Protective Effect of Artificially Enhanced Level of L-Ascorbic Acid against Water Deficit-Induced Oxidative Stress in Rice Seedlings

Yong Chool Boo, Moonjae Cho[1] and Jin Jung*

Department of Agricultural Chemistry, College of Agriculture and Life Sciences, Seoul National University, Suwon 441-744, Korea
[1]Department of Biochemistry, Cheju National University Medical School, Ara1, Cheju 690-756, Korea

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Effects of the enhanced level of L-ascorbic acid (AA) on the water deficit-induced oxidative damage were studied in rice (*Oryza sativa* L.) seedlings. The seedlings sprayed with 20 to 80 mM L-galactono-γ-lactone (GL), a putative precursor of AA, showed 2 to 5-fold higher levels of AA compared with controls. Pretreatment of the seedlings with GL prior to water stress imposition caused virtually no effect on dehydration of tissues during water deficit but substantially mitigated oxidative injury, as accessed by 2-thiobarbituric acid-reactive substances, α-tocopherol, chlorophylls and β-carotene. Proline accumulation during water stress was also significantly lowered in the treated seedlings. In a complementary experiment, AA retarded photodegradation of α-tocopherol in isolated thylakoids far more efficiently than glutathione. GL in itself did not show any noticeable reactivity toward α-tocopheroxyl radical. The results demonstrate the antioxidative function of AA in rice seedlings encountering water-limited environments, suggesting a critical role of AA as a defense against oxidative stress in plants.

Key words : *L-ascorbic acid, L-galactono-lactone, antioxidants, singlet oxygen, water stress, Oryza sativa L.*

Physiological dysfunction of plant cells incurred by severe water-deficit has been implicated to be oxidative stress, which results from a disturbance in the prooxidant-antioxidant balance in cells in favor of the former.[1,2] L-Ascorbic acid (AA) is an exceptional antioxidant that scavenges, either directly or indirectly, all of the reactive oxygen species and free radicals commonly encountered in plant cells.[3] The mechanism of antioxidant action of AA involves donation of an electron to various reactive species, eliminating their oxidative reactivity. The resultant monodehydroascorbate radical, that has been regarded as a 'sink' for oxidative radicals,[4] can be reduced back to AA by monodehydroascorbate reductase which uses NAD(P)H as an electron donor.[5] In our previous study,[6] an increased oxidation of AA and the activation of monodehydroascorbate reductase have been observed in rice seedlings during water deficit, implying an important role of AA in cells as a defensive measure.

AA is synthesized from D-glucose in eukaryotic organisms. The terminal step in the biosynthesis of AA in plants is the oxidation of L-galactono-γ-lactone (GL) catalyzed by GL dehydrogenase present in mitochondria[7,8] although another biosynthetic pathway has also been proposed.[9] Following the addition of GL to plant cells, the AA level has been observed to reach values several times larger than those of controls.[10] In the present work, rice seedlings, fed with GL to enhance endogenous cellular level of AA, were subjected to water stress treatment and examined for the protective effect of AA at an elevated level against the water deficit-induced oxidative injury.

*Corresponding author
Phone: 82-331-290-2406; Fax: 82-331-293-8608
E-mail: jinjung@plaza.snu.ac.kr

Abbreviations: AA, L-ascorbic acid; GL, L-galactono-γ-lactone; GSH, glutathione; TBARS, 2-thiobarbituric acid-reactive substances.

Materials and Methods

Plant materials and treatments. Rice (*Oryza sativa* L., cv. Hwasungbyeo) seeds were germinated under moist conditions in the dark. The germinated seeds were placed on cheese cloth stretched on plastic supports in a plastic tray (20×20×5 cm^3) and kept in a growth chamber at relative humidity of 60~65% and at 25±1°C. The seedlings were grown under continuous illumination (30 W/m^2). The tray was supplied with Hoagland solution just enough to cover the seeds once a day. On the 10th day of growth, the seedlings were sprayed with aqueous solution of GL (20~80 mM, 50 ml per tray). Water stress treatment was done by immersing roots in 30% polyethylene glycol 6000 solution. Control seedlings were kept in distilled water.

Metabolite analyses. Shoot extracts prepared by grinding 1 g of freshly harvested shoots in a mortar with addition of 5 ml of 5% metaphosphoric acid were analyzed for AA by reversed phase HPLC as in Anderson *et al.*[11] Tocopherols, chlorophylls and carotenoids were extracted with water-saturated n-butanol from shoot homogenates, prepared in 50 mM K-phosphate buffer (pH 7.0) containing 5 mM AA, and analysed by reversed phase HPLC with isocratic

elution of either 95% aqueous methanol for α-tocopherol and chlorophylls or chloroform/methanol (4:96) for β-carotene. α-Tocopherol was detected by using a fluorescence detector set at 298 nm for excitation and 328 nm for emission, and chlorophylls and β-carotene were detected by absorbance at 450 nm. Lipid peroxidation was estimated by measuring the content of 2-thiobarbituric acid-reactive substances (TBARS) in shoot homogenates, prepared in 10% trichloroacetic acid containing 0.25% 2-thiobarbituric acid and heated at 95°C for 25 min as in Heath and Packer.[12] Free proline extracted in 5% sulfosalicylic acid was measured according to Bates *et al.*[13]

Preparation of thylakoid membranes and photolysis. Thylakoids were prepared from rice leaves as described by Leegood and Malkin[14] and suspended in a suspension medium (0.3 M sucrose, 50 mM Hepes, 10 mM mannitol, 5 mM $MgCl_2$, 5 mM NaCl, 2 mM EDTA, adjusted to pH 7.6). Thylakoid suspensions (33 μg chlorophyll/ml) with additives as necessary were irradiated with white light (300 W/m^2) from a 1 kW Xe lamp under aerobic conditions at 25°C. The irradiated samples were partitioned with an equal volume of water-saturated n-butanol and analyzed for the metabolites as described above.

Photochemical production and detection of α-tocopheroxyl radical. A quartz cell containing ethanolic solution of 40 mM α-tocopherol plus additives was placed in a UV-visible spectrophotometer, and white light (30 W/m^2) from a 500 W-halogen lamp was supplied *in situ* to the sample through an optical fiber. The formation of α-tocopheroxyl radical was monitored at 425 nm. The absorption spectrum of α-tocopheroxyl radical was measured when the radical content reached a steady state level using a non-irradiated sample as a reference.

Results

Elevation of cellular AA level by GL treatment. GL treatment of rice seedlings induced a remarkable accumulation of AA in shoots as indicated by typical HPLC profiles of the shoot extracts from GL-treated and control seedlings. AA peak was identified by coinjection with authentic AA, which was further confirmed by a complete disappearance of the peak after incubation of the extract with AA oxidase (Fig. 1). Changes in AA level as a function of time in the seedlings treated with GL revealed that, although the rate of AA formation during the initial 6 h period seemed independent of the dose of GL, there was a reasonable dose dependency in the maximum AA level attained (Fig. 2), indicating slow but efficient penetration of GL into the plant cells and its quantitative conversion into AA. The AA content in the seedlings treated with 80 mM GL reached values 5-fold higher than the controls. Nonetheless, such high dose of GL did not cause any observable morphological change such as wilting that has been observed in detached bean shoots.[15] AA

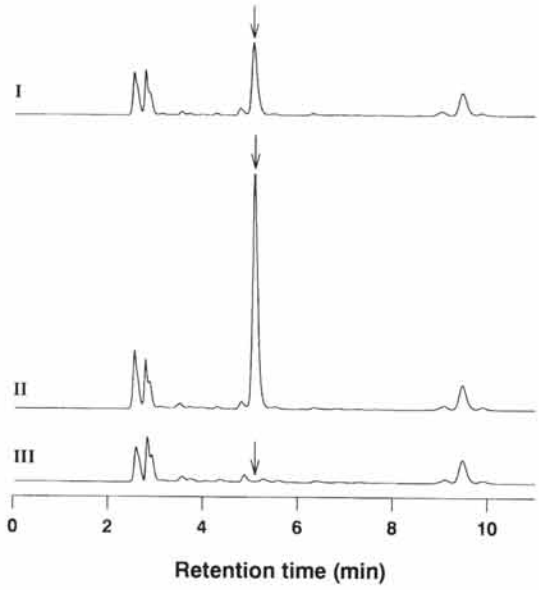

Fig. 1. Typical HPLC profiles of the shoot extracts from rice seedlings sprayed with either distilled water (I) or 80 mM GL (II) 24 h before sampling. An aliquot of the extracts from GL-treated seedlings was incubated with AA oxidase (0.1 unit/ml) prior to analysis (III). AA peaks are indicated by arrows.

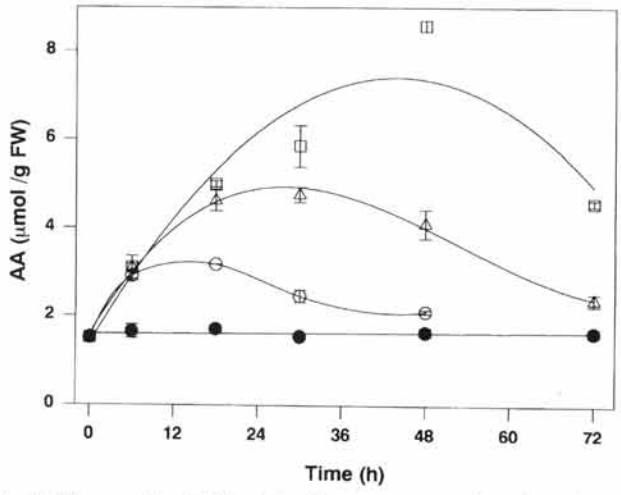

Fig. 2. Changes in AA level in rice shoots as a function of time from GL treatment. Rice seedlings were sprayed with aqueous solutions of 20 mM (○), 40 mM (△) and 80 mM GL (□) or distilled water (●), and kept in a growth chamber. Shoots were harvested from the seedlings at indicated time for the analysis of AA. Mean±SD, n=3.

level in GL-treated seedlings remained elevated for several days, which enabled us to study the influence of increased level of AA on water stress in rice plants.

Mitigation of water stress by GL treatment. The effects of GL treatment on various parameters relevant to the occurrence of oxidative reactions in plant cells during water deficit was investigated by using rice seedlings pretreated with 80 mM GL for 24 h and subsequently subjected to water stress for additional 48 h. Although tissue dehydration by water limitation was not curved by

Table 1. Effects of GL-pretreatment on water deficit-induced oxidative stress in rice seedlings.

Parameters	Control (A)	Water deficit		Change by water deficit (%) 100 (B-A)/A	Suppression by GL (%) 100 (B-C)/(B-A)
		-GL (B)	+GL (C)		
Water content (n=5)	85.5±0.46	51.2±5.37	54.4±5.29	-40.1**	n.s.
Ascorbate (n=6)	10.5±2.14	0.595± 0.43	3.23±1.33	-94.3**	26.6**
Chlorophyll a (n=5)	7.69±0.55	4.14±0.40	4.98±0.36	-46.1**	23.7**
Chlorophyll b (n=5)	2.79±0.14	1.80±0.22	2.16±0.28	-35.5**	36.4*
β-Carotene (n=6)	0.744±0.075	0.305±0.017	0.354±0.026	-59.0**	11.1**
α-Tocopherol (n=5)	0.329±0.030	0.188±0.013	0.227±0.020	-42.9**	27.7**
TBARS (n=8)	0.048±0.002	0.101±0.006	0.085±0.004	+111**	31.3**
Free proline (n=3)	3.59±0.29	21.8±2.39	16.4±1.16	+506**	29.3*

Rice seedlings were sprayed with either aqueous solution of 80 mM GL or distilled water 24 h prior to water stress treatment. The stress treatment was done by immersing roots in 30% polyethylene glycol 6000 solution for additional 48 h. Values (Mean±SD) are in units of μmol/g DW except for water content which is expressed in %. Asterisks denote the p values from t-test as follow: *0.01<p<0.05, **p<0.01. n.s., not significant.

GL treatment, all other parameters examined clearly indicated that incorporation of the AA precursor into plant tissues caused a mitigation of water deficit-induced oxidative damage to cells. As summarized in Table 1, the degrees of loss of photosynthetic pigments, such as chlorophylls and β-carotene, and small molecular antioxidants, such as AA and α-tocopherol, were significantly decreased by GL treatment. Further, membrane peroxidation reaction and proline formation became considerably lowered in the GL-treated seedlings as assessed by TBARS and free proline. Since there was no GL remaining in the tissues (data not shown), the effects of GL treatment in rice seedlings under water stress conditions are thought to be closely associated with those of an elevated AA level in shoots.

Suppression of light-induced decomposition of α-tocopherol in thylakoids by exogenous AA. Protective role of AA against oxidative decomposition of α-tocopherol in plant membranes was studied, using isolated intact thylakoids. Exposure of thylakoids to bright light resulted in a loss of α-tocopherol in the membranes, whose extent increased with increasing concentrations of salt in the suspension buffer (Fig. 3). This observation conformed to the notion that, under high osmotic conditions assumed to be achieved in water-stressed plant cells, light-dependent oxidative processes are accelerated in photosynthetic membranes. As also shown in Fig. 3, the decay of α-tocopherol in irradiated thylakoids was markedly inhibited by the presense of AA. In contrast, glutathione (GSH) was found to be far less effective in protecting α-tocopherol under the same conditions, which places serious doubts on the proposition regarding antioxidant role of GSH in recycling oxidized α-tocopherol.[16]

Interaction of AA with α-tocopheroxyl radical *in vitro*. The electron transfer reaction between AA and α-tocopheroxyl radical that was photochemically formed and spectrophotometrically identified was studied in ethanol (Fig. 4). Our preparation of α-tocopheroxyl radical showed an absorption spectrum which was essentially the same as has been measured by pulse radiolysis system.[17] The photoproduction

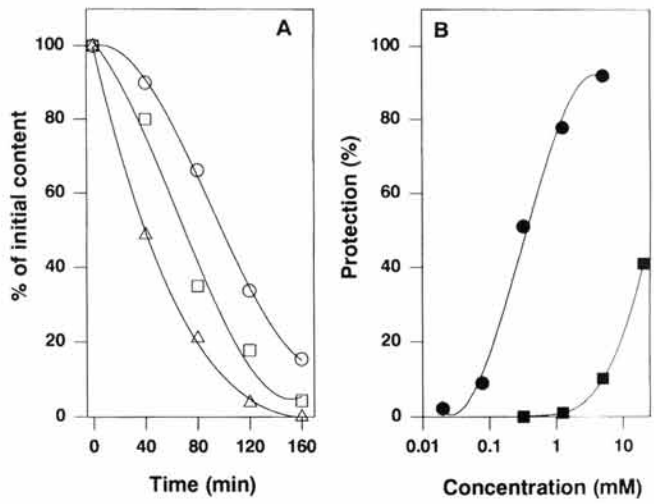

Fig. 3. Changes in the content of α-tocopherol in thylakoids as a function of irradiation time under high osmotic conditions (A) and effects of AA and GSH on the degradation of α-tocopherol (B). In (A), thylakoids were suspended in an ordinary suspension medium (○) or that supplemented with 0.75 M (□) or 1.5 M NaCl (△) and then exposed to light (300 W/m^2) for indicated time at 25°C. In (B), thylakoids sus-pended in the medium containing 1.5 M NaCl and various concentrations of either AA (●) or GSH (■) were irradiated for 120 min under the same conditions as above.

of α-tocopheroxyl radical, when spectrophotometrically monitored from the onset of photolysis of the ethanolic α-tocopherol solution, was markedly retarded by AA, indicating a rapid reduction of α-tocopheroxyl radical to α-tocopherol and thereby an efficient recycling of the radical. Since GL did not show any reactivity toward the radical even at a very high concentration, the possibility of the AA precursor acting as an antioxidant by itself in our GL treatment experiments with rice seedlings was completely ruled out.

Discussion

Our previous study[6] on the underlying mechanism of water stress in rice plants has produced some results that

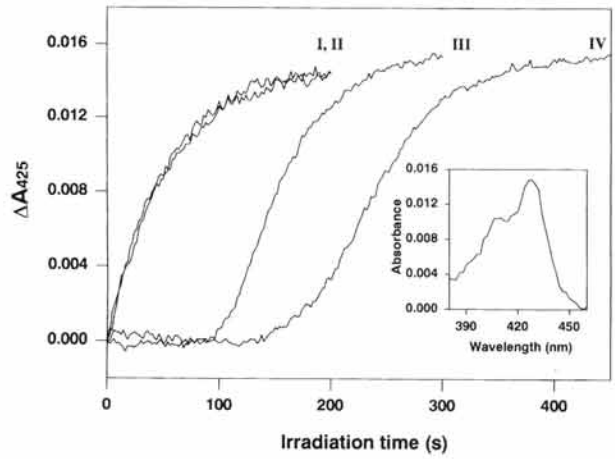

Fig. 4. Effects of AA and GL on photochemical formation of α-tocopheroxyl radical. Air-saturated ethanolic solutions (1.0 ml) of 40 mM α-tocopherol containing no additive (I), 20 mM GL (II), 2 μM AA (III) or 4 μM AA (IV) were irradiated in situ with a 500 W halogen lamp at 25°C. The formation of α-tocopheroxyl radical was monitored at 425 nm. Inset shows the absorption spectrum of α-tocopheroxyl radical.

are clearly indicative of an important role of AA in conjunction with α-tocopherol, as a major defense against excessive production of a certain prooxidant in shoot cells encountering water-limited environments. A marked increase in the capacity of AA regeneration system in cells is therefore thought to be one of primary responses of plants to water deficit so as to mitigate oxidative injury. If such is indeed the case, it would naturally be expected that artificial enhancement of cellular AA level by a proper manipulation can result in a decrease in the susceptibility of plants to water deficit-induced oxidative damage. The results presented herein are well in line with this expectation, as implicated by various parameters measured that are widely regarded as being closely associated with the occurrence of oxidative processes in photosynthetic cells (Table 1).

Although the reactive species immediately responsible for initiating or mediating oxidative processes in plant cells during water deficit has yet to be identified, there are many lines of circumstantial evidence indicating that singlet oxygen is the responsible species,[1,6] which may be excessively formed in thylakoid membranes when they suffer alterations in their structural integrity. In fact, changes in the conformations of proteins as well as the positions of various molecular species in lipid bilayers of the thylakoids have been implicated to occur in leaves under water stress conditions.[18]

There exist several types of antioxidants in plant membranes, of which α-tocopherol is probably the most important. α-Tocopherol by itself can scavenge singlet oxygen.[19] However, the major function of this lipophilic antioxidant is found in its active role in scavenging lipid peroxyl and lipoxyl radicals that are involved in propagating chain reactions of lipid autooxidation.[16] Singlet oxygen formed in membranes would interact primarily with polyunsaturated lipids because of their abundance and reactivity. This interaction gives rise to formation of lipid hydroperoxides that appear to subsequently breakdown, leading to production of the chain-propagating lipid radicals. In such situations, the defensive action of α-tocopherol on the effects of singlet oxygen could be related more to the termination of the chain reactions by reacting with lipid radicals rather than to direct quenching of singlet oxygen *per se*. The resulting α-tocopheroxyl radical can then be reduced back to its original quinol form by reaction with AA.[17,20] A high reactivity of α-tocopheroxyl radical toward AA and thereby a rapid regeneration of active α-tocopherol in the presence of AA is evidenced, indirectly *albeit*, by the effective suppression of photochemical formation of the radical in *in vitro* systems used in the present study (Fig. 4).

AA and α-tocopherol are vulnerable to attack by singlet oxygen, being transformed into some oxidized forms that seem highly unstable under physiological conditions. For instance, dehydroascorbate, an oxidized form of AA, decomposes to yield tartarate and oxalate at pH higher than 6.0.[21] Therefore, the sizes of both the ascorbate pool and the α-tocopherol pool would inevitably become smaller in plant cells in situations where an excess formation of singlet oxygen takes place unless biosynthesis of the antioxidant molecules keeps pace with their degradation. Such conjecture seems to conform to our observation as shown. Since the vulnerability of the antioxidants to singlet oxygen attack provides in itself a basis for protection of biological systems against singlet oxygen-mediated damage, decreases in the pool sizes of both AA and α-tocopherol in rice seedlings during water stress seem to be a natural consequence of the ongoing defensive processes.

In conclusion, the results may suggest that AA play a crucial role as an efficient defense against water stress in plants. Further, this study provides a working hypothesis that a highly drought-resistant variety of rice might be developed by increasing AA biosynthesis capacity of plant cells by appropriate biological manipulations, either by breeding or by molecular biological approaches.

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