This

symptom alleviation is supposed to attributed to the higher ascorbate content in SA-deficient plants [94]. Early and high-dose ascorbate treatment alleviates the symptom, and eventually inhibits virus replication. ROS eliminators could not imitate the effect of ascorbate, and could neither alleviate symptom nor inhibit virus replication, which indicates ascorbate may help plant defense via a unique machinery rather than the hydrogen peroxide signal [94].

Abundance of ascorbate in plants also influences their susceptibility to insect feeding. These effects may be mediated by ascorbate roles as an essential dietary nutrient, as an antioxidant in the insect midgut, or as a substrate for plant-derived ascorbate oxidase, which can lead to generation of toxic ROS. In addition, ascorbate can influence the efficacy of plant defenses such as myrosinases and tannins, and alter insects' susceptibility to natural enemies [95].

References

1. Iqbal Y, Ihsanullah I, Shaheen N, Hussain I (2009) Significance of vitamin C in plants. *J Chem Soc Pak* 31:169–170
2. Nishikimi M, Fukuyama R, Minoshima S, Shimizu N, Yagi K (1994) Cloning and chromosomal mapping of the human nonfunctional gene for l-gulono-gamma-lactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man. *J Biol Chem* 269:13685–13688
3. 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 that control development through hormone signaling. *Plant Cell* 15:939–951
4. Smirnoff N (1996) The function and metabolism of ascorbic acid in plants. *Ann Bot* 78: 661–669
5. Horemans N, Foyer CH, Asard H (2000a) Transport and action of ascorbate at the plant plasma membrane. *Trends Plant Sci* 5:263–267
6. Horemans N, Foyer CH, Potters G, Asard H (2000b) Ascorbate function and associated transport systems in plants. *Plant Physiol Biochem* 38:531–540
7. Loewus FA, Loewus MW (1987) Biosynthesis and metabolism of ascorbic-acid in plants. *Crit Rev Plant Sci* 5:101–119
8. Loewus FA (1999) Biosynthesis and metabolism of ascorbic acid in plants and of analogs of ascorbic acid in fungi. *Phytochemistry* 52:193–210
9. Smirnoff N (2011) Vitamin C: the metabolism and functions of ascorbic acid in plants. *Adv Bot Res* 59:107–177
10. Stevens R, Buret M, Duffe P, Garchery C, Baldet P, Rothan C, Causse M (2007) Candidate genes and quantitative trait loci affecting fruit ascorbic acid content in three tomato populations. *Plant Physiol* 143:1943–1953
11. Cordoba F, Gonzalezreyes JA (1994) Ascorbate and plant-cell growth. *J Bioenerg Biomembr* 26:399–405
12. Kerk NM, Feldman LJ (1995) A biochemical-model for the initiation and maintenance of the quiescent center—implications for organization of root-meristems. *Development* 121: 2825–2833
13. Potters G, Horemans N, Caubergs RJ, Asard H (2000) Ascorbate and dehydroascorbate influence cell cycle progression in a tobacco cell suspension. *Plant Physiol* 124:17–20
14. Arrigoni O (1994) Ascorbate system in plant development. *J Bioenerg Biomembr* 26: 407–419

15. Veljovic-Jovanovic SD, Pignocchi C, Noctor G, Foyer CH (2001) Low ascorbic acid in the *vtc-1* mutant of Arabidopsis is associated with decreased growth and intracellular redistribution of the antioxidant system. *Plant Physiol* 127:426–435
16. Tabata K, Oba K, Suzuki K, Esaka M (2001) Generation and properties of ascorbic acid-deficient transgenic tobacco cells expressing antisense RNA for L-galactono-1,4-lactone dehydrogenase. *Plant J* 27:139–148
17. Johkan M, Oda M, Mori G (2008) Ascorbic acid promotes graft-take in sweet pepper plants (*Capsicum annuum* L.). *Scientia Horti* Amsterdam 116:343–347
18. Torabinejad J, Donahue JL, Gunesekera BN, Allen-Daniels MJ, Gillaspay GE (2009) VTC4 is a bifunctional enzyme that affects myoinositol and ascorbate biosynthesis in plants. *Plant Physiol* 150:951–961
19. Hidalgo A, Gonzalez-Reyes JA, Navas P (1989) Ascorbate free-radical enhances vacuolization in onion root-meristems. *Plant Cell Environ* 12:455–460
20. deCabo RC, Gonzalez-Reyes JA, Cordoba F, Navas P (1996) Rooting hastened in onions by ascorbate and ascorbate free radical. *J Plant Growth Regul* 15:53–56
21. Takahama U (1993). Regulation of peroxidase-dependent oxidation of phenolics by ascorbic-acid—different effects of ascorbic-acid on the oxidation of coniferyl alcohol by the apoplastic soluble and cell wall-bound peroxidases from epicotyls of *Vigna angularis*. *Plant Cell Physiol* 34:809–817
22. Davey MW, Van Montagu M, Inze D, Sanmartin M, Kanellis A, Smirnoff N, Benzie IJJ, Strain JJ, Favell D, Fletcher J (2000) Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. *J Sci Food Agric* 80:825–860
23. Horemans N, Asard H, Caubergs RJ (1994) The role of ascorbate free-radical as an electron-acceptor to cytochrome b-mediated trans-plasma membrane electron-transport in higher-plants. *Plant Physiol* 104:1455–1458
24. Asard H, Horemans N, Caubergs RJ (1995) Involvement of ascorbic-acid and a b-type cytochrome in plant plasma-membrane redox reactions. *Protoplasma* 184:36–41
25. Padh H (1990) Cellular functions of ascorbic-acid biochemistry and cell biology. *Biochimie et Biologie Cellulaire* 68:1166–1173
26. Fry SC (1998) Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. *Biochemistry Journal* 332:507–515
27. Kato N, Esaka M (1999) Changes in ascorbate oxidase gene expression and ascorbate levels in cell division and cell elongation in tobacco cells. *Physiol Plant* 105:321–329
28. Pignocchi C, Fletcher JM, Wilkinson JE, Barnes JD, Foyer CH (2003) The function of ascorbate oxidase in tobacco. *Plant Physiol* 132:1631–1641
29. Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. *Ann Rev Plant Physiol Plant Mol Biol* 49:249–279
30. Smirnoff N, Wheeler GL (2000) Ascorbic acid in plants: biosynthesis and function. *Crit Rev Plant Sci* 19:267–290
31. Alhaghdow 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
32. Zhang CJ, Liu JX, Zhang YY, Cai XF, Gong PJ, Zhang JH, Wang TT, Li HX, Ye ZB (2011) Overexpression of *SIGMEs* leads to ascorbate accumulation with enhanced oxidative stress, cold, and salt tolerance in tomato. *Plant Cell Rep* 30:389–398
33. Gilbert L, Alhaghdow M, Nunes-Nesi A, Quemener B, Guillon F, Bouchet B, Faurobert M, Gouble B, Page D, Garcia V, Petit J, Stevens R, Causse M, Fernie AR, Lahaye M, Rothan C, Baldet P (2009) GDP-D-mannose 3,5-epimerase (GME) plays a key role at the intersection of ascorbate and non-cellulosic cell-wall biosynthesis in tomato. *Plant J* 60:499–508
34. Johkan M, Mori G, Mitsukuri K, Mishiba K, Morikawa T, Imahori Y, Oda M (2008) Effect of ascorbic acid on in vivo organogenesis in tomato plants. *J Horti Sci Biotechnol* 83:624–628

35. Olmos E, Kiddle G, Pellny TK, Kumar S, Foyer CH (2006) Modulation of plant morphology, root architecture, and cell structure by low vitamin C in *Arabidopsis thaliana*. *J Exp Bot* 57: 1645–1655
36. Liu YH, Yu L, Wang RZ (2011) Level of ascorbic acid in transgenic rice for l-galactono-1, 4-lactone dehydrogenase overexpressing or suppressed is associated with plant growth and seed set. *Acta Physiol Plant* 33:1353–1363
37. Kotchoni SO, Larrimore KE, Mukherjee M, Kempinski CF, Barth C (2009) Alterations in the endogenous ascorbic acid content affect flowering time in *Arabidopsis*. *Plant Physiol* 149:803–815
38. Miller G, Suzuki N, Rizhsky L, Hegie A, Koussevitzky S, Mittler R (2007) Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses. *Plant Physiol* 144:1777–1785
39. Yamamoto A, Bhuiyan NH, Waditee R, Tanaka Y, Esaka M, Oba K, Jagendorf AT, Takabe T (2005) Suppressed expression of the apoplastic ascorbate oxidase gene increases salt tolerance in tobacco and *Arabidopsis* plants. *J Exp Bot* 56:1785–1796
40. 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
41. Barth C, Moeder W, Klessig DF, Conklin PL (2004) The timing of senescence and response to pathogens is altered in the ascorbate-deficient *Arabidopsis* mutant vitamin c-1. *Plant Physiol* 134:1784–1792
42. Navabpour S, Morris K, Allen R, Harrison E, A-H-Mackerness S, Buchanan-Wollaston V (2003) Expression of senescence-enhanced genes in response to oxidative stress. *J Exp Bot* 54:2285–2292
43. 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
44. Lin LL, Shi QH, Wang HS, Qin AG, Yu XC (2011) Over-expression of tomato GDP-Mannose pyrophosphorylase (GMPase) in potato increases ascorbate content and delays plant senescence. *Agric Sci Chin* 10:534–543
45. Conklin PL, Barth C (2004) Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. *Plant Cell Environ* 27:959–970
46. Arrigoni O, DeGara L, Paciolla C, Evidente A, dePinto MC, Liso R (1997) Lycorine: A powerful inhibitor of L-galactono-gamma-lactone dehydrogenase activity. *J Plant Physiol* 150:362–364
47. Fry SC (1986) Cross-linking of matrix polymers in the growing cell walls of angiosperms. *Ann Rev Plant Physiol Plant Mol Biol* 37:165–186
48. Zancani M, Peresson C, Patui S, Tubaro F, Vianello A, Macri F (2007) Mitochondrial ferritin distribution among plant organs and its involvement in ascorbate-mediated iron uptake and release. *Plant Sci* 173:182–189
49. Munne-Bosch S, Alegre L (2002) Interplay between ascorbic acid and lipophilic antioxidant defences in chloroplasts of water-stressed *Arabidopsis* plants. *FEBS Lett* 524:145–148
50. Ranieri A, Lencioni L, Schenone G, Soldatini GF (1993) Glutathione-ascorbic acid cycle in pumpkin plants grown under polluted air in open-top chambers. *J Plant Physiol* 142:286–290
51. Batini P, Ederli L, Pasqualini S, Antonielli M, Valenti V (1995) Effects of ethylenediurea and ozone in detoxificant ascorbic-ascorbate peroxidase system in tobacco plants. *Plant Physiol Biochem* 33:717–723
52. Ranieri A, Castagna A, Soldatini GF (2000) Differential stimulation of ascorbate peroxidase isoforms by ozone exposure in sunflower plants. *J Plant Physiol* 156:266–271
53. Wang WX, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* 218:1–14

54. Yabuta Y, Motoki T, Yoshimura K, Takeda T, Ishikawa T, Shigeoka S (2002) Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems under photo-oxidative stress. *Plant J* 32:915–925
55. Ivanov BN, Sacksteder CA, Kramer DM, Edwards GE (2001) Light-induced ascorbate-dependent electron transport and membrane energization in chloroplasts of bundle sheath cells of the C4 plant maize. *Arch Biochem Biophys* 385:145–153
56. Smirnoff N (2000) Ascorbic acid: metabolism and functions of a multi-faceted molecule. *Curr Opin Plant Biol* 3:229–235
57. Talla S, Riazunnisa K, Padmavathi L, Sunil B, Rajsheel P, Raghavendra AS (2011) Ascorbic acid is a key participant during the interactions between chloroplasts and mitochondria to optimize photosynthesis and protect against photoinhibition. *J Biosci* 36:163–173
58. Zhang JX, Kirkham MB (1996) Enzymatic responses of the ascorbate-glutathione cycle to drought in sorghum and sunflower plants. *Plant Sci* 113:139–147
59. Takahama U, Oniki T (1997) A peroxidase/phenolics/ascorbate system can scavenge hydrogen peroxide in plant cells. *Physiol Plant* 101:845–852
60. Yamasaki H, Grace SC (1998) EPR detection of phytophenoxyl radicals stabilized by zinc ions: evidence for the redox coupling of plant phenolics with ascorbate in the H₂O₂-peroxidase system. *FEBS Lett* 422:377–380
61. Mehlhorn H, Lelandais M, Korth HG, Foyer CH (1996) Ascorbate is the natural substrate for plant peroxidases. *FEBS Lett* 378:203–206
62. Battke F, Ernst D, Halbach S (2005) Ascorbate promotes emission of mercury vapour from plants. *Plant Cell Environ* 28:1487–1495
63. Huang GY, Wang YS, Sun CC, Dong JD, Sun ZX (2010) The effect of multiple heavy metals on ascorbate, glutathione and related enzymes in two mangrove plant seedlings (*Kandelia candel* and *Bruguiera gymnorhiza*). *Oceanol Hydrobiol Stud* 39:11–25
64. Fridovich I (1998) Oxygen toxicity: a radical explanation. *J Exp Biol* 201:1203–1209
65. Asada K (1999) The water–water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. *Ann Rev Plant Physiol Plant Mol Biol* 50:601–639
66. Zheng X, Vanhuysen RB (1992) Anionic peroxidase catalyzed ascorbic acid and IAA oxidation in the presence of hydrogen peroxide: a defense system against peroxidative stress in peanut plant. *Phytochemistry* 31:1895–1898
67. Pignocchi C, Foyer CH (2003) Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. *Curr Opin Plant Biol* 6:379–389
68. Sanmartin M, Drogoudi PD, Lyons T, Pateraki I, Barnes J, Kanellis AK (2003) Over-expression of ascorbate oxidase in the apoplast of transgenic tobacco results in altered ascorbate and glutathione redox states and increased sensitivity to ozone. *Planta* 216:918–928
69. Fotopoulos V, De Tullio MC, Barnes J, Kanellis AK (2008) Altered stomatal dynamics in ascorbate oxidase over-expressing tobacco plants suggest a role for dehydroascorbate signalling. *J Exp Bot* 59:729–737
70. Parsons HT, Fry SC (2010) Reactive oxygen species-induced release of intracellular ascorbate in plant cell-suspension cultures and evidence for pulsing of net release rate. *New Phytol* 187:332–342
71. Lee EH (1991) Plant resistance mechanisms to air pollutants: rhythms in ascorbic acid production during growth under ozone stress. *Chronobiol Int* 8:93–102
72. Al-Hakimi AMA, Hamada AM (2001) Counteraction of salinity stress on wheat plants by grain soaking in ascorbic acid, thiamin or sodium salicylate. *Biol Plant* 44:253–261
73. Teklemariam TA, Sparks JP (2006) Leaf fluxes of NO and NO₂ in four herbaceous plant species: the role of ascorbic acid. *Atmos Environ* 40:2235–2244
74. Michael PI, Krishnaswamy M (2011) The effect of zinc stress combined with high irradiance stress on membrane damage and antioxidative response in bean seedlings. *Environ Exp Bot* 74:171–177

75. Younis ME, Hasaneen MNA, Kazamel AMS (2009) Plant growth, metabolism and adaptation in relation to stress conditions. XXVII. Can ascorbic acid modify the adverse effects of NaCl and mannitol on amino acids, nucleic acids and protein patterns in *Vicia faba* seedlings? *Protoplasma* 235:37–47
76. Hemavathi, Upadhyaya CP, Young KE, Akula N, Kim HS, Heung JJ, Oh OM, Aswath CR, Chun SC, Kim DH, Park SW (2009). Over-expression of strawberry D-galacturonic acid reductase in potato leads to accumulation of vitamin C with enhanced abiotic stress tolerance. *Plant Sci* 177:659–667
77. Hemavathi, Upadhyaya, CP, Akula N, Young KE, Chun SC, Kim DH, Park SW (2010). Enhanced ascorbic acid accumulation in transgenic potato confers tolerance to various abiotic stresses. *Biotechnol Lett* 32:321–330
78. Chen Z, Gallie DR (2005) Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. *Plant Physiol* 138: 1673–1689
79. Kwon SY, Choi SM, Ahn YO, Lee HS, Lee HB, Park YM, Kwak SS (2003) Enhanced stress-tolerance of transgenic tobacco plants expressing a human dehydroascorbate reductase gene. *J Plant Physiol* 160:347–353
80. Ushimaru T, Nakagawa T, Fujioka Y, Daicho K, Naito M, Yamauchi Y, Nonaka H, Amako K, Yamawaki K, Murata N (2006) Transgenic Arabidopsis plants expressing the rice dehydroascorbate reductase gene are resistant to salt stress. *J Plant Physiol* 163:1179–1184
81. Eltayeb AE, Kawano N, Badawi GH, Kaminaka H, Sanekata T, Morishima I, Shibahara T, Inanaga S, Tanaka K (2006) Enhanced tolerance to ozone and drought stresses in transgenic tobacco overexpressing dehydroascorbate reductase in cytosol. *Physiol Plant* 127:57–65
82. Yin LN, Wang SW, Eltayeb AE, Uddin MI, Yamamoto Y, Tsuji W, Takeuchi Y, Tanaka K (2010) Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco. *Planta* 231:609–621
83. Adak S, Datta AK (2005) Leishmania major encodes an unusual peroxidase that is a close homologue of plant ascorbate peroxidase: a novel role of the transmembrane domain. *Biochem J* 390:465–474
84. Hossain Z, Lopez-Climent MF, Arbona V, Perez-Clemente RM, Gomez-Cadenas A (2009) Modulation of the antioxidant system in citrus under waterlogging and subsequent drainage. *J Plant Physiol* 166:1391–1404
85. Jimenez A, Hernandez JA, delRio LA, Sevilla F (1997) Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Physiol* 114:275–284
86. Mittler R, Zilinskas BA (1992) Molecular-cloning and characterization of a gene encoding pea cytosolic ascorbate peroxidase. *J Biol Chem* 267:21802–21807
87. Yoshimura K, Yabuta Y, Ishikawa T, Shigeoka S (2000) Expression of spinach ascorbate peroxidase isoenzymes in response to oxidative stresses. *Plant Physiol* 123:223–233
88. Agrawal GK, Jwa NS, Iwahashi H, Rakwal R (2003) Importance of ascorbate peroxidases OsAPX1 and OsAPX2 in the rice pathogen response pathways and growth and reproduction revealed by their transcriptional profiling. *Gene* 322:93–103
89. Wang YJ, Wisniewski M, Meilan R, Cui MG, Webb R, Fuchigami L (2005) Overexpression of cytosolic ascorbate peroxidase in tomato confers tolerance to chilling and salt stress. *J Am Soc Hortic Sci* 130:167–173
90. Mehlorh H (1990) Ethylene-promoted ascorbate peroxidase-activity protects plants against hydrogen-peroxide, ozone and paraquat. *Plant Cell Environ* 13:971–976
91. Lopez F, Vansuyt G, CasseDelbart F, Fourcroy P (1996) Ascorbate peroxidase activity, not the mRNA level, is enhanced in salt-stressed *Raphanus sativus* plants. *Physiol Plant* 97: 13–20
92. Al-Ghamdi AA (2009) Evaluation of oxidative stress tolerance in two wheat (*Triticum aestivum*) cultivars in response to drought. *Int J Agric Biol* 11:7–12

93. de Pinto MC, Lavermicocca P, Evidente A, Corsaro MM, Lazzaroni S, De Gara L (2003) Exopolysaccharides produced by plant pathogenic bacteria affect ascorbate metabolism in *Nicotiana tabacum*. *Plant Cell Physiol* 44:803–810
94. Wang SD, Zhu F, Yuan S, Yang H, Xu F, Shang J, Xu MY, Jia SD, Zhang ZW, Wang JH, Xi DH, Lin HH (2011) The roles of ascorbic acid and glutathione in symptom alleviation to SA-deficient plants infected with RNA viruses. *Planta* 234:171–181
95. Goggin FL, Avila CA, Lorence A (2010) Vitamin C content in plants is modified by insects and influences susceptibility to herbivory. *BioEssays* 32:777–790

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