Comparison of Antioxidant Enzyme Activities between *Solanum tuberosum* L. Cultivars Danshaku and Kitaakari during Low-Temperature Storage

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We compared the antioxidant enzyme activities between *Solanum tuberosum* L. cultivars Kitaakari and "Danshaku" during storage at 1 °C and 20 °C. The Kitaakari and Danshaku plants contained approximately 330 μM and 120 μM ascorbic acid (AsA) immediately after the harvest, respectively. At 1 °C, the activity of ascorbate peroxidase (APx) in the Kitaakari plants showed the tendency to increase, while in the Danshaku its activity increased temporally by 9 weeks and thereafter returned to basal levels. Superoxide dismutase (SOD) activity increased after 12 weeks in the case of the Kitaakari at 1 °C. Catalase did not show any difference in both cultivars at each temperature. The contents of AsA, which was one of the substrates of APx, decreased more rapidly at 1 °C than at 20 °C in both cultivars. Particularly in the case of the Danshaku, AsA contents were already less than 30 μM at 9 weeks, which confirmed that APx was inactivated.

Keywords: *Ascorbic acid; ascorbate peroxidase; superoxide dismutase; catalase; potato tubers; low temperature; ascorbate recycling*

INTRODUCTION

Plants vary in their responses to cold temperatures. Species of tropical and subtropical origin are sensitive to chilling and are therefore usually damaged or killed when exposed to low temperatures. In contrast, plants from temperate regions are resistant to colder temperatures and can withstand winter freezing. This includes an increase in resistance to freezing when exposed to very low temperatures (Levitt, 1980). Acclimatized to cold comprises numerous physiological and metabolic changes in plants; changes in lipids, carbohydrate composition, and membrane structure have been correlated (Uemura and Steponkus, 1994; Webb et al., 1992). Furthermore, there is increasing evidence that chilling elevates the levels of active oxygen species (Wise and Naylor, 1987; Prasad et al., 1994; Omran, 1980), which likely contribute significantly to the damage caused by the chilling. Species of active oxygen, H_2O_2 (hydrogen peroxide), O_2^- (superoxide), $OH^\bullet$ (hydroxyl radical), and $^1O_2$ (singlet oxygen), are present in all the plants at various degrees; this is the result of normal aerobic metabolism. When allowed to accumulate, active oxygen can cause damage to cellular components and thereby severely disrupt metabolic function (Elstner, 1987). Plants possess active oxygen scavenging systems, and they can usually keep active oxygen below harmful levels (Loewus, 1988). When a plant is stressed, the production of oxygen can exceed the capacity of the scavenging systems, resulting in oxidative damage. Under prolonged oxidative conditions, active oxygen would cause lipid peroxidation, DNA damage, and protein denaturation (Fridovich, 1978; Halliwell and Gtteridge, 1986; Pacifici and Davies, 1990; Scandalions, 1993; Tottempudi and Prasad, 1997).

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There are enzymatic and nonenzymatic free radical scavenging systems in plants. The following are a few examples as enzymatic scavengers: superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APx). SOD reacts with superoxide radicals ($O_2^{\bullet -}$) and converts them to O_2 and H_2O_2. CAT detoxifies H_2O_2 into water, and APx scavenges H_2O_2 using AsA as an electron donor. Furthermore these enzymes have a functional cooperation (Mizuno et al., 1998). The nonenzymatic scavengers such as lipid-soluble antioxidants, AsA, carotenoids, and glutathione play an important role in biochemical adaptation (Kendall and McKersie, 1989; Walker and McKerise, 1993). Plants contain abundant AsA, which has important antioxidant and metabolic functions. However, some reports have shown that AsA content decreases rapidly during storage (Esteve et al., 1995; Mizuno et al., 1998). It has been ascertained that APx activity is inactivated at the concentration of less than 30 μM of the endogenous AsA contents (Miyake and Asada, 1996). In this paper, we used two potato cultivers with different AsA contents in order to compare the influence of endogenous AsA contents on antioxidant enzymes under low-temperature stress.

MATERIALS AND METHODS

Growth and Storage Conditions. Tubers of the *Solanum tuberosum* L. cultivars, Kitaakari and Danshaku, were grown at the Kobe University experimental farm. Both cultivars were stored at 1 °C and 20 °C, and each group was sampled at the 3rd, 6th, 9th, and 15th weeks of storage.

Quantitative Analysis of AsA by HPLC. The sample (10 g) was homogenated in a sufficient amount of cold 5% metaphosphoric acid (20 mL) and filtrated through four layers of gauze. The filtrate was centrifuged at 10 000*g* for 20 min at 4 °C, and the supernatant was passed through a Millipore filter (0.22 μm). HPLC analysis was performed with slight modifications according to the method of Rose and Nahrwold (1981).

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For the HPLC, the filtrate was applied to a 5 NH_2 column (4.6 × 250 mm, Nakarai tesque, Japan) using a GL Sciences model 570B; the sample was then eluted with 20 mM KH_2PO_4/CH_3-CN (20:80, v/v) at a flow rate of 2 mL/min. A UV detector was used at 254 nm. The quantity of AsA in the potato was determined using three replicates each.

Extraction. For the SOD assay, the frozen sample (10 g) was homogenized in 40 mL of 100 mM Na_2HPO_4/KH_2PO_4 (pH 7.0). The homogenate was centrifuged at 4000*g* for 15 min at 4 °C. The supernatant was passed through a PD-10 column (Pharmacia Biotech, Tokyo), which was equilibrated with 50 mM $Na_2CO_3/NaHCO_3$ (pH 10.2) to remove low molecular weight substances that interfere with enzyme assay. For the CAT assay, all procedures were the same as for the SOD assay except that the desalting step was omitted, and the centrifugation was filtrated through four layers of gauze and centrifuged at 105 000*g* for 10 min at 4 °C. For the APx assay, the sample (5 g) was homogenized in 20 mL of ice-cold extract. The iced-cold extraction medium contained 50 mM Na_2HPO_4/KH_2PO_4 (pH 7.0), 1 mM EDTA, 5% polyvinylpolypirolidone (PVPP), and 1 mM AsA. The homogenate was filtrated through four layers of gauze and centrifuged at 105 000*g* for 10 min at 4 °C. For the monodehydroascorbate reductase (MDHAR) assay and the dehydroascorbate reductase (DHAR) assay, the samples (5 g) were homogenized in 20 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 2 mM 2-mercaptoethanol. The homogenate was filtrated through four layers of gauze and centrifuged at 13 000*g* for 20 min at 4 °C. Total soluble protein content in the enzyme extracts of potato tubers was determined by DC protein assay (Bio Rad laboratories) modified with the method of Lowry et al. (1951). The Bio-Rad protein assay (Bio Rad laboratories) modified with the method of Bradford. (1976) was used for MDHAR and DHAR assays. Bovine serum albumin was used as the protein standard.

Enzyme Assays. *APx (EC 1.11.1.11).* APx activity was elicited out according to the methods of Nakano and Asada (Nakano and Asada, 1981) in 3 mL of a reaction mixture containing 50 mM potassium phosphate (pH 7.0), 2 mM AsA, 2 mM H_2O_2, and 100 μL of enzyme extract. Oxidation of AsA was determined by monitoring the decrease in absorbance at 300 nm (extinction coefficient 0.74 $mM^{-1}\cdot cm^{-1}$). One unit of APx was defined as the amount of enzyme oxidizing 1 μmol of AsA per min.

CAT (EC 1.11.1.6). CAT activity was measured at 25 °C according to the method described by Aebi (1984). The assay contained 3.125 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 200 μL of enzyme extract in a total volume of 3 mL. CAT activity was estimated by the decrease in absorbance of H_2O_2 at 240 nm; one unit of CAT was defined as the amount of enzyme dismuting 1 μmol of H_2O_2 per min.

SOD (EC 1.15.1.1). SOD activity was determined essentially as described by Spychalla and Desborough (Spychalla et al., 1990). The assay was performed at 25 °C in a 3 mL cuvette containing 50 mM $Na_2CO_3/NaHCO_3$ (pH 10.2), 0.1 mM EDTA, 0.015 mM ferricytochrome *c*, and 0.05 mM xanthine. The assay was initiated by the addition of sufficient xanthine oxidase to produce a basal rate of ferricytocrome *c* reduction corresponding to an increase in A550 of 0.025. After determination of the amount of xanthine oxidase, enzyme extract was added, and the resulting reaction velocity was calculated. One unit of SOD was defined as the amount of enzyme inhibiting the rate of ferricytochrome *c* reduction by 50%.

MDHAR (EC 1.6.5.4). MDHAR activity was determined as described by Hossain et al. (Hossain et al., 1984). The reaction mixture (1 mL) contained 50 mM Tris-HCl buffer (pH 7.6), 0.1 mM NADH, 2.5 mM AsA, and AsA oxidase (0.14 unit, 1 μmol Ascorbate oxidized min^{-1} being 1 unit) at 25 °C. Oxidation of NADH was determined by monitoring the decrease in absorbance at 340 nm (absorbance coefficient 6.2 $mM^{-1}\cdot cm^{-1}$)

DHAR (EC 1.8.5.1). DHAR activity was assayed at 25 °C by following the increase in absorbance at 265 nm (absorbance coefficient 6.2 $mM^{-1}\cdot cm^{-1}$) due to the GSH-dependent production of AsA (Nakano and Asada, 1981).

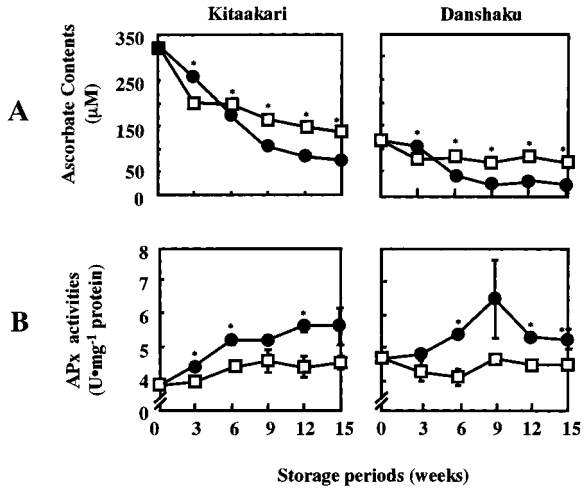

Figure 1. Changes in (A) ascorbic acid contents and (B) ascorbate peroxidase activities during storage. After tubers had been stored at 1 °C (●) and 20 °C (□) for the times indicated; samples were taken for the determination of tissue levels. Data are mean ± SE (n = 3). Asterisks indicate significant difference between the value of storage at 20 °C and at 1 °C by Student's t-test (*$p < 0.05$).

RESULTS

Kitaakari. The AsA content of Kitaakari (330 μM) was about triple that of the Danshaku plant (120 μM) at zero time storage (Figure 1A). Though it was higher at 1 °C than at 20 °C until 3 weeks, the contents decreased more rapidly at 1 °C than at 20 °C after 6 weeks. The contents within 15 weeks of storage at 1 °C and at 20 °C decreased to approximately 41% and 23%, respectively, compared with the respective storage conditions at time zero storage. The APx activity is shown in Figure 1B. In the storage at 1 °C, APx activity could maintain higher activity than at 20 °C during the storage period, and the highest activity was approximately 1.5-fold, compared with time zero storage (3.81 U). In contrast, APx activity did not show any change during the 15-week storage period at 20 °C.

SOD activity during the storage at 1 °C increased after 12 weeks (Figure 2A). In the storage at 20 °C, SOD activity barely changed during the entire storage period. During the storage period as a whole, SOD activity was higher at lower temperatures than it was at higher temperatures. CAT activity did not show any significant change throughout the storage period at 20 °C. In the storage at 1 °C, CAT activity tended to decrease temporarily and afterward it increased. Thus, CAT activity did not depend on the storage temperature, as shown in Figure 2B.

MDHAR activity gradually increased in the storage at 1 °C (Figure 3A) and reached 124.4 $nmol\cdot mg^{-1}$ $protein\cdot min^{-1}$, and it was about 37% compared with time zero storage. Its activity in the storage at 20 °C decreased to 25% of the zero time storage levels within the first 3 weeks. Thereafter, MDHAR activity remained fairly static until 15 weeks. DHAR, the other enzyme of AsA recycling system, was also measured. Though DHAR activity in the storage at 20 °C was higher than at 1 °C, its activity gradually decreased in both storage at 1 °C and 20 °C (Figure 3B). At 1 °C, DHAR activity decreased to 36% of time zero storage levels, whereas at 20 °C it decreased to 46% of the original levels.

Danshaku. The AsA levels of Danshaku plants were lower than those of the Kitaakari during the entire

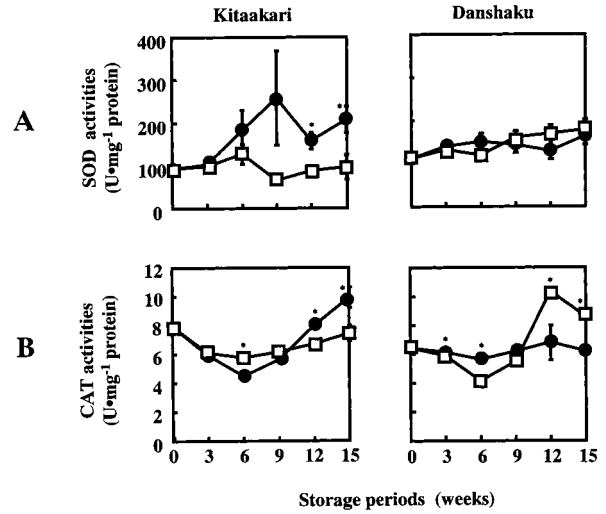

Figure 2. Changes in catalase activity and superoxide dismutase activity during storage of potato tuber at 1 °C (●) and 20 °C (□) for the times indicated; samples were taken for the determination of tissue levels. Data are mean ± SE (n = 3). The values of total protein contents (each cultivars at each of three sampling dates during storage) do not differ significantly ($P < 0.05$) through the whole storage period and storage temperature. Asterisks indicate significant difference between the value of storage at 20 °C and at 1 °C by Student's t-test (*$p < 0.05$).

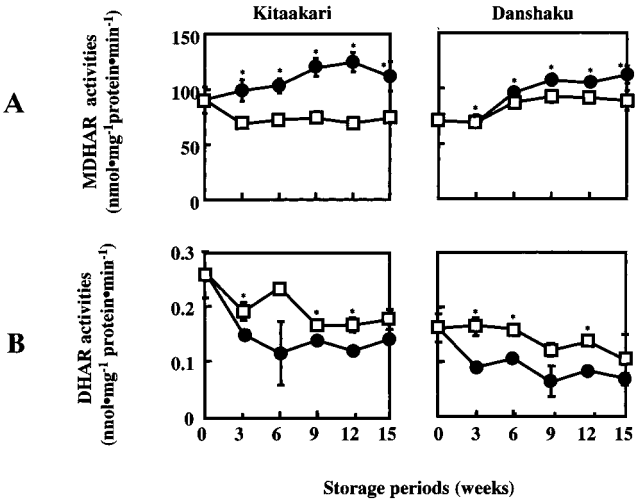

Figure 3. Changes in (A) monodehydroascorbate reductase (MDHAR) activity and (B) dehydroascorbate reductase (DHAR) activity during storage of potato tubers at 1 °C (●) and 20 °C (□) for the times indicated; samples were taken for the determination of tissue levels. Data are mean ± SE (n = 3). The values of total protein contents (each cultivar at each of three sampling dates during storage) do not differ significantly ($P < 0.05$) through the whole storage period and storage temperature. Asterisks indicate significant difference between the value of storage at 20 °C and at 1 °C by Student's t-test (*$p < 0.05$).

storage period at both temperatures (Figure 1A). Similar to Kitaakari results, the Danshaku AsA contents at 3 weeks were higher at 1 °C than they were at 20 °C; thereafter, AsA contents decreased more rapidly at the low temperature. Compared to the AsA contents at time zero storage with 1 °C and 20 °C storage, they decreased to about 18% and 28%, respectively. APx activity of Danshaku showed a different tendency than that of the Kitaakari plant. APx activity in the storage at 1 °C seemed to decrease after 9 weeks (Figure 1B). However, at 20 °C, APx activity maintained the same levels as at time zero and was lower than activity in the storage at 1 °C throughout the storage period.

No drastic change in SOD activity was observed during the entire storage period at either storage temperature (Figure 2A). SOD activity in the storage at 1 °C and 20 °C did not change throughout the storage period. As shown in Figure 2A,B, SOD and CAT activities changed very little and did not depend on the storage temperature.

MDHAR activity (Figure 3A) at time zero storage was 70.7 nmol, which was lower than that of the Kitaakari. It increased gradually in the storage at both 1 and 20 °C, but the degree of change was little higher at 1 °C than at 20 °C throughout the storage period. As compared with Kitaakari, the MDHAR activity of the Danshaku was less pronounced than that of the Kitaakari. DHAR activity (Figure 3B), at both temperatures, decreased by 36% and 57%, respectively, compared to time zero storage; DHAR showed higher activity in the storage at 20 °C than at 1 °C during the storage period. From these results, APx in the antioxidant enzymes appeared to be most sensitive to the storage temperature.

DISCUSSION

We investigated antioxidant enzymes in two cultivars of potato tubers at low and high temperatures during a short-term storage of 15-week. AsA contents in both cultivars decreased drastically during storage at a low temperature (1 °C) (Figure 1A). A similar finding was observed in asparagus too (Esteve and Farre, 1995). The tendency toward such decrease in both types of potato cultivars was much higher at 20 °C than at 1 °C for the first 6 weeks of storage. Thereafter, the trend reversed until 15 weeks. There are some reports that low-temperature storage generates toxic activated oxygen species in plants, which increase the activated oxygen amounts in inner cells (Tottempudi and Prasad, 1997; Saruyama and Tanida, 1995; Wise and Naylor, 1987). Spychalla and Desborough (1990) suggested that, in potato tubers, there is a relationship between the level of antioxidant enzymes and the rate of active oxygen production during the low-temperature storage. Moreover, it has been reported that APx is an important enzyme that detoxifies H_2O_2, generated in potato tubers at low temperatures (Mizuno et al., 1998). Therefore, it seems that the observed decrease in AsA contents at low temperatures might be associated with the generation of active oxygen. As shown in Figure 1B, APx activity seemed to increase in Kitaakari plants throughout the entire low-temperature storage period. However, APx activity seemed to decrease after 9 weeks in the Danshaku plant. It has been ascertained that APx activity is inactivated at concentrations of less than 30 μM of the endogenous AsA contents (Miyake and Asada, 1996). This is considered to be the reason the APx activity showed the tendency of increasing throughout the storage period in the case of the Kitaakari plants and the tendency of decrease after 9 weeks in the case of the Danshaku plants. Indeed, at 1 °C AsA levels in the Kitaakari plants were at least 73 μM, at 15 weeks, but the AsA levels in the Danshaku had already reached 22 μM at 9 weeks (Figure 1A). It has been reported that APx activity temporally increased and thereafter declined during low-temperature storage in "Touya", which contained almost the same levels of AsA contents as in the Danshaku plant (Mizuno et al.). In this study, we

had stored each potato tuber during short-term storage such as 15 weeks when it might not be enough to indicate the certain tendency of relationship between AsA contents and APx activity at low temperature. Because Spychalla and Desborough (1990) have reported that alteration of the increased levels of antioxidant enzymes in potato may improve the ability of potato tubers to withhold oxidative stresses during low temperature and long-term storage.

Dismutation of the superoxide anion by SOD might be the primary step in the defense against the low-temperature treatment. In this study, SOD activity in the Kitaakari showed a 2.0-fold increase in tubers stored at 1 °C compared with the time zero measurement (Figure 2A, left). It has been reported that an increase in SOD activity alone enhances cytotoxicity due to species of active oxygen (Finazzi-Agro and Di Giulio, 1986; Iwahashi and Ishii, 1988; Scott et al., 1987). In such instances, cytotoxicity was likely due to the accelerated formation of the enzyme's product, H_2O_2, and subsequent generation of the hydroxyl radical by metal. It has been suggested that in order to minimize cytotoxicity caused by active oxygen in the presence of elevated SOD activity, H_2O_2 must also be effectively scavenged (Finazzi-Agro and Di Giulio, 1986; Scott et al., 1987). Therefore, concurrent increases in other antioxidant enzymes may become necessary. As shown in Figure 1B, APx activity in the Kitaakari plant continued to increase during storage at 1 °C but not at 20 °C. This result demonstrates that, after dismutation of the superoxide anions into H_2O_2 and O_2 by SOD, APx may reduce H_2O_2 into water, thus converting AsA into monodehydroascorbate, resulting in AsA consumption. On the contrary, in the Danshaku plants storage at low and high temperatures, SOD activity showed no changes. This suggests that H_2O_2 generation in Danshaku is lower than in Kitaakari. CAT, another H_2O_2 scavenging enzyme, did not correspond to SOD activity (Figure 2A,B). The same phenomena have been observed by Spychalla and Desborough (Spychalla and Desborough, 1990). Such observations might be explained by the CAT not working until H_2O_2 concentration increased beyond a certain threshold, since CAT possesses a very low affinity for H_2O_2.

Asada and Takahashi (Asada and Takahasi, 1987) described that in the detoxification mechanism H_2O_2 is reduced to H_2O by APx. The regeneration of reduced AsA from MDHA or DHA, which are the reaction products of APx, can be catalyzed either by NADH-dependent MDHAR or by GSH-dependent DHAR, coupled with glutathione reductase. As shown in Figure 3A, MDHAR was sensitive to low-temperatures. However, DHAR activity declined during storage (Figure 3B). The cause of this decline in DHAR activity is not clear. Nevertheless, such a decline has also been found in other plant tissues exposed to low temperatures (Asada and Takahashi, 1987; Cakmak et al., 1993). Our results imply that during storage at low temperatures the regeneration of reduced AsA for scavenging H_2O_2 may be mostly catalyzed by MDHAR activity rather than by DHAR activity. However, the rate of increase in MDHAR activity was, at its maximum, equivalent to 1/45-fold of the rate of increase of the APx activity (Figure 1B). These findings suggest that the AsA levels decrease during storage, despite the regeneration of AsA activated by low temperatures. Thus, APx may be one of key enzymes against low-temperature stress, and its activity may depend on an endogenous concentration in potato tubers.

ABBREVIATIONS USED

APx, ascorbate peroxidase; SOD, superoxide dismutase; CAT, catalase; AsA, ascorbic acid; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; H_2O_2, hydrogen peroxide; $O_2^{\bullet -}$, superoxide radicals.

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