Water Deficiency-induced Changes in the Contents of Defensive Substances against Active Oxygen in Spinach Leaves

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Changes in the contents of defensive substances against the active oxygen in water-stressed spinach plants were examined. The contents of ascorbate peroxidase (AP; EC 1.11.1.7), glutathione reductase (GR; EC 1.6.4.2) and α-tocopherol increased remarkably in water-stressed spinach leaves, while those of superoxide dismutase (SOD; EC 1.15.1.1), dehydroascorbate reductase (EC 1.8.5.1), ascorbate and glutathione changed little. The content of α-tocopherol in chloroplast thylakoid membranes isolated from water-stressed leaves was higher than that from normal leaves. It is, therefore, conceivable that GR, AP and α-tocopherol might be related to the tolerance of plants to water deficiency.

Water is essential to plant life, and water-deficiency causes various physiological and biochemical effects of plants. The decrease in photosynthesis,[1,2] the closure of stomata[3] and osmotic adjustment[4] appear to be typical plant responses to water deficiency at the first stage. Plant-growth regulators have been suggested to play a role in the adaptive strategies of plants to water deficiency, and increases in the abscisic acid content have been reported in a number of plant species experiencing water deficiency.[5] Changes in protein and gene levels, which may be related to the tolerance to water stress, have also been examined in water-stressed plants.[5-9]

The closure of stomata by a decrease of water content in plant leaves disturbs the supply of CO_2 for photosynthesis. When water-stressed leaves were illuminated, the reducing power from the photosystem was not consumed for CO_2 fixation but for the activation of O_2.[10] The reduced species of O_2 are very toxic to plants and cause plant injury by various kinds of environmental stress such as aging,[11] treatment with herbicides[12,13] or heavy metals,[14] air pollutants,[15-17] and a high O_2[18] or low CO_2 atmosphere.[19] It is also widely known that plants have an intrinsic defensive system against active oxygen[20] and resist the stress-induced production of active oxygen by increasing components in this system. In the present work, various components participating in the defensive system against active oxygen were determined in spinach plants, which were water-stressed for a few days in an environment-controlled growth cabinet, and it was found that the contents of AP, GR and α-tocopherol increased.

Materials and Methods

Plant materials. Spinach (*Spinacia oleracea* L. cv. New Asia) was grown at 20/15±0.5°C day/night temperatures with a relative humidity of 70±5% in pots (the available soil capacity was 1.8 liter) in a naturally lighted environment-controlled glasshouse, as has been previously described.[21] Tabacco (*Nicotiana tabacum* L. cv. Samsoun)

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Abbreviations: AP, ascorbate peroxidase; GR, glutathione reductase; SOD, superoxide dismutase.

and petunia (*Petunia hybrida*) were grown under the same conditions, except that the temperature was 25/20±0.5°C. *Polygonum cuspidatum* was cultivated in the open air. *Kalanchoe rosei* and *Pelargonium ferulaceum* were grown in a greenhouse. The nutritional conditions for the cultivation of all plants were the same as those described previously.[22]

Water stress experiments. Spinach plants grown in a glasshouse for three to four weeks were transferred to an artificially lighted (about 30,000 lux) growth cabinet (170×230×190 cm), where the temperature and the relative humidity were maintained at 25±1°C and 40±5%, respectively. Half of the plants were provided with about 400 ml of water every morning, and the remaining half were not given water at all during the experiments. At 13:30 every day the leaf segments were sampled and stored at −80°C. The fresh weight of the water-stressed spinach plants had decreased by about 50% after 5 days.

Extraction of enzymes and reductants from plant leaves. The leaf segments were homogenized in the presence of a small amount of liquid N_2 with a mortar and pestle in various extraction media and, after centrifuging the homogenates, the supernatants were subjected to a determination of such enzymes and reductants as ascorbate and GSH as described previously.[21] Ascorbate in 5% metaphosphoric acid was determined by HPLC (Nihon Bunko VL-611) with a column of Inertsil ODS-2 (4.6×250 mm) from Gasukuro Kogyo. The eluting solution was 120 mM ammonium phosphate (pH 3.2). Ascorbate was measured by using a Nihon Bunko UVIDEC-100II at 258 nm. For tocopherol, 80% ethanol was used as the extraction medium, because its efficiency was similar to that of chloroform–methanol (1:2). Leaf segments (36 cm^2 in leaf area) were homogenized in 8 ml of 80% ethanol and centrifuged at 18,000 *g* for 30 min. To 5 ml of the clear supernatant, 0.5 ml of *n*-hexane was added, and the mixture fully agitated. After standing for 2 h, the upper layer of hexane was applied to HPLC. A column of μ Bondasphere 5 μ Si-100A (3.9×150 mm) from Waters was eluted with a mixture of *n*-hexane–2-propanol–tetrahydrofuran (96:1:3). Tocopherol was detected by using a Shimadzu RF-530 instrument by measuring the fluorescence intensity at 325 nm while exciting at 298 nm. Standard tocopherol from Merck was used.

Preparation of chloroplast thylakoid membranes. Intact chloroplasts were prepared from spinach leaves by using a Percoll according to the method of Nakano and Asada.[23] After lysis of the intact chloroplasts by fifteen volumes of 10 mM Tricine–KOH (pH 7.8) and 4 mM $MgCl_2$, thylakoid membranes were isolated according to the method of Douce and Joyard.[24] Tocopherol was extracted from the thylakoid membranes with 80% ethanol and determined by HPLC.

Results and Discussion

In the analysis by HPLC of the extracts from spinach leaves, the retention time of ascorbate was 6.2 min at a flow rate of 1.4 ml/min, and no peak other than ascorbate was found (data not shown). At a flow rate of 1.4 ml/min, α-, β-, γ- and δ-tocopherols showed retention times of 2.2, 6.6, 7.8 and 10.0 min, respectively. In the extracts from spinach leaves the peaks of α-tocopherol and an unknown substance having a retention time of 3.0 min were found, and no peaks corresponding to β-, γ- and δ-tocopherol were apparent in the extracts from spinach leaves. When *Kalanchoe* was used as the plant material, no peak other than α-tocopherol was found (data not shown).

Spinach plants in pots were grown in an environment-controlled growth cabinet without supplying water. The fresh weight of the spinach leaves decreased during the water-deficient period (Fig. 2), when several enzymes and reductants in the leaves were measured (Figs. 1 and 2). Although there was little difference in the contents of ascorbate (Fig. 1b), SOD (Fig. 1c), protein (Fig. 1a), and oxidized and reduced glutathione (Fig. 1d) between the water-stressed and the normal leaves, a remarkable increase of GR activity (Fig. 2c) and α-tocopherol content (Fig. 2b), and a slight increase of AP activity (Fig. 2a) occurred in the water-stressed spinach plants, while the respective contents in normal plants changed little. Among the three antioxidants, GR activity rose rapidly at an earlier time than the others did. The fact that the length of time required to bring about the increase of antioxidants in water-stressed plants varies with the antioxidant species may be due to the different time for the biosynthesis of antioxidants in water-stressed plants. There have been several reports of evidence that plants to oxygen-rich air,[18] the herbicide acifluorfen[25] and the air pollutant O_3,[26] which might enhance the production of active oxygen in plant cells, increased the GR activity. In field-grown wheat, GR activity was increased in dry lands more than in irrigated lands.[27]

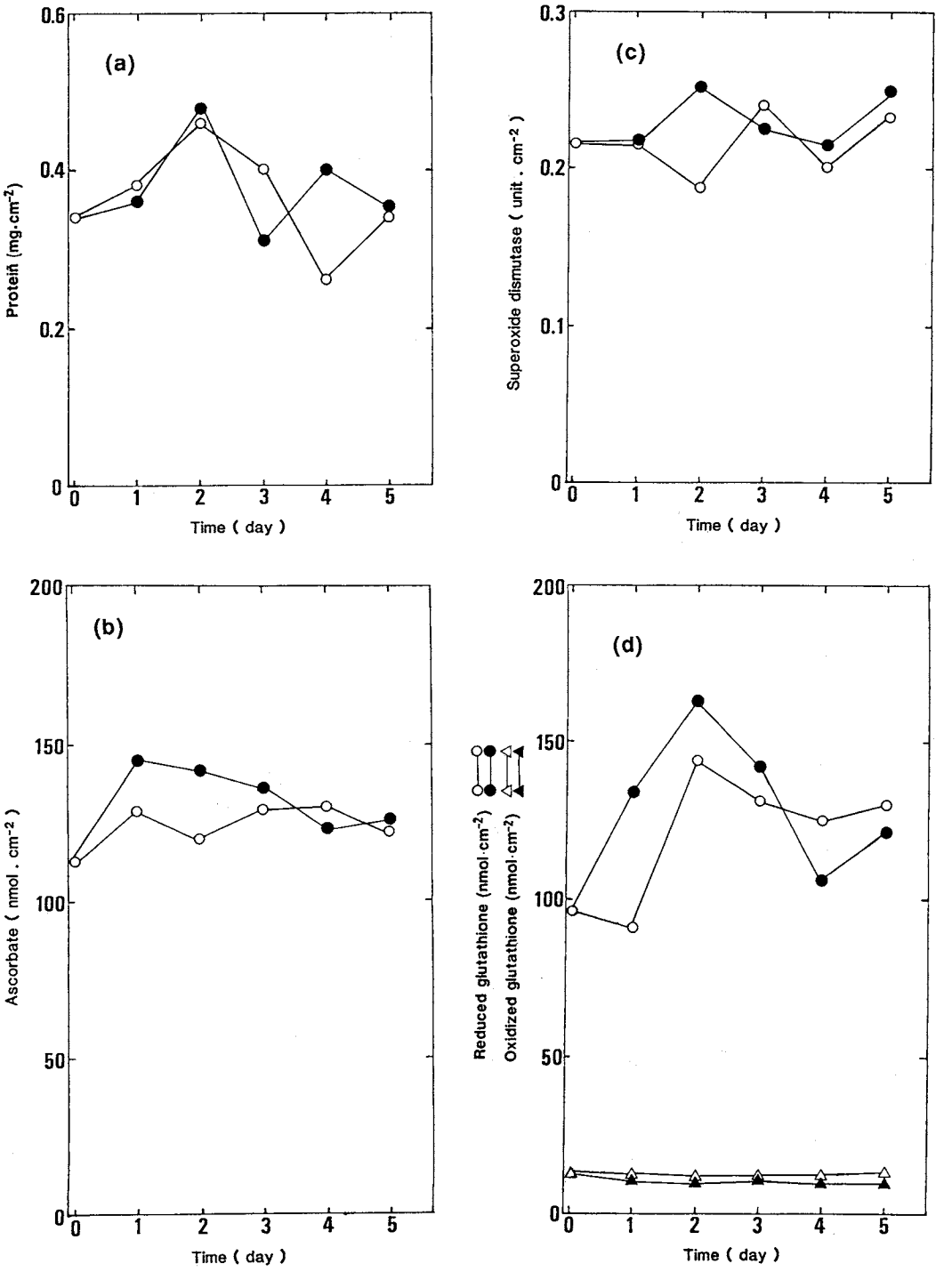

Fig. 1. Changes in Active Oxygen-defensive Substances (Protein, Superoxide Dismutase, Ascorbate and Glutathione) in Water-stressed Spinach Plants.

One group of spinach plants was supplied once a day with water (open symbols: ○—○, △—△), and the other group was not (closed symbols: ●—●, ▲—▲). At the indicated times, leaf segments (1.5 × 4 cm) were sampled by scissors and stored at −80°C.

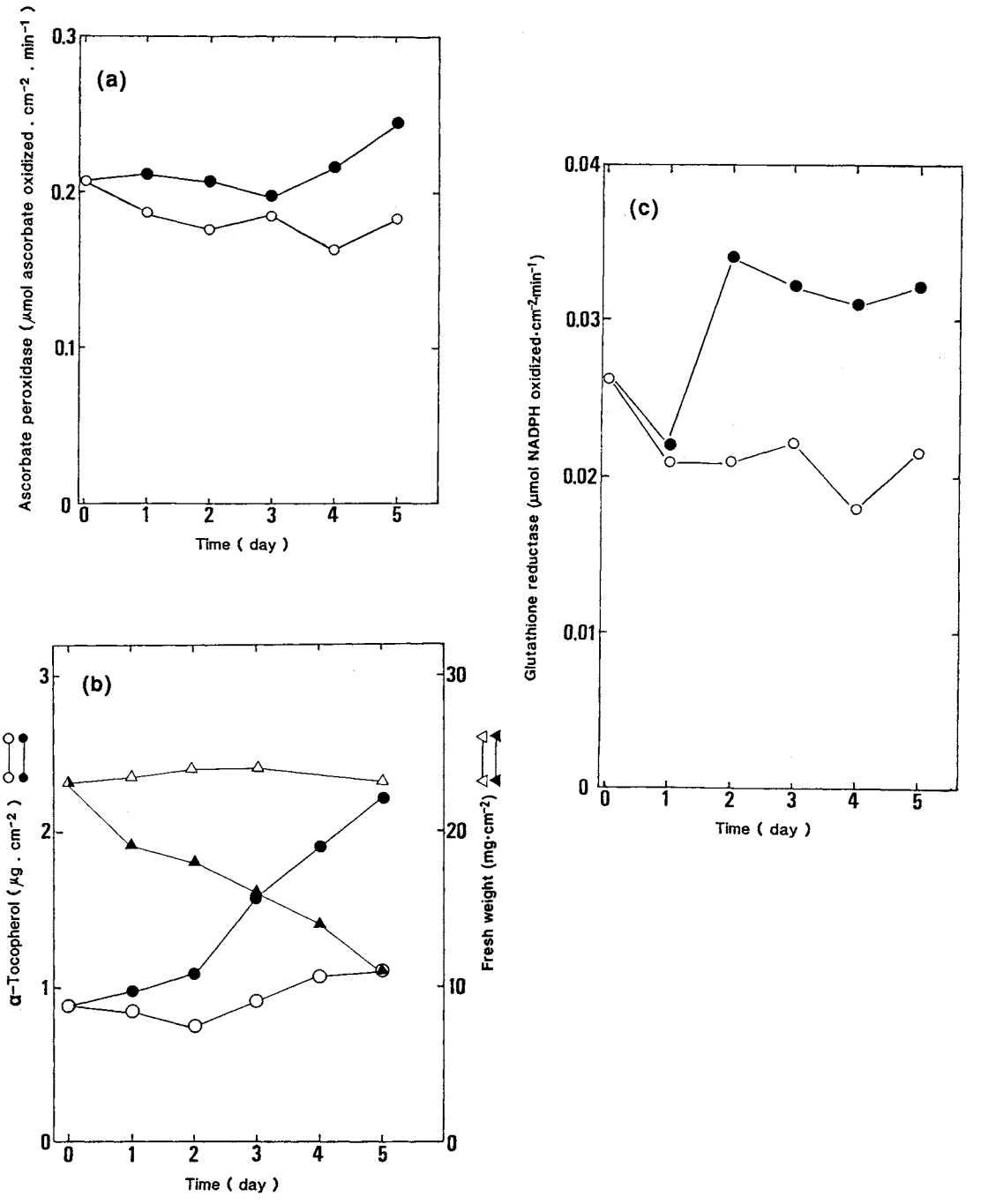

Fig. 2. Changes in Active Oxygen-defensive Substances (Ascorbate Peroxidase, Tocopherol and Glutathione Reductase) in Water-stressed Spinach Leaves.

The experimental conditions and the symbols are the same as those in Fig. 1.

Here, the increase of GR activity was observed in spinach plants water-stressed for a few days in a glasshouse. It is, therefore, conceivable that active oxygen may increase in water-stressed plants and that active oxygen may stimulate the increase of GR activity. Similarly,

since an increase of AP activity has been observed in a low cooncentration of O_3-fumigated spinach leaves,[28] the rise of AP activity in water-stressed spinach plants may show plant detoxification to water stress-induced active oxygen production.

Chloroplast thylakoid membranes were isolated both from water-stressed (5 days) and normal spinach plants, and their tocopherol content was determined. The content of α-tocopherol in the thylakoid membranes of water-stressed plants was about twice that of the normal plants (Table I). It is, therefore, conceivable that the plants might protect themselves against active oxygen toxicity by increasing the α-tocopherol content in chloroplast thylakoid membranes when plants suffer from water stress. Since active oxygen is mainly produced in chloroplast thylakoid membranes, it is also conceivable that the increase of tocopherol, the greater part of which is localized in thylakoids, may be important for the plant tolerance to water stress. It has been confirmed that the main site for the biosynthesis of tocopherol is localized in the chloroplast envelope.[29,30] (It is, therefore, possible that the increase of α-tocopherol content in the thylakoid membranes was caused by a stimulation of the biosynthesis of tocopherol in the chloroplast envelope, or by a rise of the transport rate of tocopherol from the chloroplast envelope to the thylakoid membranes.

Table I. Content of α-Tocopherol in Chloroplast Thylakoid Membranes from Water-Stressed (5 days) Spinach Plants and Normal Plants

Values indicate the mean ± standard deviation of triplicate determinations.

Treatment	α-Tocopherol content ($\mu g \cdot mg$ of protein^{-1})
Water-stressed	0.86 ± 0.14
Normal	0.42 ± 0.05

Chloroplast thylakoid membranes both from water-stressed and normal spinach plants were isolated as described in the Materials and Methods section, and their content of α-tocopherol was measured.

Table II. Content of α-Tocopherol in Six Species of Plants

Each value is the mean ± standard deviation of three plants. Mature leaves from each plants were used.

Plant species	α-Tocopherol content ($\mu g \cdot g\,fw^{-1}$)
Spinach	10.3 ± 1.4
Tobacco	14.5 ± 1.2
Petunia	18.5 ± 2.1
Polygonum cuspidatum	39.4 ± 6.8
Kalanchoe rosei	49.3 ± 4.7
Pelargonium ferulaceum	40.6 ± 5.0

The content of α-tocopherol in mature leaves was higher in the water stress-tolerant plants (*Polygonum cuspidatum*, *Kalanchoe rosei* and *Pelargonium ferulaceum*) than in the water stress-sensitive ones (spinach, tobacco and petunia) (Table II). This result also suggests that tocopherol may play an important role in defense against water stress. *Kalanchoe* and *Pelargonium* are CAM plants and live naturally in the desert. It is well-known that these plants have an outstanding ability for conserving water. Here, it was confirmed that they were equipped with a physiological defensive system against damage caused by water stress. The increases of GR activity and of the content of α-tocopherol in the water-stressed plants, and the fact that tocopherol is present more abundantly in water stress-tolerant plants than in water stress-sensitive plants, suggests the possibility that tocopherol, AP and GR are candidates for physiological indices of plant water stress-tolerance.

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