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Short Communication

Changes Induced by Abscisic Acid and Light in the Redox State of Ascorbate in the Apoplast of Epicotyls of *Vigna angularis*

Umeo Takahama

Department of Biology, Kyushu Dental College, Kitakyushu, 803 Japan

Levels of ascorbic acid (AA) and dehydroascorbic acid (DHA) were examined in epicotyl segments and intact epicotyls under various conditions. It appears that not only the redox state of an AA-DHA system but also the level of AA plus DHA in the apoplast might be affected by growth conditions.

Key words: Abscisic acid — Apoplast — Elongation growth — Light — Redox state of ascorbic acid — *Vigna angularis*.

The presence of AA and DHA in the apoplast has been reported in leaves (Castillo and Greppin 1988, Polle et al. 1990, Luwe et al. 1993, Takahama and Oniki 1992) and in stems (Takahama 1993a, Takahama and Oniki 1994). The ratio of the level of AA to that of DHA is decreased by stresses such as ozone fumigation (Castillo and Greppin 1988, Luwe et al. 1993), wounding (Takahama 1993a) and detachment of leaves from plants (Takahama and Oniki 1992). Functions of apoplastic AA have been discussed in relation to the detoxification of ozone that is taken up by plants (Castillo and Greppin 1988, Luwe et al. 1993) and the regulation of peroxidase-dependent polymerization of phenolic compounds (Takahama 1993a, b) and of peroxidase-dependent formation of cross-links between soluble molecules of extensin (Cooper and Varner 1984).

Recently, it was reported that externally added MDA radicals, generated by a redox equilibrium between AA and DHA, enhance the elongation growth of onion root cells (Hidalgo et al. 1991) and the proliferation of promyelocytic leukemia cells (Alcain et al. 1990). Externally added MDA radicals can also induce hyperpolarization of plasma membrane (Gonzales-Reyes et al. 1992) and stimulate uptake of nutrients (Gonzales-Reyes et al. 1994) by onion root cells. These observations suggested the present author that it might be of interest to estimate the apoplastic level of MDA radicals from the equilibrium constant for interconversion of AA, DHA and MDA. This communication deals with the ABA- and white light-induced changes in the levels of AA and DHA in the apoplast of epicotyls of *Vigna angularis* and includes a calculation of the level of MDA radicals.

Seedlings of *Vigna angularis* Ohwi et Ohashi were grown from seed as described previously (Takahama and Oniki 1994). Seven to ten days after sowing, epicotyls were harvested. Epicotyl segments from a region between 0.5 cm and 1.9 cm from the first leaves were used to study the effects of ABA on the levels of AA and DHA in the apoplast. Ten epicotyl segments were incubated for 20 h at 27°C (a 4-h light period followed by a 12-h dark period and another 4-h light period) in a solution (20 ml) of 10 mM sodium phosphate (pH 6.8) and 230 mM sucrose with or without 0.1 mM ABA. Light was supplied by daylight-type fluorescent lamps (maximum intensity, 6,000 lux). After incubation, the lengths and fresh weights of the epicotyl segments were measured and the segments were used to determine the levels of AA and DHA in the apoplast.

To study the effects of light on the levels of AA and DHA in the apoplast, seedlings were grown from seeds for 5 days after sowing, in the dark at 27°C. The lengths of epicotyls of the seedlings ranged from 15 to 25 mm (average, 19.5 mm). Then, growth of half of the seedlings was continued in darkness and the rest of the seedlings were grown under the continuous white light from daylight-type fluorescent lamps (maximum intensity, 6,000 lux). After incubation in darkness or in the light for 24 h at 27°C, epicotyls were harvested. Epicotyl segments from the region between 0.5 cm and 3 cm from the first leaves were used to determine the levels of AA and DHA in the apoplast.

Abbreviations: AA, ascorbic acid; DHA, dehydroascorbic acid; HPLC, high-performance liquid chromatography; IWF, intercellular washing fluid; MDA, monodehydroascorbic acid.

Table 1 Effects of ABA on levels of AA and DHA in the apoplast

Parameter	Non-incubated	Control	ABA
Increase in fr wt (mg)	—	50 ±10	28 ±8
Elongation (mm)	—	1.4 ± 0.1	0.6±0.1
IWF [(μl (g fr wt)$^{-1}$]	18 ±5	18 ± 4	17 ±2
AA+DHA [nmol (g fr wt)$^{-1}$]	2.8 ±0.3	1.2 ± 0.5	0.5±0.1
AA (AA+DHA)$^{-1}$	0.23±0.03	0.13± 0.01	—[a]

Ten epicotyl segments (14 mm long; 194±15 mg fr wt) were incubated for 20 h at 27°C. IWF was obtained by vacuum-infiltration in 100 mM KCl and centrifugation. The first column shows data from the epicotyl segments prior to incubation. Values are the means±SE; n=3.

[a] AA was not detected.

IWF, which contained the apoplastic components was obtained from the epicotyl segments by centrifugation at 1,000×g for 5 min after vacuum-infiltration in 100 mM KCl for 3 min at 4°C. AA in IWF was quantitated by HPLC immediately after the preparation of IWF to eliminate the possibility of the ascorbate oxidase-dependent oxidation of AA after the preparation of IWF (Takahama and Oniki 1994). DHA in IWF was also quantitated by HPLC after its reduction to AA by addition of 3 μl of 100 mM DTT to 3 μl of IWF. HPLC to quantitate AA was performed on a Shimpack CLC-C_8 column (6 mm i.d.×16 cm; Shimadzu, Kyoto) equipped with a spectrophotometric detector with a photodiode array (SPD-M1A; Shimadzu). The mobile phase was a mixture of methanol and 25 mM KH_2PO_4 (1 : 9, v/v) and the flow rate was 1 ml min^{-1} [for details, see Takahama and Oniki (1994)]. It has been reported that almost all of the AA and DHA in IWF is derived from the apoplast of epicotyls (Takahama and Oniki 1994). The levels of AA and DHA in the apoplast per g fresh weight were calculated from concentrations of AA and DHA in IWF and the volume of IWF per g fresh weight.

The ratio of the level of AA to that of AA plus DHA was more than 0.9 to 1 in whole epicotyls of *V. angularis*. It has been shown that the redox state is not affected even when the apoplastic levels of AA and DHA are changed by ozone fumigation (Luwe et al. 1993), wounding (Takahama 1993a) or detachment of leaves from plants (Takahama and Oniki 1992). Table 1 shows the effects of ABA on the increases in fresh weight and in length of epicotyl segments after the incubation for 20 h. ABA suppressed increases in both fresh weight and length by about 50%. The volume of IWF was not affected by ABA, suggesting that the phytohormone did not affect the volume of intercellular gas space. The level of AA plus DHA decreased to about 40% of the initial level and the ratio of the level of AA to that of AA plus DHA decreased from 0.23 to 1 to 0.13 to 1 after the incubation of epicotyl segments for 20 h in the absence of ABA (Table 1). ABA stimulated these decreases. No AA was detected in IWF of epicotyl segments that had been incubated in the presence of ABA. It has been demonstrated that, in IAA-treated epicotyl segments of *V. angularis*, the level of AA plus DHA in the apoplast increases but the redox state becomes more oxidized

Table 2 Effects of white light on levels of AA and DHA in the apoplast

Parameter	Dark-grown seedlings	Light-grown seedlings
Length of epicotyls (mm)	56.4 ±6.1	28.7 ±3.6
Fr wt of ten epicotyl segments (g)	0.31±0.03	0.34±0.01
IWF [μl (g fr wt)$^{-1}$]	27 ±7	11 ±4
AA+DHA [nmol (g fr wt)$^{-1}$]	4.0 ±0.6	0.9 ±0.4
AA (AA+DHA)$^{-1}$	0.19±0.06	0.28±0.09

Seedlings were grown for 5 days after sowing in darkness and then they were grown for a further 24 h in darkness (dark-grown seedlings) or in the light (light-grown seedlings). The length of epicotyls of seedlings grown for 5 days in darkness was 19.5±3 mm. After harvesting of epicotyls, segments (25 mm long) were obtained as described in Materials and Mathods and weighed. To obtain IWF, segments were vacuum-infiltrated in 100 mM KCl and centrifuged. Values are the means±SE; n=3.

Table 3 Effects of phytohormones and light on level of MDA radical in the apoplast

Compound	Phytohormone				Light/Dark	
	Non-incubated	Control	ABA	IAA[b]	Dark	Light
AA [nmol (g fr wt)$^{-1}$]	0.64	0.16	—[a]	0.37	0.76	0.25
DHA [nmol (g fr wt)$^{-1}$]	2.16	1.04	0.50	4.93	3.24	0.65
MDA radical [pmol (g fr wt)$^{-1}$]	0.12	0.04	—[a]	0.14	0.16	0.04
MDA radical [nM in IWF]	6.5	2.2	—[a]	2.7	5.9	3.6

The levels of AA, DHA and MDA were calculated from the data in Tables 1 and 2.

[a] Almost zero.

[b] Calculated from the data reported by Takahama and Oniki (1994).

(Takahama and Oniki 1994).

The elongation growth of epicotyls was greatly inhibited by illumination (Table 2). However, there was no significant difference in fresh weight between the light-grown and the dark-grown epicotyls. The volume of IWF per g fresh weight was smaller in the case of epicotyls of seedlings grown in the light than in the case of those grown in darkness, suggesting a light-dependent inhibition of the increase in the intercellular gas space. The levels of AA and DHA in the apoplast of epicotyls were much higher in seedlings grown in darkness throughout than in those transfered in the light after initial growth in darkness. The level of AA relative to that of AA plus DHA increased somewhat by illumination.

From the data in Tables 1 and 2, it appeares that levels of AA and DHA in the apoplast change depending on changes in physiological conditions. The data also indicate that there is a system(s) for translocation of AA and DHA across the plasma membrane, the activity of which may be dependent on phytohormone and light. Such a system for translocation of AA and DHA across the plasma membrane has been suggested (in *Sedum*, Castillo and Greppin 1988; in spinach, Takahama and Oniki 1992). The chloroplast envelope can also transport AA and DHA (Beck et al. 1983). Animal species that require AA as vitamin C absorb it, via an intestinal transport system, against a concentration gradient using the electrochemical potential of sodium ions (Rose 1988). Simple diffusion across biomembranes seems to be impossible for the translocation of either AA or DHA under normal conditions, given to their oil/water distribution coefficients (Rose 1987). Changes in the redox state of the AA-DHA system may be due, in part, to changes in activities of redox enzymes in the apoplast (Takahama and Oniki 1994).

There was no relationship between the redox state of the apoplastic AA-DHA system and the growth of epicotyls. However, the level of AA plus DHA was correlated with elongation growth. Recently, it was demonstrated that externally added MDA radicals (20–100 nM) stimulate the elongation growth of onion root cells (Hidalgo et al. 1991) minicking the effects of auxin (Rubinstein and Luster 1993). The level of MDA radicals in the apoplast of epicotyls of *V. angularis* was calculated from the data in Tables 1 and 2 using an equilibrium constant, $[MDA]^2[AA]^{-1}[DHA]^{-1}$, of 10^{-8} (Alcain et al. 1990, Hidalgo et al. 1991, v. Foerster et al. 1965) and on the assumption that the equilibrium constant for the interconversion of AA, DHA and MDA is similar to that in solutions even in the apoplast (Table 3) since the level of MDA radicals may be too low for detection by electron spin resonance. The results obtained from the calculation demonstrated that the level of MDA radicals in the apoplast was higher under enhanced growth conditions than under suppressed growth conditions. The concentration of MDA radicals in IWF was calculated roughly to be several nanomolar from the volume of IWF per g fresh weight under various conditions (Table 3). The concentration of MDA radicals in the apoplast must be higher than in the IWF since IWF consists of vacuum-infiltrated water and apoplastic water. ABA, which participates in various physiological functions that include growth inhibition, decreased the apoplastic level of AA plus DHA and that of MDA radicals (Tables 1 and 3). Some stresses also cause a decrease in the level of AA plus MDA (Luwe et al. 1993, Takahama 1993a, Takahama and Oniki 1992) that may result in a decrease in the level of MDA radicals. In this way, the AA-DHA system in the apoplast seems to be affected by the physiological condition of the plant. It is postulated that the MDA radical in the apoplast is a physiological acceptor of electrons in an electron transfer reaction from the symplast to the apoplast across the plasma membrane (Morre et al. 1988, Gonzales-Reyes et al. 1992).

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