Function of Ascorbate Peroxidase Against Active Oxygen Species in Potato Tubers During Storage at Low Temperature

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FUNCTION OF ASCORBATE PEROXIDASE AGAINST ACTIVE OXYGEN SPECIES IN POTATO TUBERS DURING STORAGE AT LOW TEMPERATURE

低温貯蔵中におけるジャガイモ塊茎に生成する活性酸素種に対する
アスコルビン酸ペルオキシダーゼの機能

SACHIKO KAWAKAMI

川上佐知子

CONTENTS

CHAPTER I

GENERAL INTRODUCTION

Although a variety of temperate plant species can undergo processes leading to cold hardening, many plants of tropical origin do not tolerate cold and can be damaged at quite modest temperatures. Various mechanisms have been proposed to account for chilling injury in plants (Levitt, 1980). A critical element in chilling-sensitive plants appears to relate to cellular membrane integrity. As the temperature is reduced, lipids in cell membranes change from a liquid crystalline condition to a solid state at a transition temperature that is determined by the ratio of saturated to unsaturated fatty acids (Quinn, 1988). This critical change occurs at a temperature that is equivalent to the temperature that elicits chilling damage. Development of frost hardiness in chilling- and frost-resistant plants appears to involve changes in the ratio between the different classes of fatty acids. An increase in the proportion of unsaturated fatty acids results in the membrane functioning at low temperature. The change in the physicochemical state of plant cell membranes brought about by reduced permeability may result in ionic and pH imbalance together with the malfunction of complex membrane-based systems such as mitochondria and chloroplasts. Indeed, products of glycolysis have been observed to accumulate and less ATP is formed (Levitt, 1980). Such effects may be related to cell injury and killing. However, some plants are more sensitive to chilling injury than others, although their fatty acid ratios are similar, suggesting that other mechanisms are also important. It has been suggested that oxidative stress may be a significant factor to chilling injury in plants (Burdon, *et al.*, 1994). An evidence for oxidative stress in chilled *Arabidopsis thaliana* came from studies on tissue levels of H_2O_2 and changes in levels of reduced and oxidized glutathione (O'kane, *et al.*, 1996). Evidence for chilling induced oxidative stress has also been observed in maize seedlings (Prasad, *et al.*, 1994). In these studies H_2O_2 levels were elevated during acclimation, and catalase (CAT) and guaicol peroxidase

(POX) activities were also increased.

The production of active oxygen species, particularly H_2O_2, appears to be a ubiquitous defense mechanism of eukaryotes. In plants, the production of active oxygen species has been evidenced during pathogen infection (Baker and Orlandi, 1995) and in photooxidative processes induced during abiotic stress conditions such as chilling, drought, salt and ozone stress (Foyer, and Mullineaux, 1994). By virtue of the chemical properties of active oxygen species, they are highly reactive and have the potential to damage membrane lipids, proteins, chlorophyll, and nucleic acids, thus disrupting the homeostasis of the organism (Shaaltiel and Gressel, 1986; Scandalios, 1993). Plants have evolved several mechanisms to prevent or alleviate the damage from active oxygen species. These mechanisms include scavenging the active oxygen species by natural antioxidants such as ascorbate and α-tocopherol, and the use of an enzymatic antioxidant system including superoxide dismutase (SOD), CAT, POX, ascorbate peroxidase (APx), and glutathion reductase (GR), many of which act in tandem (Scandalios, 1993; Foyer and Mullineaux, 1994; Allen, 1995; Anderson *et al.*, 1995; Rao *et al.*, 1996). The existence of multiple molecular forms of antioxidant enzymes, their location within tissues, cells and organelles, as well as any changes they may undergo in response to various environmental signals or development implies potential roles for these isozymes in the detoxification of active oxygen species. Several isozymes of SOD, CAT, GR, POX, and APx are present in plants and their relative compositions change during exposure to stress. Acclimation and chilling of maize (Scandalios, 1990; Anderson *et al.*, 1995), air adaptation of submerged rice seedlings (Ushimaru *et al.*, 1995), and exposure of *Arabidopsis thaliana* to UV-B light (280-320 nm) or ozone (Rao *et al.*, 1996) have been shown to result in changes in antioxidant isozymes composition. Additionally, increases in the activity of different antioxidant enzymes under stress appear to occur simultaneously. For example, in tobacco and maize subjected to paraquot treatment, an increase in SOD activity is accompanied by an increase in GR activity (Malen *et al.*, 1990). Moreover, there were some reports that overexpression of SOD

results in improved oxidative stress tolerance (Bowler *et al.*, 1991; McKersie *et al.*, 1993; Sen *et al.*, 1993; Pitcher and Zilinskas, 1996). Regarding the other antioxidant enzyme against H_2O_2, APx, its importance was demonstrated in APx-antisense transgenic tobacco which was highly susceptible to ozone injury compared with the wild type (Örvar and Ellis, 1997). Many authors have reported the enhanced expression of APx in response to environmental and biotic stresses such as drought, salt, high light, chilling, UV irradiation, ozone, oxygen tension, wounding, and pathogen attack (Mittler and Zilinskas, 1994; Hernández *et al.*, 1995; Mishra, *et al.*, 1993; Rao *et al.*, 1996; Tanaka *et al.*, 1985; Willekens *et al.*, 1994; Conklin and Last, 1995; Ushimaru *et al.*, 1992; Örvar *et al.*, 1997; Zahaby *et al.*, 1995). Thus, in the detoxification APx seemed to mediate mainly the reduction of H_2O_2 to H_2O. Of course, CAT, which is mostly localized in peroxisomes, may be the other antioxidant enzyme to detoxify H_2O_2. However, CAT possesses a very low affinity (*Km* value of more than 1.1 M) for H_2O_2, and shows either no or very low activities in cytosol, mitochondria and chloroplasts.

Ascorbate (AsA) and glutathione (GSH) are the major water-soluble antioxidants in plants, occurring in millimolar concentrations in most tissues. AsA participates in the removal of hydrogen peroxide via APx. The resulting primary oxidation product, the monodehydroascorbate (MDHA) radical, is reduced by NAD(P)H-dependent monodehydroascorbate reductase (MDHAR) to AsA. Any MDHA escaping reduction disproportionates to form AsA and dehydroascorbate (DHA). DHA is unstable at physiological pH and is reduced to AsA by GSH, the reaction being catalyzed by DHA reductase. The resulting oxidized glutathione is reduced to GSH by NADPH-dependent glutathione reductase. This series of reactions are known as the Halliwell-Asada pathway (Asada, 1994). The AsA-GSH cycle has particular importance in

chloroplasts (which do not contain CAT), in which H_2O_2 and other potentially damaging forms of active oxygen species are formed as a consequence of photosynthesis (Asada, 1994). Particularly, APx allows scavenging of H_2O_2 at its site of production in photosystem I (PSI) (Asada, 1994).

AsA can also react non-enzymically with superoxide and singlet oxygen (Asada, 1994). Because AsA can directly and indirectly accept electron from photosystem I, it has been proposed that it has an important role in the regulation of photosynthesis and as a photoprotectant. Furthermore, it is also a cofactor for violaxanthin de-epoxidase, which forms zeaxanthin when leaves are exposed to excess excitation energy. Zeaxanthin is proposed to quench excitation energy in the light-harvesting pigment bed and radiate it as heat (Siefermann and Yamamoto, 1975). Therefore, AsA has a central role in photosynthesis and can reach concentrations of 10-50 mM in the stroma.

Potato (*Solanum tuberosum* L.) tubers are an important source of vitamin C (AsA) for many people because much AsA remains in potato tubers even after a long storage time. Low temperature storage of potato tubers can have the beneficial results of lowered respiration rates, slowed physiological aging, inhibition of sprouting, reduced evaporative water loss, and minimized microbial pathogenesis. However, the contents of AsA decrease more rapidly during storage at low temperature than those at high temperature. This phenomenon results in the deterioration of nutritional quality of potato tubers. Although potato tubers are non-photosynthetic tissue, the antioxidants and antioxidant enzymes are presumed to play the same role in scavenging active oxygen species in the chloroplasts. However, the behaviors of antioxidants and antioxidant enzymes are not fully understood in potato tubers stored at low temperature.

In **Chapter II**, the changes of AsA content and antioxidant enzymes were investigated at low and high temperatures in *Solanum tuberosum* L. cultivar 'Touya'. As it was ascertained that APx was a key enzyme to detoxify active oxygen species in antioxidant enzymes, molecular cloning of APx was carried out in **Chapter III**. In **Chapter IV**, the oxidative stress response of cytosolic

APx using a DNA probe encoding potato tubers cytosolic APx and anti-APx antibody was investigated. As APx activities were regulated by the concentration of endogenous AsA (Miyake and Asada, 1996), the changes of AsA contents and antioxidant enzymes were investigated using two potato tubers with different AsA contents, *Solanum tuberosum* L. cultivars 'Danshaku' and 'Kitaakari', in order to compare the influence of endogenous AsA contents on antioxidant enzymes under low temperature stress in **Chapter V**. In **Chapter VI**, as in **Chapter IV**, molecular mechanism of APx was investigated using two potato cultivars with different endogenous AsA contents. So far it was demonstrated that H_2O_2 was generated more in potato tubers stored at low temperature and APx was induced *de novo* synthesis to detoxify H_2O_2. In **Chapter VII**, the author cytochemically investigated the localizations of H_2O_2 and APx in potato tubers stored at low temperature mainly using transmission electron microscopy by its reaction with cerium chloride to produce electron-dense deposits of cerium perhydroxides and by the immunostaining with anti-APx antibody. In **Chapter VIII**, taking all data in this study in consideration, the author discussed the defense mechanism by APx against generation of active oxygen species, particularly H_2O_2, and the localization of H_2O_2 and APx in tissue of potato tubers stored at low temperature.

CHAPTER II

THE BEHAVIOR OF ANTIOXIDANT ENZYMES IN POTATO TUBERS DURING STORAGE AT LOW TEMPERATURE

II-1 INTRODUCTION

During normal aerobic metabolism, ground state dioxygen can be chemically activated by a series of univalent reductions, yielding superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH). Oxygen can also be photochemically activated with an input of energy, yielding singlet oxygen ($^1O_2$) (Elstner, 1987). Activated oxygen is highly reactive and can cause deleterious oxidations of lipids, proteins, and nucleic acids (Halliwell, 1981; Fridovich, 1986; Halliwell, 1987; Harper and Harvey, 1978; Liebler and Reed, 1986; Niehaus, 1978; Price and Hendry, 1987; Wise and Naylor, 1987; Armstrong and Buchanan, 1978; Asada and Takahashi, 1987; Davies, 1987). Excessive oxidative damage to such molecules can seriously perturb normal cell metabolism.

Superoxide radicals are produced continuously in plant chloroplasts as O_2 is reduced to $O_2^{\bullet-}$ by electrons from the photosystems (the Mehler reaction) (Boveris *et al.*, 1978; Rabinowitch and Fridovich, 1983) or in mitochondria as electron transport chains leak electrons to oxygen (Elstner, 1987; Fridovich, 1986; Bowler *et al.*, 1994). Superoxide dismutase (SOD) scavenges the superoxide radical, the one electron reduced form of oxygen: $2\ O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$. Most plants contain a number of SOD isozymes that are located in various cellular compartments. In pea, Cu,Zn-SOD isoforms are found in chloroplasts and in the cytosol, whereas Mn-SOD is located in mitochondria.

H_2O_2 is highly toxic. Detoxification of H_2O_2 is mediated by catalase (CAT) which is mostly localized in peroxisomes (Elstner, 1987). However, CAT possesses a very low affinity (*Km* value is more than 1.1 M) for H_2O_2 and its activities are either low or not detectable in cytosol, mitochondria and chloroplasts (Halliwell, 1981). In the case of mammalian cells, glutathione

peroxidase plays an important role of detoxification of H_2O_2 in the cytosol and mitochondria (Chance *et al.*, 1979). In plant cells, an alternative and more effective detoxification mechanism against H_2O_2 exists, operating both in chloroplasts and the cytosol (Asada, 1992). It has been reported that this detoxification mechanism is ascorbate peroxidase (APx) by which H_2O_2 is reduced to H_2O (Asada and Takahashi, 1987).

Ascorbate (AsA), one of the important components used to estimate the quality of potato tuber, decreases more during storage at low temperature (1°C) than at higher temperature (5 or 20°C). Spychalla and Desborough (1990) demonstrated the possible relationship between the increases in antioxidants such as SOD, CAT and α-tocopherol and the rate of activated oxygen production in the potato tubers. However, little has been reported on the effects of the low temperature during the storage of potato tubers on AsA decrease. In this chapter, the behavior of antioxidant enzymes in potato tuber during storage at low temperatures will be described.

II-2 MATERIAL AND METHODS

II-2-1 Growth and Storage Conditions.

Tubers of *Solanum tuberosum* L. cultivar 'Touya' (an hybrid between WB77025-2 and R392-50) were grown in the experimental farm of Kobe University and stored at 1, 5 and 20°C every 3 weeks for 15 weeks. At various times after storage, tubers were immediately frozen in liquid nitrogen and stored at -80°C until use.

II-2-2 Protein 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 4,000 x *g* for 15 min at 4°C. The supernatant was passed through a PD-10 column (Pharmacia Biotech, Tokyo) equilibrated with 50

mM Na_2CO_3/$NaHCO_3$ (pH 10.2) to remove low molecular weight substances which interfere with the enzyme assay. For the CAT assay, all procedures were the almost same as for SOD except a desalting step by column was omitted and the centrifugation was carried out at 105,000 x *g*. For the APx assay, the sample (5 g) was homogenized in 20 ml of ice-cold extractant. The extraction medium contained 50 mM Na_2HPO_4/KH_2PO_4 (pH 7.0), 1 mM EDTA, 5% polyvinylpyrrolidone (PVP-40) and 1 mM AsA. The homogenate was filtrated through four layers of gauze, and was centrifuged at 105,000 x *g* for 10 min at 4°C. For monodehydroascorbate reductase (MDHAR) and dehydroascorbate reducatse (DHA) assay, the sample (5 g) was 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 x *g* for 20 min. For glutathione reductase (GR) assay, the sample (5 g) was homogenized with 100 mM potassium phosphate (pH 7.8) containing 2 mM EDTA and 1% PVP-40 at 4°C. The following procedures were the almost same as for DHA reductase except the centrifugation was carried out 15,000 x *g*. Total soluble protein content in the enzyme extracts of potato tubers were determined by the method of Lowry *et al.* (1951). Bovine serum albumin was the protein standard.

II-2-3 Enzyme assays.

II-2-3-1) 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 μmole of AsA per minute.

II-2-3-2) 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 μmole of H_2O_2 per minute.

II-2-3-3) SOD (EC 1.15.1.1)

SOD activity was determined essentially as described by Spychalla and Desborough (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 ferricytochrom *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 A_{550} of 0.025. After determination of the amount of xanthine oxidase, enzyme extract was added and the resulting reaction velocity calculated. One unit of SOD was defined as the amount of enzyme inhibiting the rate of ferricytochrom *c* reduction by 50%.

II-2-3-4) MDHAR (EC 1.6.5.4)

MDHAR activity was determined as described by 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 μmole 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 $mM^{-1}•cm^{-1}$)

II-2-3-5) 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 $mM^{-1}•cm^{-1}$) due to the GSH-dependent production of AsA (Nakano and Asada, 1981).

II-2-3-6) GR (EC 1.6.4.2)

GR activity was determined at 25°C according to Rao (1992), by following the rate of NADPH oxidation at 340 nm. The assay mixture contained 0.2 mM NADPH, 0.5 mM GSSG and 2 mM EDTA in 0.1 mM potassium phosphate buffer (pH 7.8). GR activity was expressed as nmole NADPH oxidized mg^{-1} protein•min^{-1}.

II-2-4 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 x *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). For the HPLC, the filtrate was applied to a 5 NH_2 column (4.6 x 250 mm, Nakarai tesque, Japan) using a GL Sciences model 570B; the sample was then eluted with 20 mM KH_2PO_4/CH_3CN (20:80, v/v) at a flow rate of 2 ml/min. An UV detector was used at 254 nm. The quantity of AsA in the potato was determined using three replicates each.

II-3 RESULTS

SOD activity was low in tubers at zero time storage (**Figure II-1A**), peaked at 6 weeks after harvest in tubers stored at 1 and 5°C and returned to basal levels by 15 weeks. It was increased approximately 2.5-fold during the first 6 weeks of storage at 1 and 5°C. On the contrary, tubers stored at 20°C tended to increase gradually by 12weeks 1.7-fold, and thereafter reached to the almost same level as the tubers stored at 1 and 5°C. Over the course of the entire storage, tubers stored at 1 or 5°C seems to have higher SOD activity than at 20°C.

CAT activity decreased once by 6weeks and then increased by 15 weeks again (**Figure II-1B**). Tubers during storage at 1 and 5°C for 15 weeks storage had higher CAT activity than tubers at 20°C. Moreover, CAT activity after the storage for 15 weeks at 1 and 5°C returned to the levels at zero time storage. However, in the case of storage at 20°C, its levels at 15 weeks decreased just to 0.7-fold original levels.

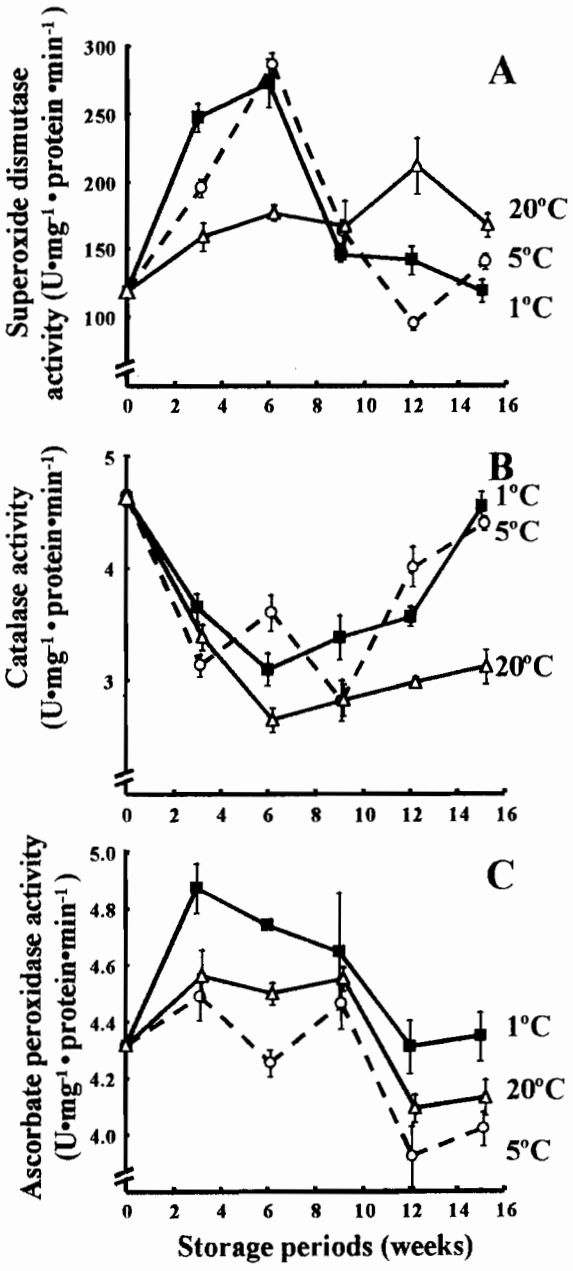

Figure II-1. Changes during storage in (A) superoxide dismutase activity, (B) catalase activity and (C) ascorbate peroxidase activity. After tubers had been stored at 1°C (■), 5°C (○) or 20°C (△) for the times indicated samples were taken for the determination of tissue levels of each antioxidant enzyme activity. Data are mean SE (n=3).

APx activity shows almost the same behavior as SOD activity (**Figure II-1C**). During the

first 3 weeks of storage at 1°C potato tubers increased their APx activities, and thereafter declined and returned to basal levels by 15 weeks. All over of the entire storage, tubers stored at lower temperature had higher activity than at higher temperature. Thus, when potato tubers were stored at low temperature, antioxidant enzymes such as SOD, CAT and APx were induced. These demonstrated that active oxygens were generated more at lower temperature than at high temperature during the storage.

Because the metabolism of H_2O_2 involving APx uses AsA as a substrate, cellular levels of AsA were investigated in potato tubers stored at 1°C as compared with at 5 and 20°C. As shown in **Figure II-2**, in tubers stored at 1°C AsA contents rapidly decreased by 6weeks and thereafter continued to dwindle by 15weeks whereas in tubers at 20°C they remained fairly static. While AsA contents at 5°C storage after 15 weeks were similar to that at 1°C for 15 weeks, differences were nevertheless evident at moderate periods of storage between 3 to 12 weeks at lowest temperature.

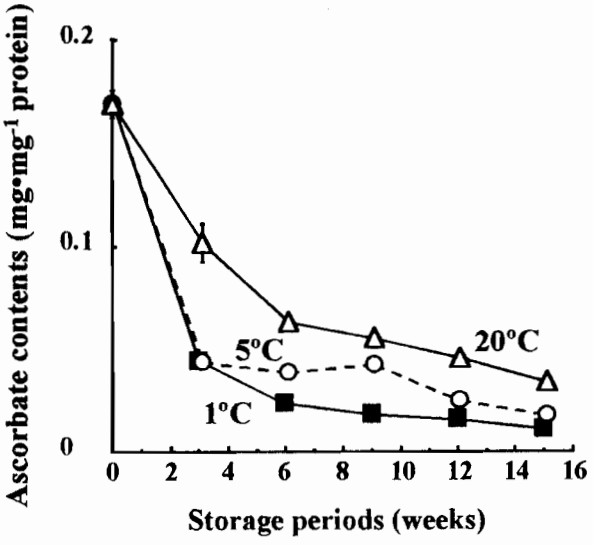

Figure II-2. Changes of ascorate contents during storage. Used symbols were the same in Figure 1. Data are mean SE (n=3).

It has recently reported that APx was inactivated at a low concentration of AsA resulting in the instability of Compound I to H_2O_2 (Miyake and Asada, 1996). This might cause APx to inactivate during the storage after 6weeks. Then, we measured each enzyme associated with ascorbate recycling (Halliwell-Asada pathway). **Figure II-3A** shows that during storage at 1°C, MDHAR activity increased by 12 weeks and then returned to baseline levels but in storage at 5 and 20°C it not change so much. Particularly, at 5°C, it tended rather to decrease. In the other two enzymes, DHA and GR, no drastic changes were observed during storage at each temperature (**Figure II-3 B and C**).

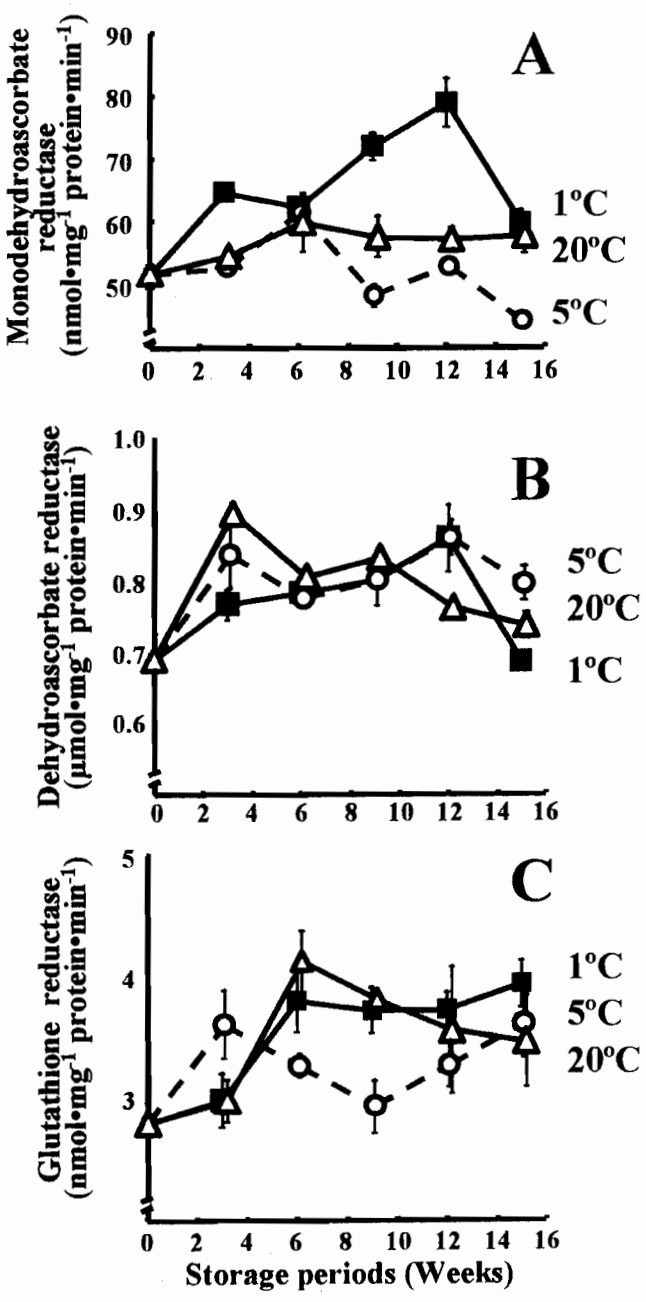

Figure II-3. Changes during storage in (A) MDHAR activity, (B) DHAR activity and (C) GR activity. After tubers had been stored at 1°C (■), 5°C (○) or 20°C (△) for the times indicated samples were taken for the determination of tissue levels of each antioxidant enzyme activity. Data are mean SE (n=3).

II-4 DISCUSSION

In this chapter, the behaviors of antioxidant enzymes such as SOD, CAT and APx in potato tubers were investigated during storage at low temperatures, because oxygen solubility in water is a function of temperature. There is 40% more dissolved oxygen in water at 9°C than 25°C, and 16% more at 3°C than at 9°C (Forstner and Gnaiger, 1983). This is important since electron transport chains leak electrons to oxygen forming superoxide radicals (Elstner, 1987; Fridovich, 1986; Boveris *et al.*, 1978). The rate of this leakage reflects proportionally to the local oxygen concentration (Halliwell, 1987).

Dismutation of $O_2^{\bullet-}$ by SOD might be the primary step for the defense against the low temperature treatment. SOD activity was a dramatic 2.5-fold increase in tubers placed into 1°C storage compared to the zero time measurement with a corresponding increase in APx activity during the storage at 1°C (**Figure II-1A and C**). This result seems to demonstrate that following dismutation of $O_2^{\bullet-}$ into H_2O_2 and O_2 by SOD, APx reduces H_2O_2 into water by converting AsA into monodehydroascorbate. Okuda *et al.* (1991) have previously observed levels of H_2O_2 rise in leaves of winter wheat when subject to cold treatments. A similar situation also exists in chilled cucumber plants (Omran, 1980)) and in maize seedlings (Prasad *et al.*, 1994). Such procedures resulting in increased APx at low temperatures may be the protection mechanism against H_2O_2. In fact, overproduction of SOD can be toxic to cells because excess H_2O_2 reacts with Fe^{2+} to produce highly toxic •OH (Mao *et al.*, 1993).

CAT, another H_2O_2 scavenging enzyme, did not correspond to SOD increase (**Figure II-1B**). CAT activity decreased first until APx activity tended to decrease and then increased slowly to basal levels during the storage at 1 and 5°C. Spychalla and Desborough (1990) also reported the same results in CAT activity when potato tubers were stored at low temperature such as 3 or 9°C. This might be caused by either that CAT does not work until H_2O_2 concentration increases more than certain

threshold because CAT possesses a very low affinity for H_2O_2 or that CAT is localized in peroxisomes.

Gupta *et al.* (1993) have observed that there is a compensatory increase in the expression of APx in transgenic tobacco plants that overexpress Cu,Zn-SOD. This seems to suggest that APx was induced to protect against hydrogen peroxide generated by SOD. It was suspected that an increase of SOD activity at low temperatures could be inducing increased levels of hydrogen peroxide that can diffuse freely between organelles and cytosol. Thus, the newly accumulated hydrogen peroxide in turn may be triggering a mechanism that increases the activities of APx.

It has been reported that mitochondria generated oxidative stress was the major source of the cytosolic H_2O_2 (Puntarulo, 1991). Indeed, APx which is located only in cytosol in potato tubers (Elia *et al.*, 1992), was activated within 6 weeks. It seems that APx which has the higher affinity for H_2O_2 works at low temperatures to detoxify H_2O_2, resulting the consumption of AsA as an electron donor. It is generally said that oxidized ascorbate produced by APx is reduced to AsA by reactions that are catalyzed by MDHAR, DHAR and GR in a series of reactions known as the Halliwell-Asada pathway (Bowler *et al.*, 1992). It was demonstrated that MDHAR was the most sensitive to low temperatures (**Figure II-3A**). This phenomena was also reported by Gupta *et al.* (1993). However, MDHAR activity was just 2% at the maximum to APx activity, suggesting that the contents of AsA decreased during storage even if the recycling system of AsA was activated by low temperature. Recently, it has reported that APx was inactivated at a low concentration of AsA resulting in the instability of Compound I to H_2O_2 (Miyake and Asada, 1996). As shown in **Figure II-2**, the concentration of AsA decreased more rapidly in the storage at 1°C than at higher temperatures. Therefore, the decrease of APx activity after storage for 3 weeks might be caused by the depletion of AsA as an electron donor in potato tubers at low temperatures.

CHAPTER III

CLONING OF ASCORBATE PEROXIDASE IN POTATO TUBER

III-1 INTRODUCTION

In **Chapter II**, it was demonstrated that APx was the most sensitive in potato tuber under low-temperature stress and it was suggested that this enzyme played an important role of detoxifying H_2O_2 germinated in potato tuber under low-temperature stress. Indeed there are many reports that APx responds to a wide range of environmental stresses. Ushimaru *et al.* (1997) suggested that APx played important roles in the protection against oxidative stress after exposure of rice (*Oryza sativa*) seedlings germinated under water to air. In infected tomato leaves, it was suggested that APx was mobilized as a detoxification mechanism for active oxygen spices (Kuźniak and Skłodowska, 1999). In *Arabidopsis thaliana*, APx was synthesized when the plant was irradiated with UV-B (Rao *et al.*, 1996).

APx in higher plants exists in at least three forms: cytosolic, stromal and thylakoid membrane-bound isoforms (Chen and Asada, 1989; Tanaka *et al.*, 1991; Miyake and Asada, 1992). Recently, some reports mentioned that the other types of APx were localized in mitochondria from pea leaves (Jimémez *et al.*, 1997; 1998), in membranes from oilseed glyoxysome (Yamaguchi *et al.*, 1995; Bunkelmann and Trelease, 1996), and in peroxisomes (Yamaguchi *et al.*, 1995). Several cDNAs which encode cytosolic APx of pea (Mittler and Zilinskas, 1992), *Arabidopsis* (Kubo *et al.*, 1992; Santos *et al.*, 1996), and fruits of bell pepper (Schants *et al.*, 1991) have been isolated. However, potato APx cDNA has not been isolated and it was not known which form of APx efficiently detoxified H_2O_2 in potato tuber under low-temperature stress. In this chapter, in order to determine the sequence and the form of APx in potato tuber, cloning from potato cDNA library was performed.

III-2 MATERIAL AND METHODS

III-2-1 Total RNA preparation.

Total RNA was prepared essentially as described by Yoshioka *et al.* (1995). Stored tubers were ground to fine powder in a mortar with a pestle under liquid nitrogen. The powdered cells were immediately transferred to the mixtures of phenol/chloroform/isoamyl alcohol (25:24:1, v/v, 2 ml/g tissues), to which an equal volume of RNA extraction buffer (0.1 M Tris-HCl, pH 9.0, 1% SDS, 0.1 M NaCl, 1 mM EDTA) and 1/5 volume of 2-mercaptoethanol were added. After the centrifugation at 2,000 x *g* for 20 min the resulting aqueous phase was adjusted to 0.25 M NaCl and subjected to RNA precipitation with an equal volume of isopropanol at -80°C for 30 minutes. After the centrifugation at 2,000 x *g* for 40 min at 4°C, the total RNA was isolated from the pellet according to the method of Chomczynski and Sacchi (1987) with a slight modification. The denaturing stock solution was composed of 4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), and 0.5% sarcosyl. Denaturing solution (solution D) was fleshly prepared by adding 2-mercaptoethanol (final concentration at 0.1 M) into the above solution. The pellet was completely dissolved in 500 μl of solution D by drawing the solution up and down several times through a 18G needle fitted on a 1 ml syringe. Sequentially, 50 μl of 2 M sodium acetate (pH 4.0), 500 μl of phenol (water saturate), and 500 μl of chloroform:isoamyl alcohol mixture (49:1) were added to the tube, with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 sec and cooled on ice for 15 minutes. The tubes were centrifuged at 15,000 rpm for 20 min at 4°C. After centrifugation, the aqueous phase was transferred to a fresh tube, mixed with 1 volume of isopropanol (or 2.5 volume of ethanol) and 1/10 volume of 3 M sodium acetate (pH 5.2), and then placed at –80°C for 15 min to precipitate RNA. Centrifugation at 15,000 rpm was again performed and the resulting RNA pellet was resuspended in 75% ethanol. After centrifugation, the supernatant was sucked off and the pellet was dried for 5 min, and RNA precipitation was dissolved in 200 μl of

diethyl pyrocarbonate (DEPC) treated water. The absorbance at 260 nm was measured by spectrophotometer and total RNA concentration was calculated (1 OD_{260} was considered as 40 μg/ml). Finally, total RNA was dissolved in DEPC treated water to adjust the concentration to 11 μg/5 μl.

III-2-2 cDNA synthesis.

Total RNA from potato tubers was transcribed into single-stranded cDNA, following the protocol of 1st Stand cDNA Synthesis Kit for RT-PCR (AMV) (Boehringer Mannheim). The reaction solution , consisting of 10x Reaction buffer (2.0 μl), 25 mM $MgCl_2$ (4.0 μl), deoxy nucleotide mix (2.0 μl), Oligo-pd(T)15 Primer (2.0 μl), RNase Inhibitor (1.0 μl), AMV Reverse Transcriptase-Polymerase (0.8 μl), sterile water (7.7 μl), and 1 μg of total RNA (0.5 μl) was incubated at 25°C for 10min and then at 42°C for 60 min in a thermocycler (GeneAmp PCR System 9700). AMV Reverse Transcriptase was denatured by incubating the reaction at 99°C for 5min and afterward cooling to 4°C for 5 minutes.

III-2-3 Amplification of APx DNA fragment by polymerase chain reaction (PCR).

The sense primer (APx1) and the antisense primer (APx2) (each synthesized 20 mer primer described in table III-1) were designed as a part of high homology among three plants APx cDNAs; *Arabidopsis thaliana*, soybean (Glycine max *L.*), and radish (Raphanus *L.*). APx DNA fragment was amplified from potato cDNA by PCR, following the protocol of Expand™ Hight Fidelity (Boehringer Mannheim). Two master mixes were set up as follows. Master mix 1 contained 1.6 μl of 25 mM *d*NTP mixture (consisting of each 25 mM *d*ATP, *d*CTP, *d*TTP, and *d*GTP, respectively, Pharmacia Biotech), 0.5 μl of each two primer (100 pmol/μl APx1, and APx2, respectively), 3 μl of cDNA (synthesized by reverse transcription), and 44.4 μl of sterile water. Master mix 2 contained 10 μl of 10x Expand HF buffer with 15 mM $MgCl_2$,

Table III-1. The sequence of APx1 and APx2

Primer	sequence
APx1	5'-GCATGGCACTCTGCTGGTAC-3'
APx2	5'-TCATCCGCAGCATATTTCTC-3'

0.75 μl of Expand enzyme mix and 39.25 μl of sterile water. Equal volume of master mix 1 and 2 were mixed in a PCR tube and placed in a thermocycler. Template was denatured at 94℃ for 2min and then PCR was performed, 25cycles. One cycle was composed of 3; degeneration (at 94℃ for 15 sec), annealing (at 45℃ for 30 sec) and elongation (at 72℃ for 1 minutes). Finally, the sample was kept at 72℃ for 7 min with a final extension (**Figure III-1**).

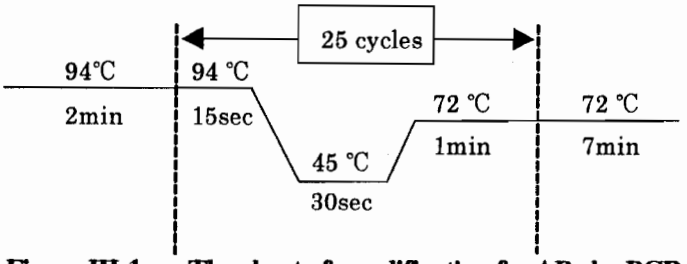

Figure III-1. The chart of amplification for APx by PCR.

III-2-4 Check of PCR product size.

The size of PCR product was checked by agarose electrophoresis. The mixture of sample (5 μl), 5x dye [5 μl; composed of 1.25 μl of 100% autoclaved glycerol, 0.1 μl of 0.25 M EDTA (pH 8.0), 0.25 μl of 10% SDS (w/v), 6.25 μg of bromophenol blue, 6.25 μg of xylene cyanol, and 3.4 μl of autoclaved water] and molecular maker (10 μl; composed of 0.5 μg of λDNA treated by Eco T14 I) was analyzed on 0.9% agarose gel containing 1x TAE [8 mM Tris, 8 mM acetic acid, and 10 mM EDTA (pH 8.0)] in 1x TAE electrophoresis buffer. The gel was stained for 10 min by etidium bromid (1.0 μg/ml) and then washed in distilled water for 10 minutes. PCR product could be observe on the tranceilluminater.

III-2-5 DNA purification from agarose gel.

Equal volume of phenol was added in final PCR solution and mixed vigorously. After the centrifugation at 6,000 x *g* for 3 min, the resulting aqueous phase was transferred to new tube. Three molar sodium acetate (pH 5.2, 1/10 volume of this aqueous phase) and 100% ethanol (2.5-fold of this aqueous phase) were added and mixed. After it was placed for 10 min at room temperature, it was centrifuged at 15,000 rpm for 10 min at 4℃. The pellet was washed with 70% ethanol and dried for 5 minutes. DNA precipitation was redissolved in appropriate volume of 1x TE [consisting 0.5 M

NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA].

After checking the size of the PCR product, the gel containing the band of PCR product was excised on tranceilluminater. The gel slice was put in a centrifugation tube with DNA recovery filter (SPREC^{TM}-01, Takara) and the tube was placed at -80℃ for 30 minutes. After cooling, the tube was placed at 37℃ for 5 min, and centrifuged at 10,000 rpm for 10 min at 4℃. 1x TE (200 μl) was added to the agarose gel remaining on filter and mixed, and then the tube was centrifuged again at 10,000 rpm for 10 min at 4℃. The filter was removed and 1 μl of glycogen (20 mg/ml), double volume of 100% ethanol, and 1/10 volume of 3 M sodium acetate were added to the filtrated solution, and then the tube was placed at -80℃ for 10 minutes. The sample was centrifuged at 15,000 rpm for 10 min at 4℃ and the pellet was resuspended with 80% ethanol. Centrifugation was performed again at 15,000 rpm for 10 min at 4℃, then the pellet was dried and dissolved in 20 μl of 1x TE. Electrophoresis was performed to check the size of PCR product.

III-2-6 Subcloning of APx.

III-2-6-1) Ligation.

The PCR product was cloned into the vector pT-Adv (Clontech; AdvanTAge^{TM}PCR Cloning Kit). The ligation reaction mixture was composed of 2.9 μl of PCR product, 1 μl of 10x Ligation buffer, 2 μl of pT-Adv Vector (25 ng/μl), 1 μl of T4DNA ligase (4.0 Weiss units/μl), and 3.1 μl of sterile water. The ligation reaction was incubated at 14℃ for overnight.

III-2-6-2) Transformation.

The ligation reaction solution (20 μl) and the 100 μl of thawed JM109 competent cell (Toyobo) were gently mixed with the pipette tip, and then the tube was incubated on ice for 30 minutes. The tube was placed for exactly 30 sec in a 42℃ water bath to produce heat shock, and then the tube was placed on ice for 2 min immediately. SOC medium (500 μl, consisting of 2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, and 2.5 mM KCl) preheated at 37℃ was added to the tube, and the

tube was shook horizontally at 37℃ for 1 h in a rotary shaking incubator. The tube containing the transformed cell was placed on ice, and the transformed cell solution (50 μl and 200 μl) was spread on LB / X-Gal / IPTG plate [consisting 1% Bacto tryptone, 0.5% Yeast extract, 0.5% NaCl, 1.5% Bacto agar, 0.4% X-gal, 0.1 mM IPTG (Isopropyl-β-D-thiogalactopyanoside)] containing 50 μg/ml of ampicillin. These plates were incubated in at 37℃ overnight and at 4℃ for an additional 2 h to allow proper color development.

III-2-6-3) CTAB-boiling plasmid miniprep.

The white colonies were selected and these colonies were grown over night in 2-5 ml LB plate (consisting 1% Bacto tryptone, 0.5% Yeast extract, 0.5% NaCl and 1.5% Bacto agar) containing 50 μg/ml of ampicillin. The solution was centrifuged at 15,000 rpm for 1 min at 4℃. The precipitate was dissolved vigorously in 200 μl of STET consisting 8% sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0) and 0.1% Triton X-100. Then 4 μl of lysozyme solution consisting 10 mg lysozyme in 200 μl STET was added to 5 μl RNase A (25 mg/ml in TE), and mixed vigorously. The tube was placed at room temperature for 5 min and confirmed jelly in the tube, and then incubated in boiling water for 45 sec. After the centrifugation at 15,000 rpm for 10 min at 4℃, the white pellet was removed by pick. Five percentage of hexadecyltrimetylammoniumbromide (CTAB) solution (10 μl) was added to the tube, and the tube was placed for 5min at room temperature. After a centrifugation at 15,000 rpm for 10 min at 4℃, the pellet was dissolved in 300 μl of 1.2 M NaCl and added 750 μl of 95% ethanol was added, and then placed at -80℃ for 10 minutes. Centrifugation at 15,000 rpm for 10 min was again performed and the resulting pellet was resuspended in 300 μl of 70% ethanol to wash. After a centrifugation, the pellet was dried for 15 min, and the precipitate was dissolved in 20 μl of 1x TE.

III-2-6-4) Incision of inserted PCR product from plasmid by restriction enzyme.

As PCR product was flanked on each side by EcoRI, it was excised from plasmid by

restriction enzyme EcoRI. The plasmid (150 ng) was digested completely at 37℃ for 1 h by 0.04 μl of EcoRI (10 unit/μl, Fermentas) in H-Buffer. Electrophoresis was performed to check excised PCR product, and then PCR product was purified as in **III-2-5.**

III-2-6-5) Partial APx sequencing.

DNA sequencing reaction was performed following the protocol of AutoCycle™ Sequencing Kit (Pharmacia Biotech). Master Mix (18 μl) contained DNA 0.2-0.5 pmol (plasmid 0.5-2 μg),

Table. III-2. The sequence of fluorescence primer

Primer	Sequence
(Cy5) M13 Universal Primer	5'-F CGACGTTGTAAAACGACGGCCAGT-3'
(Cy5) M13 Reversal Primer	5'-F TTTCACACAGGAAACAGCTATGAC-3'

fluorescence primers (2 μl of 1 pmol/ μl; shown in **Table III-2**), reaction buffer (2 μl), *d*NTP solution (5 μl), *Taq* DNA polymerase (2 μl, 2.5 unit/2 μl), DMSO (2 μl), and distilled water. Each of *dd*ATP, *dd*CTP, *dd*GTP and *dd*TTP was added at a volume of 2 μl to 4 tubes. And then Master Mix (4 μl) was added each tube, and mixed. PCR was performed following chart (**Figure III-2**). After all cycles were completed, tubes were placed on ice and 4 μl of stop solution were added. In order to degenerate the DNA , these solutions were heated at 95℃ for 2-3 min and immediately placed on ice. Thereafter these solutions were applied to the sequencer (ALFexpress DNA Sequencer).

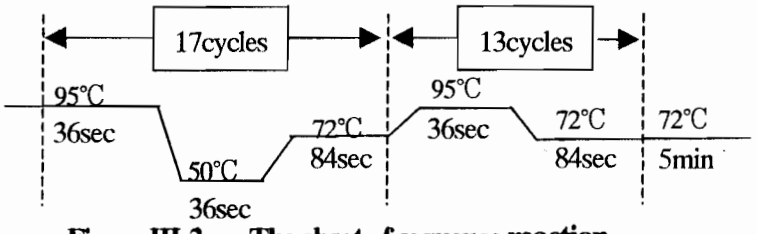

Figure III-2. The chart of sequence reaction.

II-2-7 Plaque hybridization (Screening of the cDNA library).

A potato tuber cDNA library constructed with the Zap-cDNA synthesis kit was a gift from Dr. H. Yoshioka, Nagoya University.

III-2-7-1) Preparation of the host bacteria.

The bacteria XL1-Blue MRF' glycerol stock were grown at 37℃ shaking over night in 3 ml

of NZY broth adjusted with NaOH to pH 7.5, composed of 0.5% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract, and 1.0% NZ amine (casein hydrolysate) supplemented with tetracycline (final concentration of 12.5μg/ml). Bacteria (3 ml) were added into 100 ml of NZY medium supplemented with tetracycline, and grown again at 37℃ shaking until an OD_{550} of 0.5. After centrifugation at 5,000 rpm for 5 min at 4℃, the pellet was gently resuspended in 40 ml of 10 mM $MgSO_4$, and used as host cell.

III-2-7-2) Titer of the cDNA library.

Various dilutions (from 10^{-1} to 10^{-7}) of the cDNA library were prepared in SM buffer consisting 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, and 0.01% gelatin. Each 10 μl of the dilution of the cDNA library were added to 200μl of host cell and this mixture was incubated at 37℃ for 30 min to allow the phage to attach to the cell. The solution (210 μl) and 2.5 ml of NZY top agar (NZY broth and 0.7% agarose were autoclaved and supplemented at the final concentration 100 μg/ml ampicillin) were mixed and plated immediately on prewarmed NZY agar plates (NZY broth and 15% Bacto agar were autoclaved and supplemented at the final concentration of 12.5 μg/ml Tetracycline). After incubation at 37℃ for 10 h, the plaques were counted and the titer in plaque-forming units per milliliter (pfu/ml) was determined.

III-2-7-3) Plaque lifting.

Plaques lifting was performed flowing the protocol of Colony/Plaque screen™ Hybridization Transfer Membranes (NEN™ Life Science Products). Approximately 3 x 10^9 plaques were screened. Plates were chilled to 4℃ for 1 h prior to lifting. The dry membrane was placed on to the agar for 3 min and position was marked by magic marker at appropriate position. The membrane was carefully removed from the plate and was placed with plaque side up onto a pool of 0.5 N NaOH (0.75 ml) at evenly place. After 2 min, the membrane was removed and placed on blotting paper (plaque side up). The membrane was placed again onto the pool of fresh 0.5 N NaOH for more 2 minutes. After

removing the excess NaOH on blotting paper, the membrane was placed with plaque side up on to the pool of 1.0 M Tris-HCl buffer (0.75 ml, pH 7.5) for 2 minutes. The membrane was removed and placed on blotting paper (plaque side up), and placed again onto the pool of the same buffer. Excess buffer was removed and the membrane was wiped up with filter paper and UV-crosslinked for 3 minutes. Before prehybridization, the membrane was wet in 2x SSC (consisting 33.3 mM NaCl and 33.3 mM $C_6H_5O_7Na_3$, pH 7.0).

III-2-7-4) Preparation of labeled probe.

The subcloned DNA (described at **III-2-6**) was used as probe. Labeling was performed following the protocol of AlKPhos Direct labeling kit (Amersham Life Science). DNA was diluted to be labeled to a concentration of 10 ng/μl using the water supplied. Diluted DNA sample (10 μl) was placed in a microcentrifuge tube and denatured by heating for 5 min in a vigorously boiling water bath. The DNA was immediately cooled on ice for 5 min and the cooled DNA was added to an equal volume of reaction buffer, 1/5 volume of labeling reagent, and equal volume of the cross-linker working solution in turn and the solution was mixed thoroughly but gently at each addition. The reaction mixture was incubated for 30 min at 37℃. The probe can be immediately used or kept on ice for up to 2 h.

III-2-7-5) Plaque hybridization.

Hybridization was performed following the protocol of AlKPhos Direct labeling kit (Amersham Life Science Ltd). The membranes were placed at 55℃ in 2x SSC before prehybridization. The required volume (0.125 ml/cm^2 membrane) of AlKPhos Direct hybridization buffer (0.5 M NaCl and 4% Blocking reagent) was pre-heated to 55℃. The membrane was placed into plastic bags containing the hybridization buffer and prehybridized for more than 1 h at 55℃ in hybridization oven. The labeled probe (10 μg/ml buffer) was added to the buffer used for the pre-hybridization step. Hybridization was performed at 55℃ overnight in hybridization oven. After hybridization, the membrane was washed twice at 55℃ for 10 min in primary wash buffer

consisting of 2 M Urea, 0.1% SDS, 50 mM Na phosphate buffer (pH 7.0), 150 mM NaCl, 1 mM $MgCl_2$, and 0.2% Blocking reagent (Amersham Life Science), and then washed twice at room temperature for 5 min in secondary wash buffer containing 50 mM Tris, 100 mM NaCl, and 2 mM $MgCl_2$.

III-2-7-6) Detection.

Detection was performed following the protocol CDP-Star™ detection kit (Amersham Life Science). After removing the excess secondary wash buffer on filter paper, the membrane was placed with DNA side up on Saran Wrap™, and detection reagent was pipetted on to the membrane and left on for 5 minutes. Excess detection reagent was drained off by touching the corner of the membrane on the non-absorbent surface. The membrane was warped in Saran Wrap™, and exposed to film with DNA side up for 1 h in the cassette.

III-2-7-7) The extraction of phage from plaque.

The putative clones with the strongest signal on film were determined. The putative clones that lined up with the film spot were cut out from the stock plate using an inverted 1000 μl tip. These putative clones were put into 1 ml of SM buffer, and vortexed vigorously at 4℃ for 2 hours. The supernatant (500 μl) centrifuged at 15,000 rpm for 10 min at 4℃ was stored at –80℃ and used after experiments.

III-2-7-8) Amplification of APx DNA fragment from phage.

In order to select the phage (extracted at **III-2-7-7**) that inserted the longest APx DNA fragment, PCR was performed following the same protocol described in **III-2-3**. The sequences of the sense primer (T3 primer) and the antisense primer (APx2) were listed in **Table III-3**. The size of PCR product was checked by electrophoresis following the protocol described in **III-2-4.**

Table III-3. The sequence of T3 primers and APx2

Primer	sequence
T3	5'-AATTAACCCTCACTAAAGGG-3'
APx2	5'-TCATCCGCAGCATATTTCTC-3'

III-2-7-9) Second Screening.

The selected phage and the bacteria (XL1-Blue MRF') were mixed and incubated at 37℃ for 30 min to allow the phage to attach to the cell. The plaque lifting, prehybridization, and hybridization were performed similarly as described in **III-2-7-3** to **III-2-7-6**.

III-2-8 *In vivo* excision.

III-2-8-1) Preparing the Host cell.

XL1-Blue MRF' and SOLR cells were cultured in LB broth, 0.2% maltose and 10 mM $MgSO_4$ at 30℃ over night. After centrifugation at 5,000 rpm for 5 min at 4℃, the collected cells were diluted with 10 mM $MgSO_4$ in order to adjust to OD_{600}=1.0

III-2-8-2) *In Vivo* excision using ExAssist™ helper phage with SOLR strain.

In vivo excision was performed following the protocol of Zap-cDNA synthesis kit. The ExAssist/SOLR system is designed to allow efficient excision of the pBluescript phagemid from the Uni-Zap vector. The ExAssist helper phage contains an amber mutation that prevents replication of the phage genome in a nonsuppressing *E. coli* strain such as SOLR cells. This allows only the excised phagemid to replicate in the host, removing the possibility of productive co-infection from the ExAssist helper phage. Since the ExAssist helper phage cannot replicate in the SOLR strain, single-stranded rescue cannot be performed in this strain using this helper phage.

XL1-Blue MRF'(200 μl), ExAssist™ helper phage (1 μl), and phage stock (250 μl, prepared at **III-2-7-9**) were incubated at 37℃ for 15 min in 50 ml tube. LB broth (3 ml) was added and incubated for 3 h at 37℃ with shaking. The tube was heated at 70℃ for 20 min, and then centrifuged for 15 min at 1,000 x *g*. The supernatant (1.5 ml) was transferred to a new tube and stored as phage stock at 4℃. SOLR cells (200 μl) prepared at **III-2-8-1** were added to two 1.5 ml tubes, and 100 μl of the phage stock was added to one tube and 10 μl of the phage stock to the other tube. The tubes were incubated at 37℃ for 15 min and 200 μl from each tube was plated on LB-ampicillin plate, and

then these plates were incubated overnight at 37°C. Colonies appearing on the plate contain the pBluescript double-stranded phagemid with the cloned DNA insert. Helper phage will not grow, since they are unable to replicate in nonsuppressing SOLR strains and do not contain ampicillin-resistance genes.

III-2-8-3) Incision of DNA fragment from phagemid by restriction enzyme.

The preparation of phagemid was preformed following the same protocol described at **III-2-6-3.** Insert DNA fragment was excised from phagemid by EcoRI and Xho I, and the size of insert DNA fragment was checked by electrophoresis similarly as described in **III-2-4**.

III-2-9 DNA sequencing.

DNA sequencing reaction was performed following the protocol ABI PRISM^{TM}310 Genetic Analyzer (Applied Biosystems). Each primer (1.6 μl of 2 pmol/ μl) , premix (4.0 μl, Applied Biosystems), plasmid (1.0 μl of 100 ng/ μl), and autoclaved water (3.4 μl) were mixed and placed in a thermocycler. PCR was performed following chart in **Figure. III-3**. After reaction, 10 μl of autoclaved water were added. Three molar sodium acetate (2 μl) and 95% ethanol (50 μl) were added to the solution and the tube was placed at room temperature for 15 minutes. The sample was centrifuged at 15,000 rpm for 20 min at room temperature and the pellet was resuspended with 70% ethanol. Centrifugation was performed again at 15,000 rpm for 10 min at room temperature and then the pellet was dried. Moreover, ethanol was completely removed by Kimwipe. The pellet was dissolved in 20 μl of TSR (Template Suppression Reagent, Applied Biosystems) and heated at 95°C for 2 minutes. Immediately, the solution was placed on ice and then applied to the sequencer (ABI PRISM^{TM}310 Genetic Analyzer, Applied Biosystems).

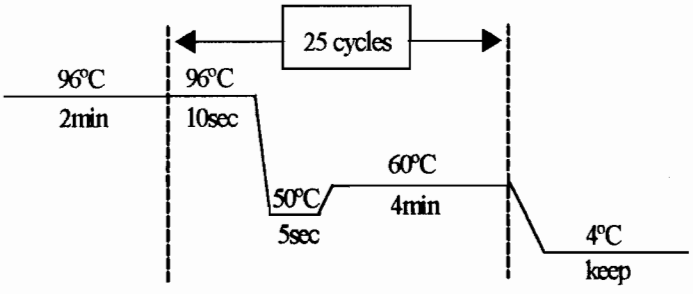

Figure III-3. The chart of sequence reaction.

III-3 RESULTS

III-3-1 Subcloning of APx.

The size of RT-PCR products was checked by electrophoresis (**Figure III-4**). The band showed 588-bp that corresponded to the size of APx DNA fragments. After subcloning, the plasmid in each clone was digested with EcoRI to check the insert DNA fragment. As shown in **Figure III-5**, clone 3, 7 and 11 indicated just two bands that corresponded with pT-Adv vector (3,900-bp) and APx DNA fragment (588-bp), respectively.

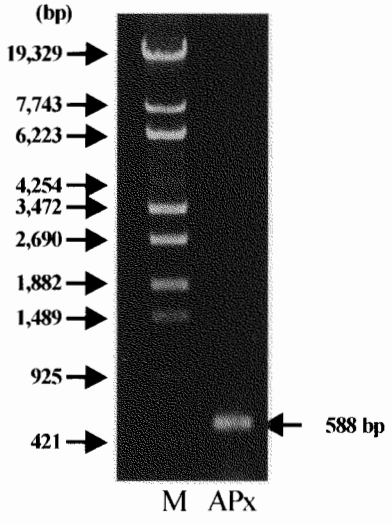

Figure III-4. Electrophoresis of APx DNA probe amplified by PCR.
M: molecule marker.

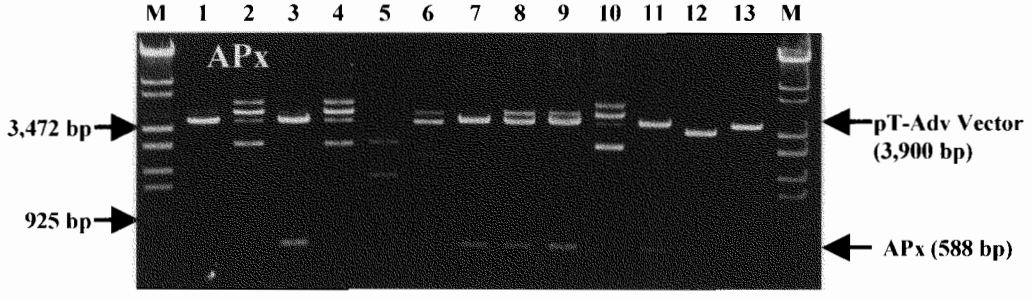

Figure III-5. Electrophoresis of plasmid containing APx DNA fragment in pT-Adv vector from each clone restricted by Eco RI. M: molecule marker. 1～13: clone number

III-3-2 Partial APx sequence.

Clone 3, 7, and 11 were used for sequence reaction. Since the results from 3 clones indicated the same sequence, a partial APx sequence from clone 7 is shown in **Figure III-6.** This sequence showed high homology with tobacco (90%) and *Arabidopsis* (80%) and spinach (81%), and deduced amino acid sequence showed high homology. As shown in **Figure III-7,** the amino acid sequence of partial APx aligned with tobacco cytosolic APx shows the highest homology from N terminal 41 to 221 residue, being 91%. From these results, this DNA fragment seemed to be a cytosolic APx DNA fragment.

TTCATCCGCAGCATATTTCTCAACAAGGGACGGAAAGCRGGGTSACAG
AGGAGAGCCTTGTCTGATGGCAACTGTAAGAGCCCTTCTTTTTCCCSA
CTCAAAAGCTCCGTGAAGTATGAATTGTCAAAGATAAGAGGATTGGCG
GTCCAAGGTCCCTCAAAACCAGAACGCTCCTTGTGGCACCTTCCCAAG
GTATGGCCACCAGAGAGTGCAACAATATCCTTGTCGGAAAGACCCATT
TGTTTCTTGAACACATCTCTCAAGTGGTCAGAGCCCTTGGTGGCATCC
GGCAAGCGACCTTCAACTGGTGGTTCTGGCTTGTCCTYTCTGCCAGGG
TGAAAGGGAACGTCAGGTCCTCCAGTAACTTCAACAGCAACGACACCA
GCCAATTGATAGAAATCAGCATAGGAGATGGTGGGAAACTGCTCCCTA
ATGGGCTCCAAGAGTCTGAGAGCAATGTCAAGACCATTGTTTGCACCA
TGTGCTTGCTCAGCTTTGAATCTCATGGTACCAAAGGGACCACCAGTC
TTGGAACACACATCATAGGTACCAGCAGAGTGCCATCA

Figure III-6. Partial APx sequence *Solanum tuberosum* L.

```
Potato     1  *WHSA GTYDV CSKTG GPFGT MRFKA EQAHG ANNGL DIALR LLEPI REQFP  50
Tobacco   40  AWHSA GTYDV CSKTG GPFGT MRFKA EQGHG ANNGI DIAIR LLEPI KEQFP  89

Potato    51  TI-SY ADFYQ LAGVV AVEVT GGPDV PFHPG RXDKP EPPVE GRLPD ATKGS 100
Tobacco   90  -ILSY GDFYQ LAGVV AVEVT GGPDV PFHPG REDKT EPPVE GRLPD ATKGS 139

Potato   101  DHLRD VFKKQ MGLSD KDIVA LSGGH TLGRC HKERS GFEGP WTANP LIFDN 150
Tobacco  140  DHLRD VFVKQ MGLSD KDIVA LSGGH TLGRC HKERS GFEGP WTTNP LIFDN 189

Potato   151  SYFTE LLSXE KEGLL QLBSD KALLC XPAFR PL 182
Tobacco  190  SYFTE LLSGE KEGLL QLBSD KALLS DPAFR PL 221
```

Figure III-7. Amino acid sequence alignment of *Solanum tuberosum* L. APx with *Nicotiana tabacum* APx.

III-3-3 Screening of cDNA library.

Subcloned partial APx DNA fragment was used as a probe for plaque hybridization. At the first screening of plaque hybridization, 23 spots were obtained on the film. The phages corresponded to each plaque in plates that were extracted. After the phages attached to XL1-Blue MRF, DNA fragments were amplified by PCR (**Figure III-8**). Various sizes of DNA fragments were amplified at first screening. Clone 11, which showed the longest size, was used for the second screening. As shown in **Figure III-9**, the size of amplified DNA fragments from each clone was about 900-bp. Clone 14, which showed the most visible band (**Figure III-9**), was selected for the third screening. Since clone 2 showed the highest titer (**Figure III-10**), this clone was used for *In Vivo* Excision.

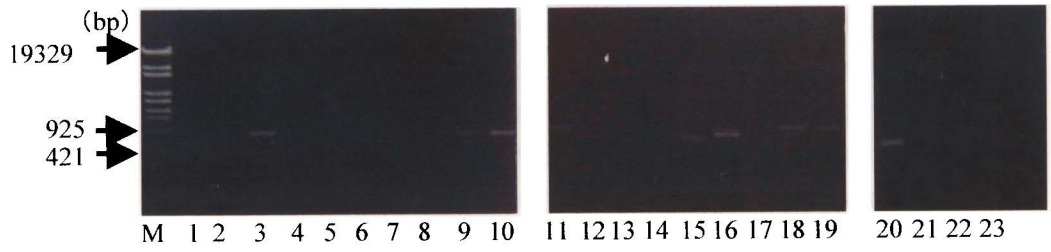

Figure III-8. Agarose electrophoresis of each clone after first screening.
M: molecular weight marker, 1～23:clone number

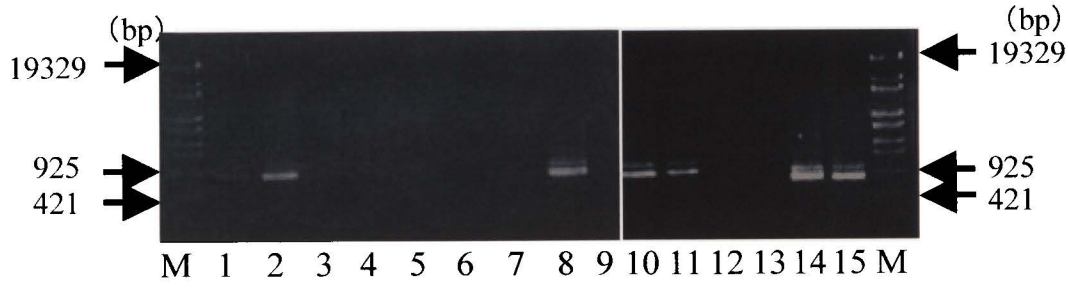

Figure III-9. Agarose electrophoresis of each clone after second screening. M: molecular weight marker, 1～15:clone number

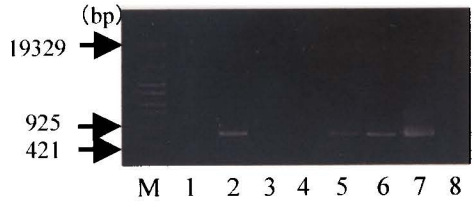

Figure III-10. Agarose electrophoresis of each clone after third screening.
M: molecular weight marker, 1～8:clone number

III-3-4 *In Vivo* Excision.

After *In Vivo* Excision, 10 single colonies were selected and plasmids were extracted by CTAB method. Digested DNA fragments from all clones were shown in two bands corresponding to pBluescript (2,958-bp) and putative full length APx (about 1,000-bp), respectively (**Figure III-11**). These results suggested that putative full length APx was inserted in multiple cloning sights.

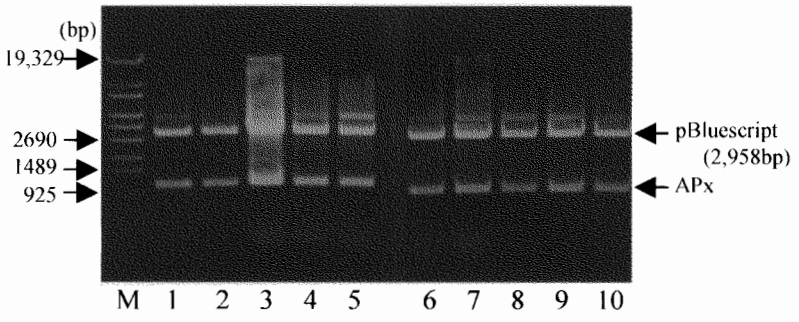

Figure III-11. Electrophoresis of plasmid containing full length APx cDNA in pBluescript vector from each clone restricted by EcoRI and Xho I.
M: Molecular weight marker, 1～10: Clone number

III-3-5 Full length APx sequence.

The first sequence reaction was performed using Reverse primer and M13 primer shown with black boxes in **Figure III-12**. However, since the result indicated that the obtained sequence included two unknown bases, two new primers (APx3 and APx4 shown in **Figure III-13**) were designed by OLIGO™ ver. 4.0 Manual soft and were synthesized to decide the correct sequence of full length APx. Sequences obtained from APx3 and APx4 primers were overlapped partially from reverse and M13 primers, respectively. In addition to the result from the sequence of partial APx cDNA shown in **Figure III-6**, the full-length sequence of APx was determined as shown in **Figure III-13**. The full-length sequence of 1039-bp contains an ORF of 750-bp coding for 250 amino acids. The cDNA contains an 84-bp 5' UTR (untranslated region) and 205-bp 3' UTR.

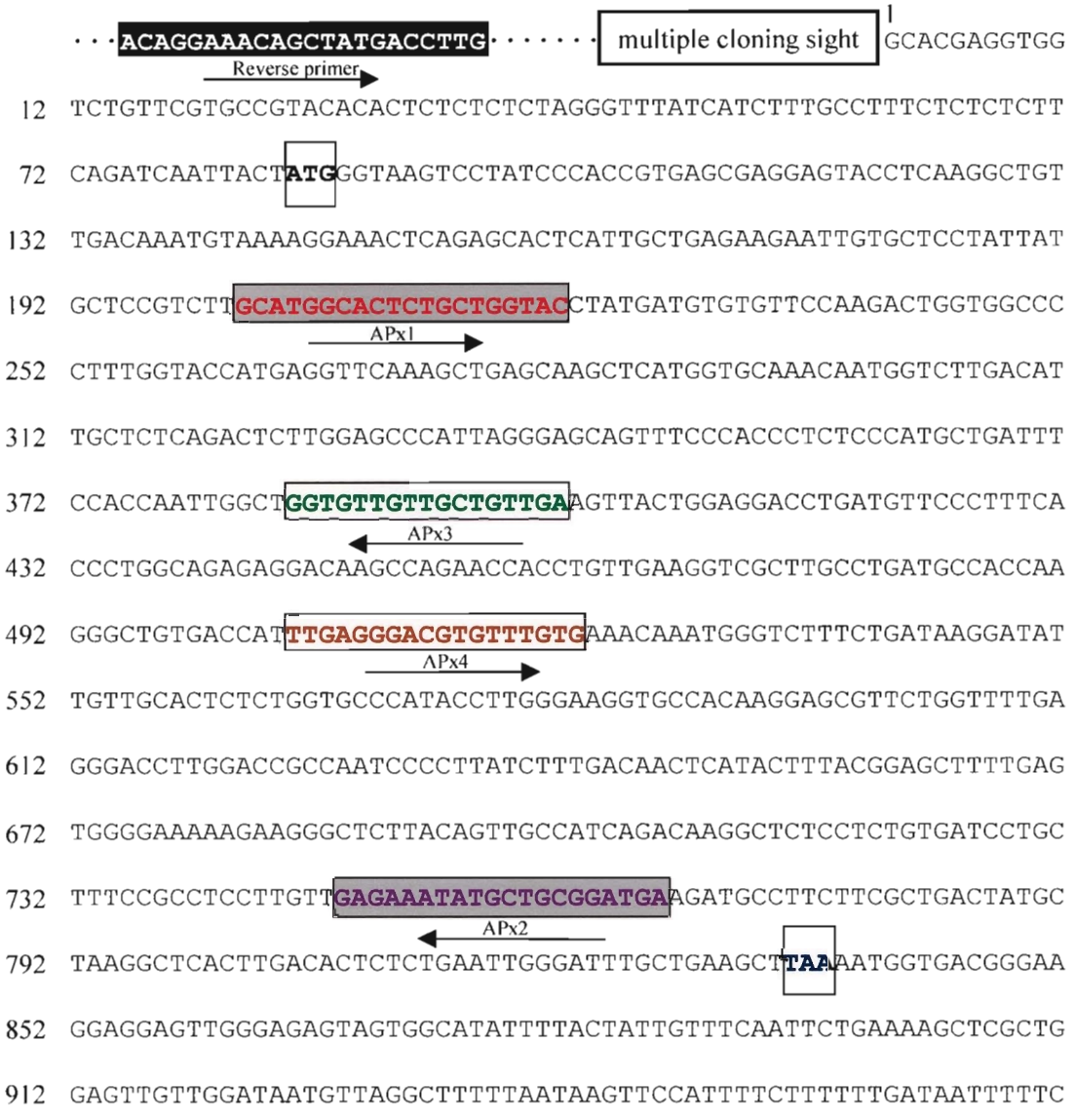

Figure III-12. Complete full length cDNA sequence of APx from *Solanum tuberosum* L., and the location of each primers (APx 1, 2, 3, 4, reverse primer, and M13 primer).
ATG:initiation codon TAA:termination codon

The deduced amino acid sequence of the full-length APx of potato tuber is shown in **Figure III-13**. The sequence is 250 residues long and was found to have conserved amino acids of APx H42 (active site), H163 (binding site), W179 (active site), and D208 (active site) (**Figure III-13, in rectangular**). These amino acids (H163, W179, and D208) are known to make up part of the

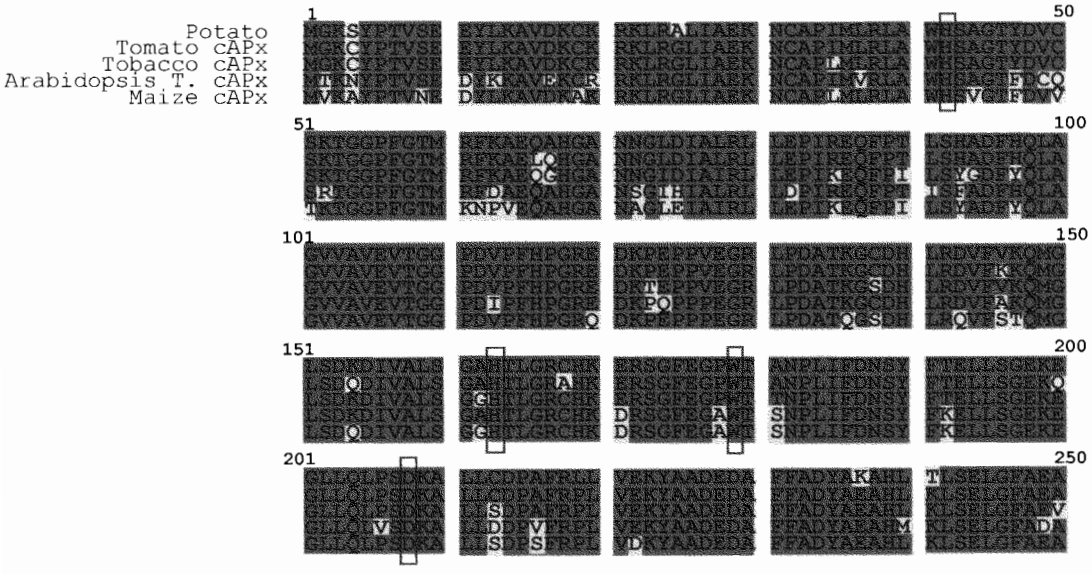

Figure III-13. Amino acid sequence homology between potato tuber (*Solanum tuberosum L.*) and other plant APx.

His-Asp-Trp catalytic triad, as determined by the three-dimensional structure (Patterson and. Poulos, 1995). To examine similarities and differences in individual amino acid sequence positions (**Figure III-13**), we aligned the deduced amino acid sequence of potato APx with other plant APx in the database of NCBI Blast Search. The APx enzymes with high similarity (tobacco, tomato, *Arabidopsis* and zea maize) were all cytosolic types. Tomato (accession No. CAB58361) and tobacco (accession No. BAA12918) which fall into the same family as potato (classed *Solanacea*) showed the highest similarity with potato, 89% and 85%, respectively. *Arabidopsis. T* (accession No. S28816) and zea maize (accession No. S49914) in a different family to potato also showed high similarity to potato (79% and 77%, respectively). Furthermore, the fact that the cloned cDNA does

not have a transit peptide supports the idea that this enzyme exists in the cytosol (Mittler and Zilinskas, 1992). From these results, it is suggested that the subcloned APx cDNA was a cytosolic type in the potato tubers.

III-4 DISCUSSION

It had been reported that most of full-length APx cDNA is about 1.1-kbp long: maize (1,042-bp, Bresegem, *et al.* 1995), strawberry (1,044-bp, Kim and Chung, 1998), spinach (1,102-bp, Webb and Allen, 1995), tobacco (1,040-bp, Örvar and Ellis, 1995), and pea (1,040-bp, Mittler and Zilinskas, 1991). However, there had been no reports about potato APx. Three different cDNA clones containing the complete coding sequence were obtained. One of them is shown in **Figure III-12**. The full-length potato APx sequence was 1,039-bp long and contained an open reading frame of 750-bp, which was large enough to encode APx. The deduced amino acid sequence (**Figure III-13**) shares nearly 90-80% similarity with other plant cytosolic APx reported to date. Furthermore, the isolated APx gene has conserved sequences or regions, such as the catalytic triad, that are important for the maintenance of structure and function in all APx. Meanwhile, it does not have any sequence similar to the transit peptide of plastidal isoforms (Yamaguchi, *et al.* 1996; Ishikawa *et al.*, 1996).

CAPTER IV

DE NOVO PROTEIN SYNTHESIS OF ASCORBATE PEROXIDASE IN POTATO TUBERS DURING LOW-TEMPERATURE STORAGE

IV-1 INTRODUCTION

In **Chapter II**, it was ascertained that the enhancement of the active oxygen scavenging system that was induced by low temperature in potato tubers could result not only in a decrease of AsA but also in increases in APx, which was important for protection against the H_2O_2 generated during low-temperature storage. Moreover, it has reported that the other antioxidant enzymes, SOD and MDHAR, in potato tuber stored at a low temperature also showed higher activity than at a high temperature. This is in agreement with Foyer *et al.*, (1994), who suggest that an increase in several components of the antioxidative defense system would simultaneously be necessary to increase oxidative stress tolerance of plants. Thus, it was demonstrated that active oxygens were more readily generated when potato tubers were stored at low temperature. However, little work has been performed on the molecular mechanism of APx gene regulation during storage at low temperature. In order to examine the detail of APx gene regulation, we investigated the oxidative stress response of cytosolic APx using a DNA probe encoding potato tubers cytosolic APx and anti-APx antibody in this chapter.

IV-2 MATERIALS AND METHODS

IV-2-1 Growth and Storage Conditions.

Tubers of *Solanum tuberosum* L. Cultivar 'Touya' (a hybrid of WB77025-2 and R392-50) were grown at the experimental farm of Kobe University. Potato tubers were stored at 1°C, 5°C, and 20°C, and each potato tuber was sampled at the 3rd, 6th, 9th, and 15th week of storage.

IV-2-2 Northern Blot Analysis.

IV-2-2-1) Total RNA Preparation.

Total RNA was prepared essentially as previously described at **III-2-1.** For Northern blot analysis, 10 μg samples were run on 1.2% agarose (containing 5.4% formaldehyde) gels and transferred to nylon membranes (Hybond^{TM}-N^{+}, Amersham pharmacia biotech).

IV-2-2-2) Subcloning of catalase.

All procedures were followed the same as APx subcloning. After total RNA from potato tubers was transcribed into single-stranded cDNA, twenty mer of CAT1 and CAT2 shown in **Table IV-1** were designed as a sense and an antisense primer for potato CAT cDNA. Amplification of CAT cDNA fragment by PCR was performed under the condition as shown in **Figure IV-1**. After checking the size of PCR product, it was purified from agarose gel, and cloned into the vector pT-Adv by the same method in **Chapter III**. Nucleotide sequences of CAT were carried out as decided in **III-2-6**.

Table. IV-1. The sequence of CAT1 and CAT2

Primer	sequence
CAT1	5'-GTTTCTTCCTTGACTGTTGG-3'
CAT2	5'-CGATGATTGTTGTGATGACC-3'

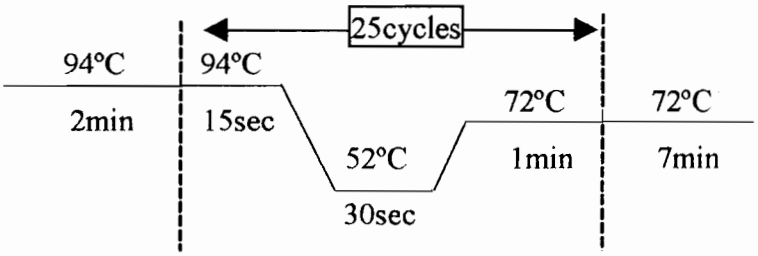

Figure IV-1. The chart of amplification for CAT by PCR.

IV-2-2-3) Hybridization.

Prehybridizaitions were carried out in prehybridization buffer [consisting 1 mM EDTA, 0.5 M NaH_2PO_4 (pH 7.2), and 7%SDS] prewarmed for more than one hour at 65°C. Hybridizations were carried out in the same buffer containing ^{32}P-labeled APx or CAT cDNA fragment for overnight at 65°C. After hybridization, membranes were washed twice in first wash buffer prewarmed to 65°C consisting 1mM EDTA, 20mM NaH_2PO_4 (pH 7.2) and 5% SDS for 15 min at 65°C. The

membranes were washed again in a second wash buffer consisting 1mM EDTA, 20mM NaH_2PO_4 (pH 7.2) and 1% SDS for 1 min at 65°C. Hybridization signals were detected by exposure to x-ray film (Fuji Medical X-Ray Film Rx-U) for overnight at –80°C.

IV-2-3 Native PAGE and Activity Staining.

IV-2-3-1) Protein Extraction.

The procedures of protein extraction were based on that described by Anderson *et al.*, (1995). Potato tubers (5 g) were homogenized in 20 ml of ice-cold extractant. The extraction medium contained 0.1 M potassium phosphate buffer (pH 7.8), 1 mM EDTA, 1 mM PMSF, and 200 mg polyvinylpyrrolidone (PVP-40). For analysis of APx, the extraction buffer also contained 5 mM AsA. Insoluble material was removed by centrifugation at 16,000 x *g* for 15 min at 4°C, and the supernatant was immediately added to 40% glycerol. For analysis of CAT, an aliquot of each sample was added to 10 mM DTT to maintain the stability of CAT.

IV-2-3-2) Native PAGE and Activity Staining.

APx: APx was visualized following the method described by Mittler and Zilinskas (1993). Protein extracts were subjected to discontinuous PAGE under nondenaturing, nonreducing conditions, essentially as described by Laemmli (1970) except SDS was omitted and the electrode buffer contained 2 mM AsA. The gels were prerun for 30 min to allow AsA, present in the carrier buffer, to enter the gel prior to the application of the samples. Following the electrophoretic separation, the gels were equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and 2 mM AsA for a total of 30 min; the equilibration buffer was changed every 10 minutes. The gels were then incubated with 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM AsA and H_2O_2 for 20 minutes. Hydrogen peroxide was added to this solution immediately prior to incubation with the gel. The gels were subsequently washed with 50 mM sodium phosphate buffer (pH 7.0) for 1 min and submerged in a solution of 50 mM sodium phosphate buffer (pH 7.8), 28 mM *N, N, N', N'*-tetramethylethylenediamine

(TEMED), and 2.45 mM nitroblue tetrazolium with gentle agitation. The reaction was stopped by a brief wash in distilled water. The APx activity was observed as an achromatic band on a purple-blue background. To inhibit APx activity in the gel, the gels were incubated in 50 mM sodium phosphate buffer (pH 7.0) containing 10 mM KCN for 20min prior to the staining of APx activity.

CAT: CAT was visualized following the method described by Anderson *et al.*, (1995). CAT was separated on nondenaturing and nonreducing polyacrylamide gels at 80 V for 22 h at 4°C using the procedures of Laemmli (1970) except SDS was omitted and the electrode buffer contained 10 mM DTT. The loading buffer contained no β-mercaptoethanol but was made to 80 mM DTT. Native gels were soaked in 3.27 mM H_2O_2 for 25 min, rinsed in water, and then stained in a solution of 1% (w/v) potassium ferricyanide, 1% ferric chloride in a method similar to that of Woodbury *et al.*, (1971).

IV-2-4 Western blot analysis.

IV-2-4-1) Preparation of antiserum to APx and purificaiton of immunoglobulin.

Polyclonal rabbit antibody rose against internal hydrophilic peptides containing 15 amino acids (CGGPDVPFHPGREDK) with identical sequences to the cDNA of potato APx were produced for the detection of the molecule in Western blot analysis (**Figure IV-2**). The peptide was chemically coupled to hemocyanin conjugated with *m*-maleinidobenzoyl-*N*-hydroxysuccinimide ester (Liu *et al.*, 1979). A rabbit (New Zealand White) was immunized with the peptide-hemocyanin emulsified in complete Freund's adjuvant, and antiserum was obtained after several of boosters with the peptide-hemocyanin. Rabbit sera was purified by affinity chromatography on cellulofine activated by 2-fluoro-1-methylpyridinium toluene-4-sulfonate, which coupled with the peptide as a ligand.

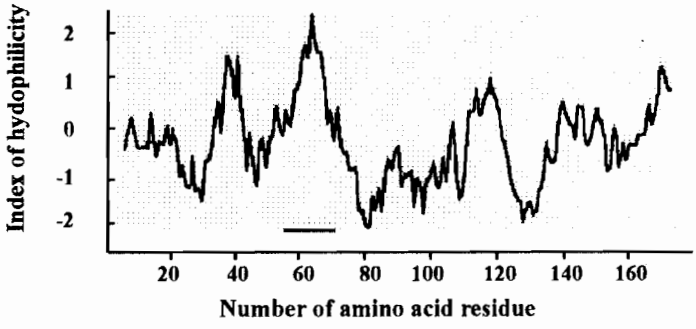

Figure IV-2. Search of hydrophilic site.

IV-2-4-2) Protein Extraction.

Protein extraction was performed following the protocol described by Elia *et al.* (1992). Potato tubers (5 g) were homogenized in 20 ml of ice-cold extraction medium [consisting 0.1 M phosphate buffer (pH 7.6), 2 mM EDTA, 2 mM 2-ME, 2 mM $MgSO_4$ and 1 mM AsA]. The homogenate was squeezed through four layers of gauze and the filtrate was centrifuged at 40,000 x *g* for 20 minutes. The supernatant was used as the crude extract.

IV-2-4-3) SDS PAGE and detection of APx.

PVDF membrane (Hybond^{TM}-P, amersham pharmacia biotech) was soaked in methanol for more than 1 min before use. After SDS PAGE, proteins were transferred to PVDF membrane using a semidry blotting apparatus (Bio-Rad Laboratories). The membranes were blocked overnight in TPBS (consisting 0.05% Tween20 in PBS) containing 5% skim milk at 4°C and then incubated for 2h with the appropriate anti-APx antibody in 5% TPBS. Enhanced chemiluminescence (ECL plus, Amersham Pharmacia Biotech Ltd.) was used to detect the relevant proteins following protocols suggested by the manufacture.

IV-3 RESULTS

IV-3-1 Subcloning of CAT.

CAT was also subcloned following the procedure as described in subcloning of APx. The size of RT-PCR products was checked (**Figure VI-3**) and it was indicated that 1,280-bp corresponded to the size of CAT DNA fragments. After subcloning, each plasmid in clones were digested with EcoRI to check the insert DNA fragment. As shown in **Figure VI-4**, most of the clones indicated just two bands that correspond with pT-Adv vector (3,900-bp) and CAT DNA fragment (1,280-bp), respectively. The sequence as shown in **Figure VI-5** was the result of sequence reaction, and this sequence was completely aligned with CAT of potato tuber in the database of NCBI Blast Search.

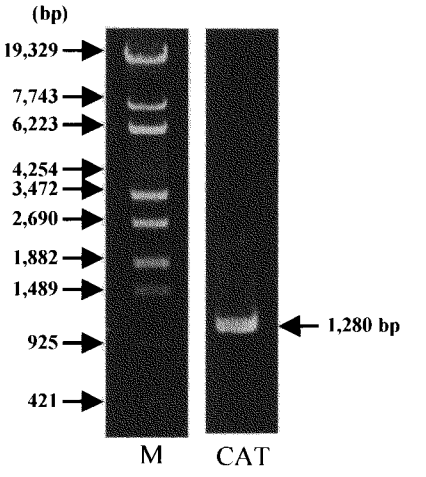

Figure IV-3. Electrophoresis of CAT DNA probe amplified by PCR. M: molecule marker.

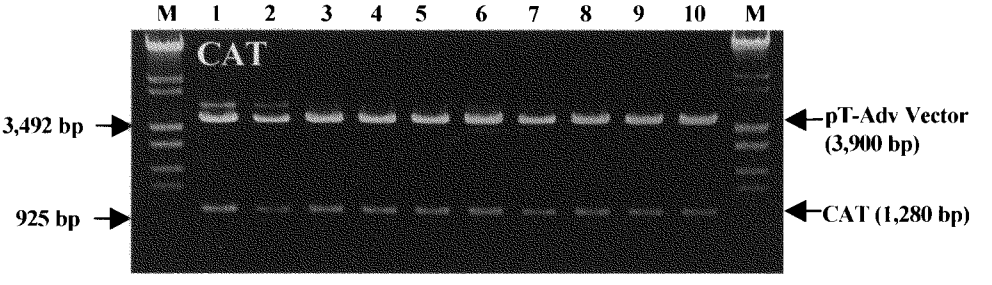

Figure IV-4. Electrophoresis of plasmid containing APx and CAT DNA fragment in pT-Adv vector from each clone restricted by Eco RI.
M: molecule marker. 1～13: clone number

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TCGATGATTGTTGTGATGACCACACTTGGGAGCATTAACTGGGAGCTGCA
TATAGTTTGGTCCAATACGGTGCCTCTGAGTATCAGCATACGCGAATATC
CTAGTCTGGAGAAGCTTATCCTCGGAATAGTAAATACCAGGGACGATATG
TCCAGGGTTAAACGCGAGCTGCTCATTCTCAGCAAAGAAGTTATCAATGT
TCCTGTTCAATACCAATCGACCAACTGGGATCAACGGCAAGAGATCCTCA
GGCCATGTCTTGGTTACATCTAGAGGRTCAAAATCGAACTTGTCTACATC
CTCAGGGTCCATTGTTTGGATGAAAAGTTTCCACTCAGGATAGTTTCCAG
CAGCAATTGAATCGTAAAGATCCTTGGTCGCGTGACTATGATTAGTACCT
CCGACTCTAATAGCTTCTTCCTCAGACATACTTTCACACCACAAGTTGSC
YCCCAGKGRMCYTCACATAATGTGCTTYCCCCCCTKTGTAATCAATTGAT
ACSCGTGAACGCCAAAMCCTCCCATGTGYCTGTAATCAGTTGGGAGACAA
ACATCATCGTAGAAGAAGGCGAATGTATGCAAACTCTCAGGAAGGAACGA
GAAGAAATCARGGAKCCTCCAGTTCTCCTGAATGTGTGACTTTGGATTTG
GTYTCAATGCACGAATCGTGTCAGGGAACGACTTAGCATCACGATTAAAG
AACACAGGGACATTGTTTCCAACAAGATCAAAGTTACCCTCTCTGGTGTA
AAACTTGACAGCAAAACCGCGAATGTCCCTGATTGACTCGGGGCTTCCAC
GTTCATGGACAACAGTAGAGAATCGACAAATAACAGGCGTTTGAGCMCCA
GGAGCTCGGAGAAATCAGCACAAGTAAGATGAGAAATGTCATGAGTAAC
TTCAAAGAATCCCTTAGCACTAGCACCTCTAGCATGAACAACACGCTCAG
GTATCTTTTCGCGATCAAAGGTGGCAAGCTTCTBDATTAGATAATAATCC
TCAAGCAGAACAGGCCCTCTAGGTCCAACAGTCAAGGAAGAAACA
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Figure IV-5. Partial CAT sequence *Solanum tuberosum* L.

IV-3-2 The expression of APx and CAT at the level of mRNA.

To examine whether the induction of antioxidant enzymes, APx and CAT, at low temperatures were derived from *de novo* protein synthesis, we subjected each enzyme to Northern bolt analysis. Although the accumulation of APx and CAT mRNAs was increased, the kinetics of them were different. APx mRNA levels during the storage at 1°C increased 2.5-fold and 1.4-fold in 3 weeks and 6 weeks, respectively (**Figure IV-6**) as compared with the initial levels. Its levels appeared to decline further during the storage and had decreased almost to the initial levels by 15 weeks. On the other hand, APx mRNA levels at 5 and 20°C storage also increased and peaked 6 weeks after storage, but the change was not as large as at 1°C and thereafter it deceased rapidly to the initial levels. Thus, the accumulations of APx mRNA were larger at low temperature than at higher temperature and their kinetics were transient in the all storage conditions.

Whereas the kinetics of the accumulation of CAT mRNA were similar at 1 and 5°C, they increased at slightly slower rate and lower at 20°C (**Figure IV-6**). Within 3 weeks CAT mRNA at 1 and 5°C increased 3-fold compared to initial levels. CAT mRNA levels at 1°C did not increase

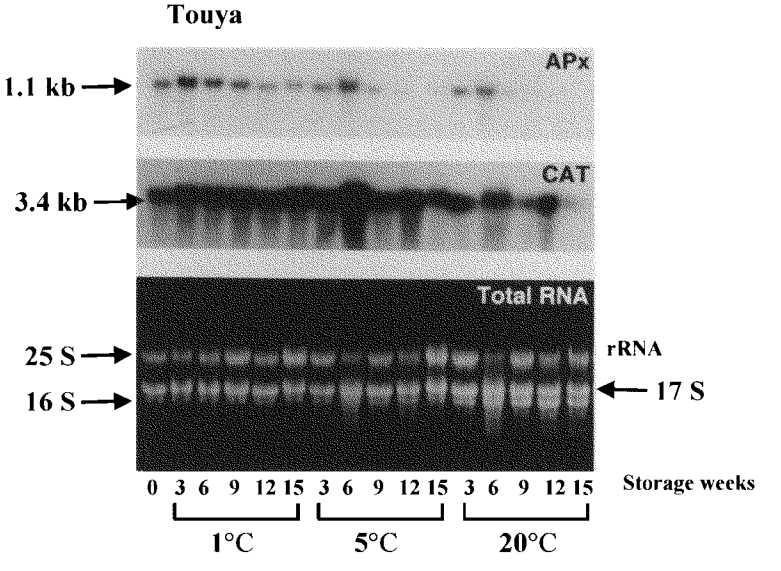


Figure IV-6. Northern blot analysis of APx and CAT mRNA by using ^{32}P-labeled DNA probe and ethidium bromide-stained rRNA band.

further during the storage by 12 weeks and had increased somewhat when the storage was terminated. They were elevated more by 6 weeks at 5°C and thereafter appeared to decline gradually by the end of storage, which was the almost same level as at 1°C. CAT mRNA levels at 20°C increased transitorily at 3 weeks to the same levels as at 1 or 5°C, but they tended to decline to constitutive levels by 12 weeks and thereafter had finally arrived to almost zero. The levels of ethidium bromide-stained rRNA bands were almost unchanged and were essentially the same in all lines (**Figure IV-6**).

IV-3-3 Activity staining of APx and CAT, and Western blot of APx.

Activity staining of APx was carried out to make sure whether APx isoforms exist in potato tubers. Because there is a possibility that the DNA probe for APx used might detect one of APx isoforms. As shown in **Figure IV-7**, some achromatic bands were detectable. However, when the activity stain was carried out in the presence of 10 mM KCN as described in Materials and Methods, one band indicated by an arrow, disappeared (**Figure IV-6** middle). Moreover, NativeWestern blot using anti-APx antibody indicated just one band (**Figure IV-7** right). Thus, potato tubers did not have any isoforms of APx. From the sequence of APx cDNA fragment, RT-PCR probe of APx was homologous to a cytosolic APx of tobacco (accession No. U15933) and spinach by a blastn research. This reflects that the DNA probe used for APx was a cytosolic type and APx

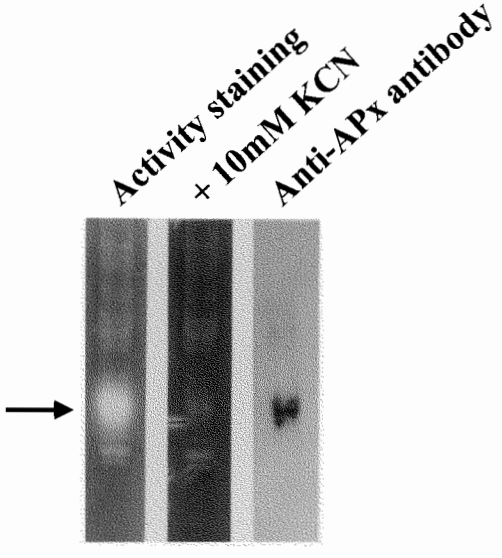


Figure IV-7. Identification of APx by activity staining with and without KCN, and Native Western blotting.

contained in potato tubers could be detected by Northern analysis using a DNA probe which was amplified by RT-PCR with two synthesized 20 mer primers.

Activity staining of APx was low in tubers at zero time storage and peaked at 12 weeks after harvest in tubers stored at 1°C (**Figure IV-8**). It was increased approximately 3.4-fold during the first 12 weeks of storage. In the contract, activity staining of APx at 20°C tended to increase gradually to a 1.9-fold increase at the same weeks, and thereafter to reach the same levels as that at 3 weeks. Over the course of the entire storage, tubers stored at 1°C had higher APx activity than at 20°C.

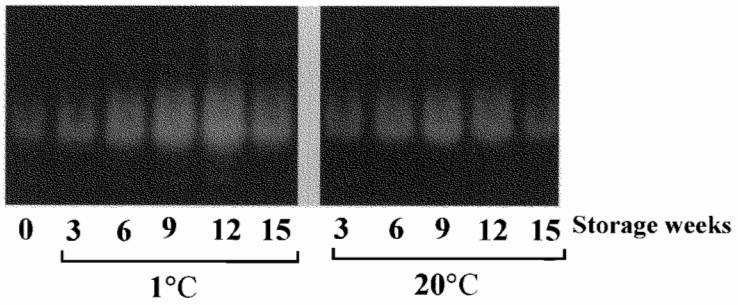


Figure IV-8. Activity staining of APx during storage at 1°C and 20°C.

Activity staining of CAT at 1°C was highest in tubers at zero time storage, and thereafter declined 9 weeks and returned to basal levels again at 15 weeks (**Figure IV-9**). However, in the case of storage at 20°C, activity staining of CAT did not show any change during storage. For the storage period as a whole, tubers stored at 1°C showed the same CAT activity except at 15 weeks as those stored at 20°C.

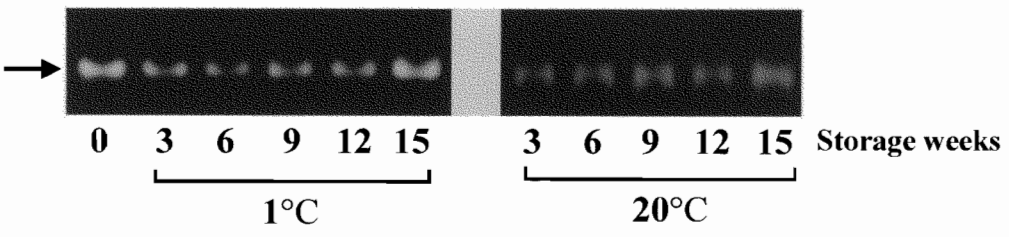


Figure IV-9. Activity staining of CAT during storage at 1°C and 20°C.

These results might indicate that active oxygen was more rapidly generated at low temperature and that *de novo* synthesis of APx was induced in low-temperature storage to detoxify them. Therefore, in order to assess the effect of low-temperature on APx expression, the amount of APx was measured by Western blot analysis. **Figure IV-10** shows that during storage at 1°C, 5°C and 20°C, contents of APx had increased by 9 weeks and then returned to the level at zero time storage, but tubers stored at 1°C showed higher contents of APx than those stored at 20°C. Thus, expressed APx levels were correlated with activity staining of APx.

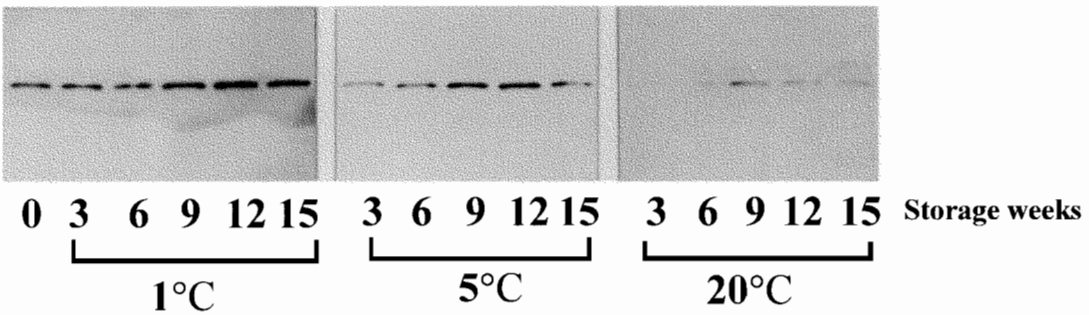


Figure IV-10. Western blotting of APx in potato tubers during storage.

IV-4 DISCUSSION

In this chapter, the effect of low temperature on expression of APx at the levels of mRNA and protein in potato tubers during storage was investigated to make clear the response of APx to low temperature stress. Transient accumulation in APx mRNA levels after low-temperature storage was observed higher than during high-temperature storage (**Figure IV-6**). The kinetics of their expression closely paralleled the results in the active staining of APx of which kinetics was delayed for 9 weeks (**Figure IV-6 and 8**). APx amounts by Western blot analysis were correlated with its active staining (**Figure IV-8 and 10**). This shows that APx was produced via *de novo* protein synthesis by

the stimulation of a low temperature stress. Moreover, it was made clear that APx isoforms do not exist in potato tuber (**Figure VI-7**) and from blastp research that APx is a cytosolic type. Elia *et al.*, (1992) had also reported that APx is located only in cytosol in potato tubers.

As shown in **Figure IV-6**, stable accumulations in CAT mRNA levels were observed at both low temperatures such as 1 or 5°C but not at 20°C. The kinetics of CAT mRNA did not parallel the active staining of CAT unlike APX mRNA (**Figure IV-6 and 9**). Moreover, it was demonstrated that CAT activity did not correspond to SOD increase in **chapter II**. CAT activities are either extremely low or not detectable in the cytosol, mitochondria and chloroplasts (Halliwell, 1981). In plant cells, an alternative and effective detoxification mechanism against H_2O_2 exists, operating both in chloroplasts and the cytosol (Asada, 1992). As described by Asada and Takahashi (1987), in this detoxification mechanism H_2O_2 is reduced by H_2O catalyzed APx which has higher affinity for H_2O_2 than CAT. Taking this evidence into consideration, newly accumulated H_2O_2 by low temperature may be detoxified by APx but not CAT because *de novo* synthesis of APx was induced by low temperature.

O'kane *et al.*, (1996) have recently reported that cellular H_2O_2 was observed to increase initially upon chilling but by day 8 cellular levels had declined to below control levels when chilling of *Arabidopsis thaliana* callus tissue to 4°C led to conditions of oxidative stress. Moreover, activity of APx increased although levels of CAT activity remained similar to those in unchilling tissue. Durner and Klessig (1995) suggested that salicylic acid (SA)-dependent inhibition of APx and CAT might result in the elevation of H_2O_2 levels. However, studies on the interaction of SA with CAT have provided evidence against the specific binding to SA to plant CAT (Ruffer *et al.*, 1995). Taken together with these data, it seems that the much higher reactivity of APx for H_2O_2 compared with CAT and the increase in its expression as a response to several stress conditions indicate a key role for this enzyme in the detoxification of oxygen-activated species. Hydrogen peroxide can cross biological

membranes because it has no unpaired electron, whereas the charged superoxide species can do so only very slowly. Therefore, H_2O_2, which enters the cell cytoplasm, might survive in sufficient concentrations to reach the plant nucleus. According to the hypothesis of Durner and Klessig (1995), the elevated level of H_2O_2 may act as a second messenger to switch on defense gene expression, especially APx. Prasad *et al.* (1994) also showed that H_2O_2 was the inducer of an antioxidant mechanism. From the results of the observed accumulation of APx mRNA in potato tubers treated only to low temperatures, it seems to indicate that H_2O_2 may function as a secondary messenger in the low temperature stress.

CHAPTER V

THE BEHAVIOR OF ACTIVE OXYGEN SCAVENGER ENZYMES IN POTATO TUBERS DURING LOW-TEMPERATURE STORAGE

V-1 INTRODUCTION

The author have demonstrated that APx is a most sensitive enzyme that is stimulated by low-temperatures in potato tuber (**Chapter II**) and newly accumulated H_2O_2 by low temperature may be detoxified by APx because *de novo* synthesis of APx was induced by low temperature (**Chapter IV**). In potato tuber it seemed that low-temperature stress led to a rapid decrease in the contents of AsA, which was used as a substrate of APx, during storage (**Chapter II**). Some reports have also shown the same phenomenon (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). Thus it seemed that the endogenous AsA contents was important factor for APx. In this chapter, we used two potato cultivars with different AsA contents in order to compare the influence of endogenous AsA contents on antioxidant enzymes under low-temperature stress.

Addition to this, the author also investigated the generation site of active oxygen. In plants photosynthesis, system of scavenging active oxygen species and the site of generating active oxygen are well documented (Asada, 1996). However, little has been reported on these mechanisms in non-photosynthetic organs. Recent work indicates that mitochondrial respiration is an important source of active oxygen *in vivo* (Longo *et al.*, 1996). Treatments, which inhibit cytochrome pathway activity, such as low temperatures, are known to induce the synthesis of alternative oxides (AOX), which was the specific enzyme of another respiration chain in plants (Wagner and Krab, 1995). The common substrate of AOX is ubiquinol [reduced Q (ubiquinone)]. AOX has been shown to remain

inactivated until the level of reduced Q reaches a high threshold value (Dry *et al.*, 1989; Moore *et al.*, 1988). Some *in vitro* studies have confirmed that progressive reduction of Q pool leads to an increase in the generation of active oxygen (Boveris and Cadenas, 1982). Therefore, we also measured the level of Q and reduced Q in mitochondria to obtain a clue about the generation site of active oxygen.

V-2 MATERIALS AND METHODS

V-2-1 Growth and Storage Conditions.

Tubers of the *Solanum tuberosum* L. cultivars, 'Kitaakari' and 'Danshaku', were grown at the Kobe University experimental farm. 'Danshaku' was an 'Early Rose' sprout mutant. 'Kitaakari', as a high AsA contents cultivar, was an interspecific hybrid between 'Danshaku' and 'Tsunica'. Both 'Danshaku' and 'Kitaakari' were stored at 1°C and 20°C and each group was sampled at the 3rd, 6th, 9th, 12th, and 15th week of storage.

V-2-2 Quantitative Analysis of AsA by HPLC.

Same procedure described at **II-2-4** was used.

V-2-3 Enzyme Extraction.

Same procedure described at **II-2-2** was used.

V-2-4 Enzyme assays.

APx (EC 1.11.1.11), CAT (EC 1.11.1.6), SOD (EC 1.15.1.1), MDHAR (EC 1.6.5.4), and DHAR (EC 1.8.5.1) were performed following the procedure described at **II-2-3.**

V-2-5 Preparation of mitochondria.

Mitochondria from potato tubers were prepared as described by Neuburger *et al.* (1982). The grinding medium consisted of a cold mixture of 20 mM pyrophpsphate buffer (pH 7.5), 0.3 M mannitol, 1 mM EDTA, 0.1% bovine serum albumin and 0.05% cysteine. The cysteine was added

immediately before using the medium. The material was homogenized in three successive batches in a Waring blender at low speed for 2 s. It is desirable to maintain the extraction medium as a semi-frozen slush. It is also critical that the grinding (mincing) process passes through eight layers of gauze and 75-μm nylon net. The crude mitochondrial pellet was further prepared as fast as possible according to the method of Bonner (1967).

V-2-6 Q-extraction.

Q-extraction was performed according to Van Den Bergen *et al.* (1994). Mitochondria (0.6-1.05 mg mitochondria protein) in a steady state were chemically quenched with 3 ml 0.2 M $HClO_4$ in MeOH (0°C). Quinones were subsequently extracted when this mixture was added to 2 ml of petroleum ether and all components were vigorously mixed with a vortex for 30 s. Centrifugation for a duration of 1.5 min at 1,500 x *g* resulted in a clear phase partitioning with the quinones present in the upper petroleum ether phase. The methanol phase (bottom phase) was rinsed once with 1 ml petroleum ether. The extract (2 ml and 1 ml petroleum ether combined) was evaporated to dryness with a stream of nitrogen and added to 200 μl ethanol in dried extract. Analysis of the redox state of the ubiquinone extract was performed by HPLC.

V-2-7 Determination of ubiquinone and ubiquinol contents.

Ubiquinol-10 and ubiquinone-10 were measured by an HPLC system (Hitachi LaChrom) equipped with an ECD detector (Shodex EC-1). The oxidation potential was 600 mV. Fifty mM sodium perchlorate in methanol/*tert*-butyl alcohol (9:1, v/v) was used as a mobile phase and its flow rate was 1 ml/min. CAPCELL PAK C18 UG120 (3 μm, 4.6x150 mm) was used for the separation column. Ubiquinone was converted to ubiquinol by an on-line reduction column (model RC-10, IRICA) before detection. The efficiency of the reduction of ubiquinone to ubiquinol was determined using standard ubiquinone (1 mole) with sodium borohydride (2 mole).

V-3 RESULTS

V-3-1 AsA contents and APx activities.

The AsA content of 'Kitaakari' (330 μM) was about triple that of 'Danshaku' (120 μM) at zero time storage (**Figure V-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 in both cultivars. In the case of 'Kitaakari' the AsA contents within 15 weeks of storage at 1°C and at 20°C decreased to approximately 41% and 23% respectively to compare with the respective storage conditions at time zero (**Figure V-1A**). In the case of 'Danshaku' compared to the AsA contents at time zero storage with 1°C and 20°C storage, they decreased to about 18% and 28% respectively (**Figure V-1A**). The APx activity is shown in **Figure V-1B**. In the storage at 1°C, APx activity of 'Kitaakari' could maintain higher activity than at 20°C during the storage period and the highest activity was approximately 1.5 folds,

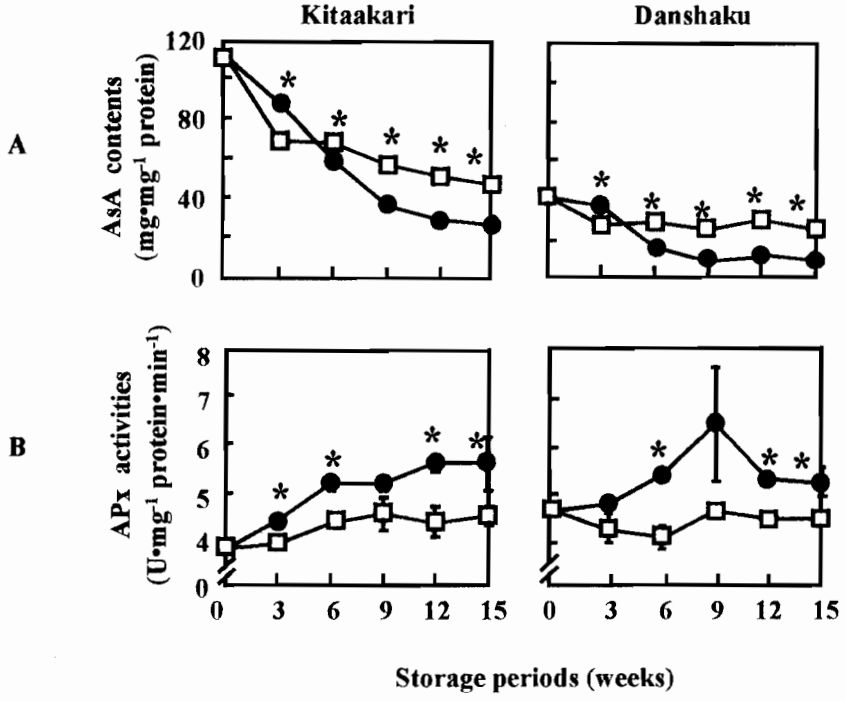


Figure V-1. Changes in AsA contents and APx activities during storage in 'Kitaakari'. After tubers had been stored at 1°C (●) and 20°C (□) for the times indicated. Samples were taken for the determination of tissue levels. Data are mean ± SE (n=3). Asterisks indicate significant difference between the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

compared with time zero storage (3.81 U). In contrast, at 20°C, APx activity of 'Kitaakari' did not show any change during the 15 weeks storage period. APx activity of 'Danshaku' showed different tendency that of the 'Kitaakari'. APx activity in the storage at 1°C seemed to decrease after 9 weeks in 'Danshaku' (**Figure V-1B**). However, at 20°C, APx activity maintained the same levels than at time zero and was lower than activity in the storage at 1°C throughout the storage period in 'Danshaku'.

V-3-2 CAT activity and SOD activity.

Though SOD activity of 'Kitaakari' barely changed during the entire storage period in the storage at 20°C, its activity increased after 12 weeks during the storage at 1°C, (**Figure V-2A**). During the storage period as a whole, SOD activity was higher at lower temperatures than it was at higher temperatures in 'Kitaakari'. While, in the case of 'Danshaku' no drastic change in SOD activity was

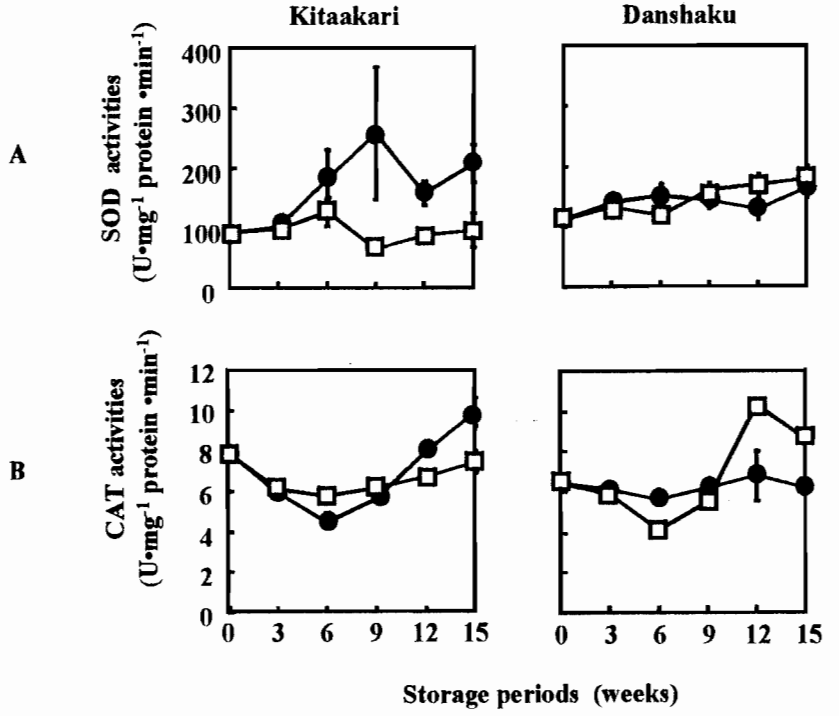


Figure V-2. Changes in CAT activity and SOD activity during storage of potato tuber at 1°C (●) and 20°C (□) for the times indicated. Samples were taken for the determination of tissue levels. Data are mean ± SE (n=3). The Values of total protein contents (each cultivars at each of 3 sampling dates during 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 (*$p<0.05$).

observed during the entire storage period at either storage temperature (**Figure V-2A**). CAT activity did not show any significant change throughout the storage period at 20°C, but in the storage at 1°C, CAT activity tended to decrease temporarily and afterward it increased in 'Kitaakari'. In the case of 'Danshaku', their tendencies at each temperature were reverse to the case of 'Kitaakari'. Thus, CAT activity did not depend on the storage temperature, as shown in **Figure V-2B**. As shown in **Figure V-2A** and **B**, SOD and CAT activities changed very little and did not depend on the storage temperature.

V-3-3 MDHAR activity and DHAR activity.

In both cultivars, MDHAR activity gradually increased in the storage at 1°C (**Figure V-3A**), and 'Kitaakari' and 'Danshaku' reached 124.4 and 70.7 nmol •mg^{-1} protein •min^{-1}, respectively. In the case of 'Kitaakari', 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 in

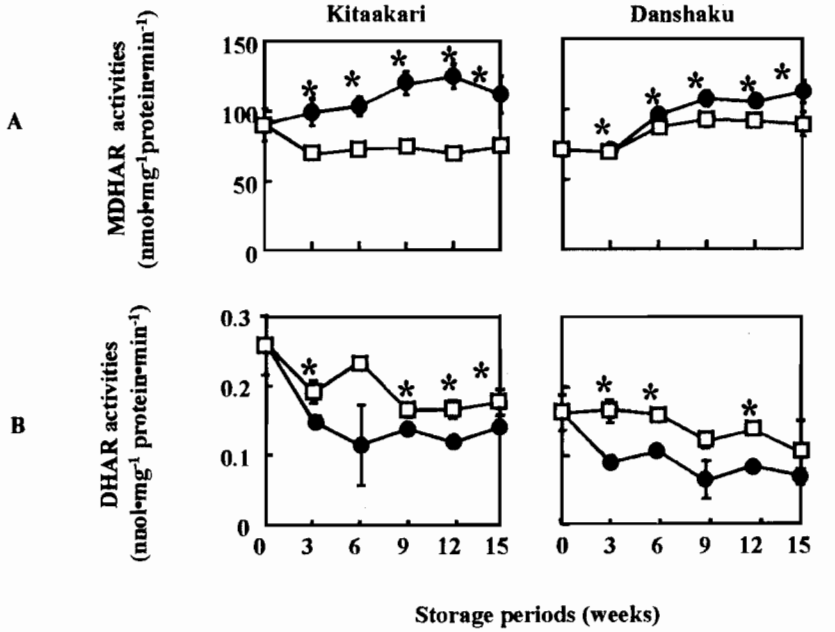


Figure V-3. Changes in MDHAR activity and DHAR activity during storage of potato tubers at 1°C (●) and 20°C (□) for the times indicated. Samples were taken for the determination of tissue levels. Data are mean ± SE (n=3). The Values of total protein contents (each cultivar at each of 3 sampling dates 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 (*$p<0.05$).

weeks. While, in the case of 'Danshaku' it increased gradually in the storage at both 1 and 20°C, but the degree of change was a 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.

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 in 'Kitaakari' (**Figure V-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 of DHAR activity in 'Kitaakari' (**Figure V-3B**). In the case of 'Danshaku', its activities decreased by 36% and 57% (1°C and 20°C, respectively) compared to time zero storage. Thus, DHAR showed higher activity in the storage at 20°C than at 1°C during the storage period in both cultivars.

V-3-4 Reduced ubiquinone (QH) / ubiquinone (Q) plus QH

As shown in **Figure V-4**, in 'Kitaakari' the ratio of reduced Q / Q plus reduced Q in the storage at 1°C increased dramatically at 6 weeks, mounted until 12 weeks and thereafter declined to the basal levels. In the storage at 20°C, this ratio temporally decreased at 6 weeks and increased gradually by 12 weeks in 'Kitaakari'. However, in 'Danshaku' throughout the storage period, this ratio was higher at low temperature than at high temperatures. The ratio of reduced Q / Q plus reduced Q was slightly different in the case of 'Kitaakari'. This ratio increased gradually through the storage period in the storage at 1°C. The behavior of this ratio at 20°C was the same as it was at 1°C

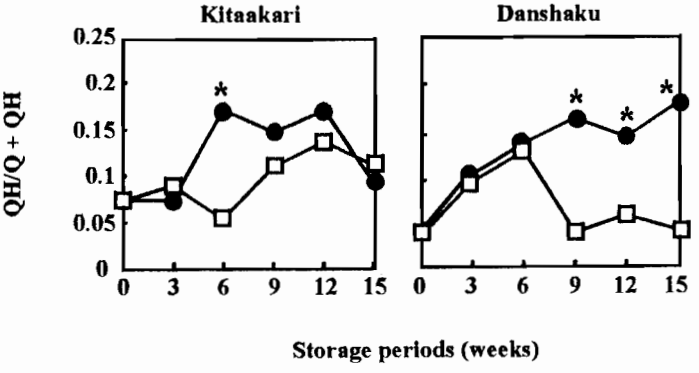


Figure V-4. Changes in reduced ubiquinone (QH) / ubiquinone (Q) plus QH during storage of potato tubers at 1°C (●) and 20°C (□) for the times indicated. Samples were taken for the determination of tissue levels. Data are mean ± SE (n=3). Asterisks indicate significant difference between the value of storage at 20°C and at 1°C by Student's *t*-test (*$p<0.05$).

until 6 weeks. However, at 9 weeks, this ratio rapidly decreased to basal levels.

V-4 DISCUSSION

In this chapter, we used two potato cultivars with different AsA contents in order to compare the influence of endogenous AsA contents on antioxidant enzymes under low-temperature stress. AsA contents in both cultivars decreased drastically during storage at a low temperature (1°C) (**Figure V-1A**). The tendency towards 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. As described at **Chapter II**, the decrease in AsA contents at low temperatures might be associated with the generation of active oxygen. As shown in **Figure V-1B**, APx activity seemed to increase in 'Kitaakari' throughout the entire low-temperature storage period. However, APx activity seemed to decrease after 9 weeks in 'Danshaku'. 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 why the APx activity showed the tendency of increasing throughout the storage period in the case of 'Kitaakari' and the tendency of decrease after 9 weeks in the case of 'Danshaku'. Indeed, at 1°C AsA levels in 'Kitaakari' were at least 73 μM, at 15 weeks, but the AsA levels in 'Danshaku' had already reached 22 μM at 9 weeks (**Figure V-1A**). This result was the same as described at **Chapter II** about 'Touya', which contained almost same levels of AsA as in 'Danshaku'.

Dismutation of the superoxide anion by SOD might be the primary step in the defense against the low-temperature treatment. SOD activity in 'Kitaakari' showed a 2.0-fold increase in tubers stored at 1°C compared with the activity at 0 weeks (**Figure V-2A**, left). As shown in **Figure V-1B**, APx activity in the 'Kitaakari' continued to increase during storage at 1°C, but not at 20°C. These

results 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 V-2A** and **2B**). The same phenomena have been observed by Spychalla and Desborough (1990). Such observations might be explained by CAT not working until the H_2O_2 concentration has increased beyond a certain threshold, since CAT possesses a very low affinity for H_2O_2.

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 V-3A**, MDHAR was sensitive to low-temperatures. However, DHAR activity declined during storage (**Figure V-3B**). 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 V-1B**). These findings suggest that the AsA levels decrease during storage, in spite of the regeneration of AsA activated by low temperatures.

The mitochondria of plant tissues, exposed to low temperatures, produces superoxide and/or H_2O_2 when electron transport through the cytochrome pathway is impaired due to the energy state of the cell or the stress-induced physical changes in membrane components (Purvis and Shewfel, 1993). In both cultivars, the ratio of reduced Q to Q plus reduced Q increased more drastically in the storage at 1°C than at 20°C (**Figure V-4).** This indicates that the redox poise of the Q pool tends to be reduced at a low temperature.

CHAPTER VI

EFFECTS OF ENDOGENOUS ASCORBATE IN POTATO TUBRES ON *DE NOVO* SYNTHESIS OF ASCORBATE PEROXIDASE DURIGN STORAGE AT LOW TEMPERATURE

VI-1 INTRODUCTION

In **Chapter IV**, mRNA and protein levels of APx in 'Touya' was investigated to clarify whether APx is produced with *de novo* protein synthesis. The kinetics of APx expression closely paralleled the results in the activity staining of APx, with a 9-week delay. APx amounts as determined by Western blot analysis using anti-APx antibody correlated well with activity staining of APx. These findings indicate that APx was produced with *de novo* protein synthesis induced by low temperature stress, and detoxified hydrogen peroxide. In **Chapter V**, in the case of 'Kitaakari' at the high concentration of AsA, APx activity continued to increase during storage at low temperature. However, in the case of 'Danshaku' at the normal concentration of AsA, its activity increased transiently until 9 weeks and thereafter declined to the almost same level as those at 3 weeks during storage at low temperature. These results demonstrated that APx activity might be affected by the concentration of endogenous AsA and AsA contents might be regulated at the level of mRNA expression. In this Chapter, 'Danshaku' and 'Kitaakari' of which contents of AsA was about triple that of 'Danshaku' were used to clarify effects of concentration of endogenous AsA on *de novo* synthesis of APx.

VI-2 MATERIAL AND METHODS

VI-2-1 Growth and Storage Conditions.

Potato tubers grown and stored under the same conditions as described in **V-2-1** were used for the following assays.

VI-2-2 Native PAGE and Activity Staining.

VI-2-2-1) Protein Extraction.

APx and CAT: The protein extraction of APx and CAT for activity staining was performed by the procedure described in **Chapter IV**.

SOD: SOD protein extraction was following the procedure described in Rao *et al.* (1996). Potato tubers (5 g) were homogenized in 100 mM potassium phosphate (pH 7.5) containing 2 mM EDTA and 1% PVP-40 at 4°C. The homogenate was filtered through four layers of gauze and centrifuged at 15,000 x *g* for 20 minutes. Protein content in the supernatant was concentrated by 45% ammonium sulfate precipitation for 30 min and centrifuged at 10,000 x *g* for 20 minutes. The supernatant was again concentrated by 90% ammonium sulfate precipitation for 30 min and centrifuged at 10,000 x *g* for 20 minutes. The pellet was in the same buffer (1 ml) and this solution was passed through a PD-10 column (Pharmacia Biotech, Tokyo), which was equilibrated with the same buffer to remove low molecular weight substances that interfere with the enzyme assay. Protein content was determined according to the method of Bradford (1976) with BSA as the protein standard.

VI-2-2-2) Activity Staining.

Activity staining of APx and CAT: The activity staining of APx and CAT were performed by the procedure described in **Chapter IV**.

Activity staining of SOD: SOD was visualized following the method described by Rao *et al.* (1996). Gels were stained for SOD isoforms by incubating in a solution containing 2.5 mM nitroblue tetrazolium for 25 min, followed by incubation in 50 mM potassium phosphate buffer (pH 7.8) containing 28 μM riboflavin and 28 mM TEMED for 20 min in the dark. The gels were placed in distilled water and exposed on a light box for 10 to 15 min at room temperature. Visualization of cyanide-sensitive and –insensitive isozymes was achieved by incubating gels in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM KCN for 30 min before staining for SOD activity.

VI-2-3-3) Northern Blot and Western Blot Analysis.

Northern Blot and Western Blot analysis were performed by the procedure described in **Chapter IV**.

VI-3 RESULTS

VI-3-1 Activity staining of APx, CAT, and SOD.

Some achromatic bands were detectable by APx activity staining in both cultivars **(Figure IV-1)**. Likely in 'Touya', the only band (indicated by arrow) disappeared when the activity staining was carried out under the same conditions in the presence of 10 mM KCN, suggesting that 'Danshaku' and 'Kitaakari' did not have any isoforms of APx.

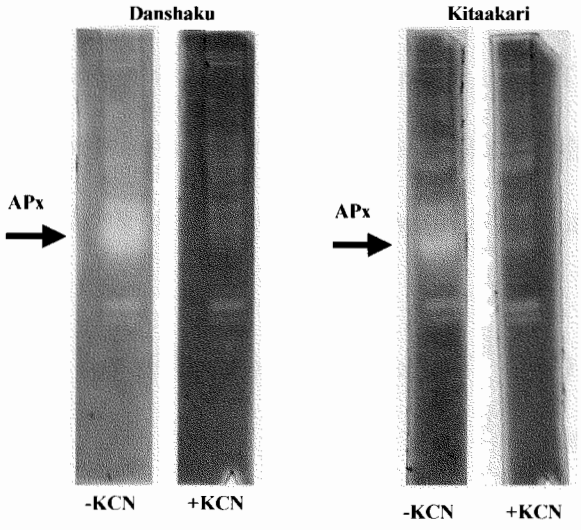


Figure VI-1. Identification of APx by activity staining with and without KCN.

The kinetics of activity staining of APx was shown in **Fig. VI-2.** In the case of 'Kitaakari', activity staining of APx at 1°C continued to increase during the storage period. In contrast, activity staining of APx at 20°C tended to increase gradually during the first 9 weeks of storage, and thereafter declined by 15 weeks. In the case of 'Danshaku', activity staining of APx at 1°C peaked at 9 weeks and seemed to decrease to the same levels as those immediately after harvest. However, in the case of storage at 20°C, activity staining of APx did not show any change during storage. These results were quite similar to the changes of APx activities in both cultivars shown in **Chapter V**.

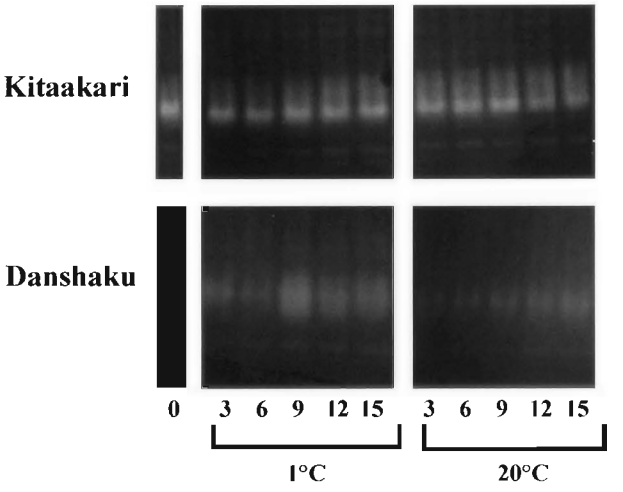


Figure VI-2. Activity staining of APx during storage at 1°C and 20°C.

As shown in **Figure VI-3**, only a single band was detected by activity staining of CAT in both cultivars. In the case of 'Kitaakari' at 1°C storage, activity staining of CAT tended to decrease temporarily and afterward it increased, while it did not show any significant change throughout the storage period at 20°C. In the case of 'Danshaku', their tendencies at each of the temperatures were reverse to the case of 'Kitaakari'. Moreover, activity staining of CAT was positively correlated with

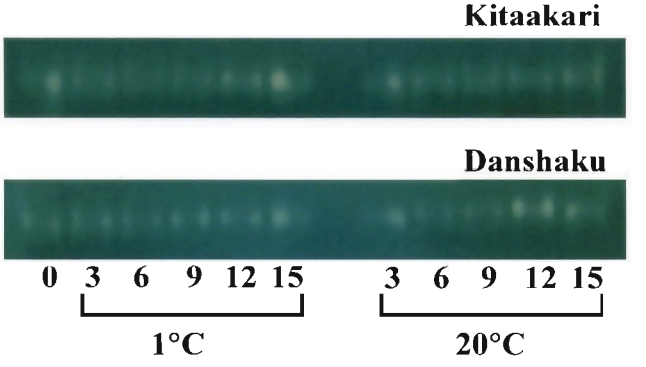


Figure VI-3. Activity staining of CAT during storage at 1°C and 20°C.

the changes of CAT activity in both cultivars shown in **Chapter V**.

Activity staining of SOD in 'Kitaakari' and 'Danshaku' showed different patterns of SOD **(Figure VI-4)**. Five isoforms of SOD were detected in 'Danshaku'.

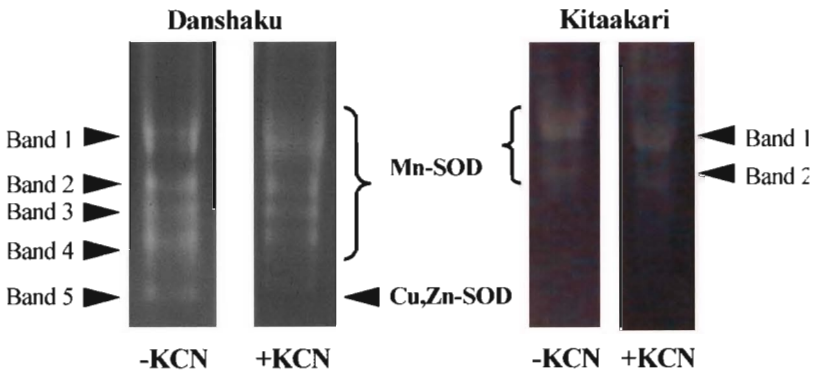


Figure VI-4. Identification of SOD by activity staining with and without KCN.

The band 5 disappeared by the treatment of KCN. Therefore band 5 was identified to be Cu,Zn-SOD, and band 1, 2, 3, and 4 were identified to be Mn-SOD because Cu,Zn-SOD was inactivated with KCN, but Mn-SOD was not. In the case of 'Kitaakari', two isoforms were detected. These bands were not inhibited by KCN, indicating that they were Mn-SOD.

The kinetics of activity staining of SOD during storage was shown in **Figure VI-5**. Cu,Zn-SOD did not change through the storage period at both storage temperatures. It was suggested that Cu,Zn-SOD did not relate to the changes of SOD activity. The kinetics of each Mn-SOD isoform in both cultivars did not relate to the changes of SOD activities measured by spectrophotometry

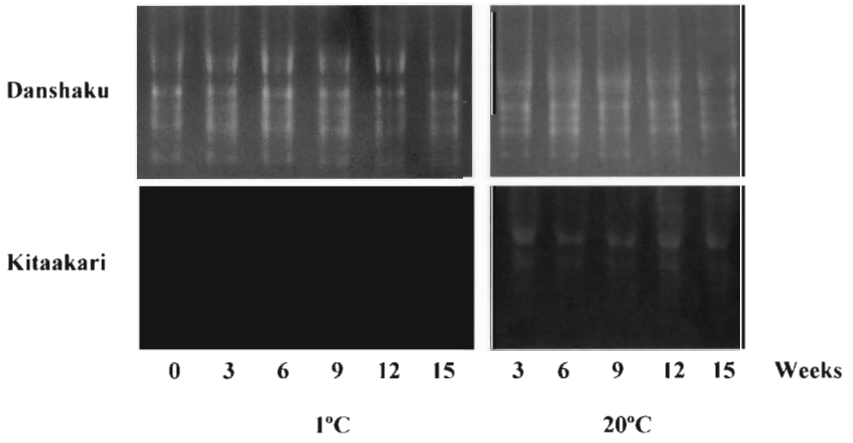


Figure VI-5. Native gels stained the superoxide dismutase activity of potato tubers during storage at 1°C and 20°C.

in **Chapter V**. Therefore it was suggested that SOD activity was not due to the reflection of activity from one of each of the Mn-SOD isoforms but the total activities of each Mn-SOD isoform.

VI-3-2 The expression of APx and CAT at the level of mRNA.

The size of APx and CAT mRNA detected by Northern blot analysis was calculated on the base of rRNA size [25 S (3.4 kb), 17 S (1.8 kb), and 16 S (1.6 kb)]. The size of APx and CAT mRNA was estimated to be 1.1 kb, which corresponds to **(Figure VI-6)** the size reported by Elia *et al.* (1992) and 4.3 kb (**Figure VI-7**) that indicated to be the same size of CAT mRNA of *S. tuberosum* reported by Niebel *et al.* (1995) and Wu *et al.* (1995), respectively.

The accumulation of Northern blot analysis of APx mRNA during storage was shown in **Figure VI-6.** In the case of 'Kitaakari' APx mRNA during the storage at 1°C continued to increase

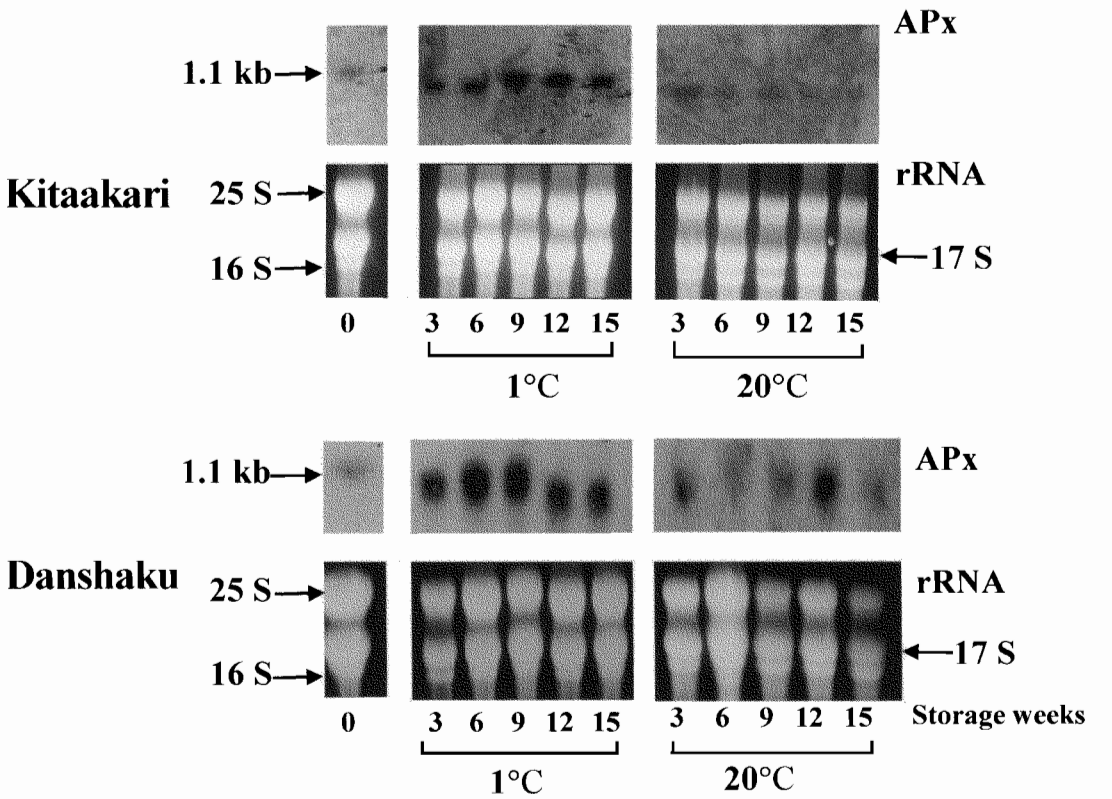


Figure VI-6. Northern blot analysis of APx mRNA by using ^{32}P-labeled DNA probe and ethidium bromide-stained rRNA band.

continuously with storage periods. While, during the storage at 20°C it was hardly induced through the storage period. In the case of 'Danshaku' in the storage at 1°C APx mRNA induced until 9 weeks and tended to decrease after 12weeks, and in the storage at 20°C, it maintained the same levels as at time zero without 12 weeks. These changes of APx mRNA levels were similar to the changes of APx activity and activity staining.

The accumulation of Northern blot analysis of CAT mRNA was shown in **Figure VI-7.** In the case of 'Kitaakari' the CAT mRNA level during storage at 1°C tended to decrease temporarily and increase afterward. While, during the storage at 20°C it hardly induced through the storage period. In the case of 'Danshaku', the tendency of CAT mRNA level during storage at 1°C was similar to that in 'Kitaakari'. On the contrary during the storage at 20°C its level induced at 3 weeks, then temporarily decreased and afterward increased by slow degrees until 15 weeks. Unlike APx mRNA, the changes of CAT mRNA levels were not similar to those of activity and activity staining of CAT.

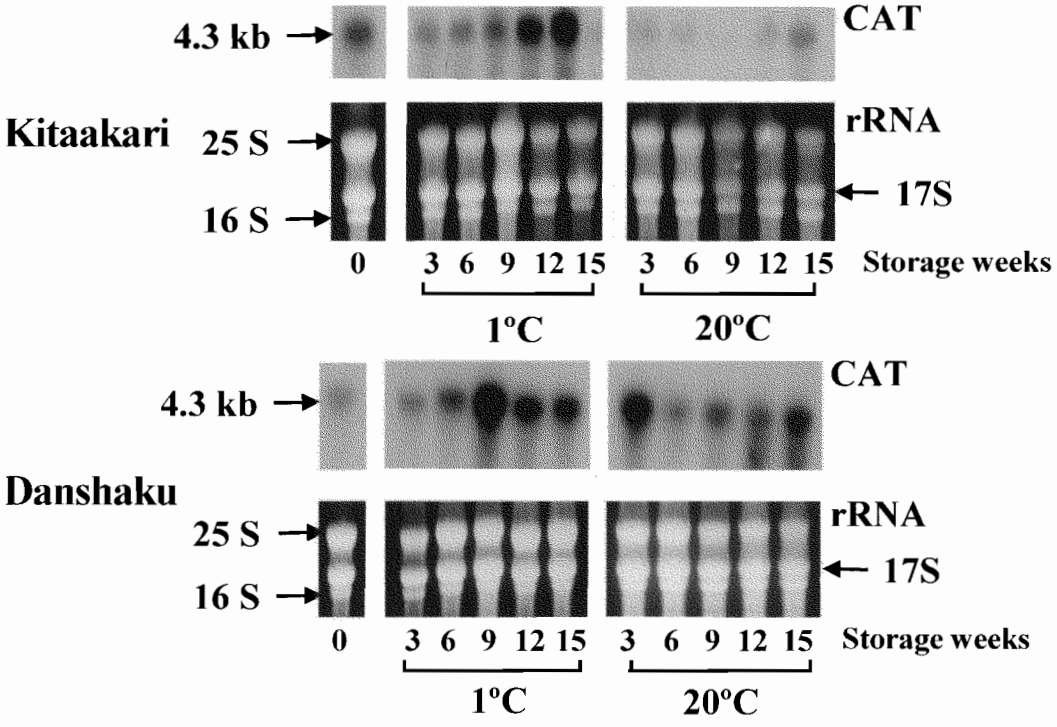


Figure VI-7. Northern blot analysis of CAT mRNA by using ^{32}P-labeled DNA probe and ethidium bromide-stained rRNA band.

VI-3-3 Western blot analysis of APx.

The result of Western blotting was shown in **Figure VI-8**. During storage at 1°C, amounts of APx in 'Kitaakari' increased gradually until 15 weeks. On the contrary, in the case of 'Danshaku' they tended to temporarily increase by 6 weeks and afterward return to the same level as at 3 weeks. Over the course of the entire storage time, amounts of APx in 'Kitaakari' were more than those in 'Danshaku'. During storage at 20°C, amounts of APx showed no change throughout storage period in both cultivars. Considering the results of activity staining, mRNA and protein levels of APx, it was demonstrated that APx is produced by *de novo* synthesis in to response to low temperature.

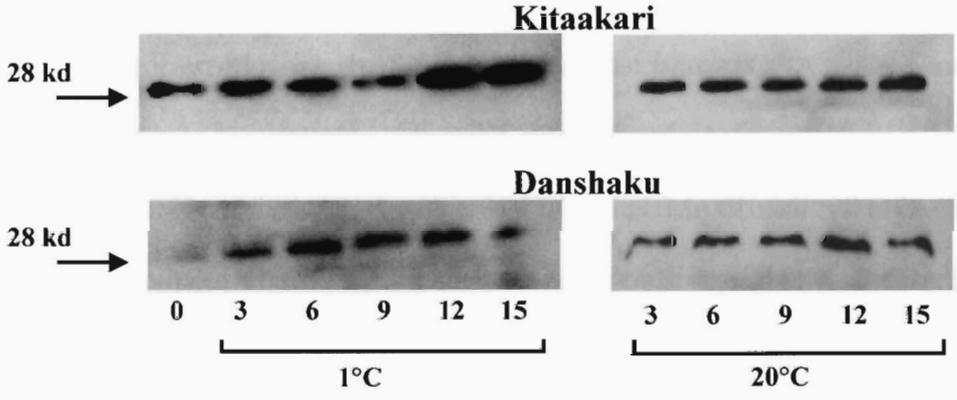


Figure VI-8. Western blotting of APx in potato tubers during storage.

VI-4 DISCUSSION

It has been reported that APx was inactivated at low concentration of AsA (less than 30 μM) due to the instability of compound I to H_2O_2 (Miyake and Asada, 1996). Indeed, it was demonstrated in **Chapter II** that endogenous AsA contents in 'Touya' had reached to less than 30 μM by 9 weeks after the low-temperature storage with a corresponding decrease in APx activity. Moreover, transient accumulation in APx mRNA levels after low-temperature storage were observed to be higher than after

high-temperature storage. The kinetics of their expression closely paralleled the results in the activity staining and Western blot analysis of APx, with a 9-weeks delay (**Chapter IV**). These findings ascertained that APx was produced with *de novo* protein synthesis in response to the stimulation of low temperature. In this Chapter, in order to make clear the effect of endogenous AsA on *de novo* synthesis of APx during low temperature stress, the expression of APx at the levels of mRNA and protein were investigated in two types of potato tubers, 'Danshaku' and 'Kitaakari' (originally about triple AsA contents of 'Danshaku'). As shown in **Figure VI-6**, accumulation in APx mRNA levels at low temperature (1°C) continued to increase in 'Kitaakari', but in 'Danshaku' it was increased transiently by 6 weeks and thereafter declined. Regarding the protein level (**Figure VI-9**) and activity staining (**Figure VI-2**) of APx, a similar tendency in mRNA levels were observed. In **Chapter V**, in 'Danshaku', APx was inactivated after 9 weeks when the concentration of the endogenous AsA contents decreased to less than 30 μM during the low-temperature storage. However, in 'Kitaakari', APx showed the tendency to increase throughout the same storage period when the concentration of the endogenous AsA contents maintained more than 30 μM. Therefore, the decrease of APx levels in 'Danshaku' after storage for 9 weeks might be caused by the depletion of AsA as an electron donor at a low temperature. Thus, concentration of endogenous AsA affected not only APx activity but also APx mRNA expression.

It was reported that mitochondria generated oxidative stress and was the major source of the cytosolic H_2O_2 (Puntarulo *et al.*, 1991). The detoxification of H_2O_2 might be mediated by catalase which is mostly localized in the peroxisome (Elstner, 1987). However, catalase possesses a very low affinity with H_2O_2 (Halliwell, 1981). Though it has already been demonstrated in **Chapter IV**, stable accumulations in CAT mRNA levels were observed at 1°C but not at 20°C. However, the kinetics of CAT mRNA did not parallel the activity staining of CAT unlike APx mRNA. A similar tendency was observed in both cultivars in this chapter. It has reported that dark-grown maize seedlings

contain two CAT isoforms, peroxisomal CAT1 and mitochondrial CAT3 (Prasad, 1997). CAT2 is present only in photosynthetic tissues, not in nonphotosynthetic tissues (Scandalios *et al.*, 1984). However, any of the isoforms of CAT were not detected by activity staining in potato tubers stored at low and high temperature (**Figure VI-3**). It was clear that potato tubers did not have any isoforms. This evidence shows that there is no possibility that the other isozymes of CAT are contributing to the detoxification of H_2O_2 generated during low temperature storage. Moreover, it was described in **Chapter V** that CAT activity does not correspond to SOD increase in both cultivars. Therefore it seemed that CAT could not detoxify H_2O_2 generated by dismutation of $O_2^{\bullet-}$ through SOD in potato tubers exposed to low temperature. When the levels of H_2O_2 levels in suspension-cultured potato tuber cells incubated at 5°C were measured using 2',7'-dichlorofluorescin diacetate, cellular H_2O_2 increased. Moreover, APx mRNA expression was induced in this case. Furthermore, APx mRNA expression was rapidly induced within 30 min when the cells were directly stimulated with exogenous H_2O_2 (data not shown). Taken together with these data, dismutation of $O_2^{\bullet-}$ by SOD into H_2O_2 might be the primary step for the defense against the low temperature treatment, and the increase in APx expression as a secondary response to several stressful conditions might indicate a key role for APx in the detoxification of H_2O_2.

CHAPTER VII

THE LOCALIZATION OF HYDROGEN PEROXIDE AND ASCORBATE PEROXIDASE IN POTATO TUBER

VII-1 INTRODUCTION

It has already been demonstrated that active oxygen species generated in potato tubers exposed to low temperature stress and APx play an important role in scavenging H_2O_2. Some investigators have reported that H_2O_2 might act as the second messenger of signal transduction for defense mechanism or an inducer of antioxidant mechanism against oxidative stress (Durner and Klessig, 1995; Prasad *et al.*, 1994). However, it has not been clarified from the biochemical data carried out so far where active oxygen species including H_2O_2 were produced in potato tubers at low temperature. It was reported that the mitochondria generated oxidative stress, was the major source of the cytosolic H_2O_2 (Puntarulo *et al.*, 1991). It appeared to be more effective that APx was located close to the site of H_2O_2 and close to the sites at high risk of oxidative stress. APx is determined to mainly locate in chloroplasts and cytosol, and it has also been found in peroxisomes or glyoxysomes by the isolation of each of organelles (Jimémez *et al.*, 1997; Jimémez *et al.*, 1998; Yamaguchi *et al.*, 1995; Bunkelmann and Trelease, 1996). This method (a density gradient centrifugation) included the possibility of the cross-contamination on the purity of the sample. Hence, in this chapter, the author proposes to determine the localization of APx and H_2O_2 cytochemically by light microscopy and transmission electron microscopy in potato tubes during low temperature storage. To examine the localization of H_2O_2 and APx, 'Kitaakari' was used because it has been demonstrated that the behavior of APx was regulated by the AsA contents and was most sensitive to low temperature among the three cultivars examined.

VII-2 MATERIALS AND METHODS

VII-2-1 Growth and Storage Conditions.

Tubers of the *Solanum tuberosum* L. cultivars, 'Kitaakari' and 'Danshaku', were grown at the KFC Colonel farm in Hokkaido. Both 'Danshaku' and 'Kitaakari' were stored at 1°C and 20°C and each group was sampled at the 3rd, 6th, 9th, 12th, and 15th week of storage.

VII-2-2 Tissue prints of H_2O_2 using cross-sections of potato.

Tissue printing assay was performed by according to the procedure described by Bestwick (1997) with a slight modification. PVDF membranes (Hybond^{TM}-P, amersham pharmacia biotech) were soaked in methanol for more than 1 min before use. H_2O_2 printing paper was prepared by soaking strips of PVDF membrane with the mixture solution containing 100 g/l soluble starch and 0.5 M KI in distilled water. The starch was solubilized by boiling before KI was added to the cooled solution. The membranes were dried in a stream of warm air (30°C) and stored in the dark before the use. To minimize background staining with autoxidation of KI, the reagent solution was used no longer than 5 h and the printing paper no longer than 2 h after preparation.

Tissue printing was performed as described by Cassab and Varner (1989) at room temperature (18-20°C). Small pieces of H_2O_2 printing paper were placed on a pad of five layers of chromatographic papers. The tissue sections with about 5 mm thickness were prepared by hand cutting and immediately transferred to the printing papers; and the displacement of sections after contacting the papers were avoided carefully. The group of sections were covered with a pad of soft cleaning tissue (16 layers) and gently pressed with four fingers for 60 s (the effective load on the sections was about 200-300g). Then the sections were carefully removed with a forceps and the prints were examined after 5 to 10 min of drying. H_2O_2 staining was developed within 0 to 5 min depending on the amount of H_2O_2 transferred. Because the prints intensify with time, it was necessary to examine them after a defined period. Experiments were repeated at least three times on different occasions

with very similar results. The pictures shown in the figures were typical examples from these experiments.

VII-2-3 Immunostaining prints of APx using tissue-sections of potato.

PVDF membranes were soaked in methanol for more than 1mim before use. The tissue sections were immediately transferred to the printing papers; the displacement of the sections after contacting the paper was carefully avoided. After preparing the membrane, the immunostaining assay for APx was performed by the procedure described in **IV-2-4.**

VII-2-4 The localization of APx in potato tuber tissues at light microscopy.

Tissues were fixed in 4% (v/v) paraformaldehyde in 1/15 M sodium phosphate buffer (pH 7.2) overnight. After the fixation, tissues were dehydrated in a graded series of 10, 15, and 20 % sucrose solution for 8 h at the each step. The tissues were frozen in OCT compound and preserved at -80°C until use. Semi-thin sections (with the thickness of 6 μm) were cut at -24°C on a Microtome Cryostat HM 505E using steel knives. The sections were mounted on APS-coated slide glasses and dried at room temperature for 30 minutes. The detection of APx was performed by immunochemical methods. The samples were incubated with immunoreagents as following orders; (1) in rabbit anti-APx polyclonal antibody (1:500, v/v) for 1 h at room temperature, (2) in biotin-conjugated goat anti-rabbit IgG (H+L) antibody (1:10, v/v) for 10 min, (3) in streptavidin-labeled HRP for 10 minutes. In the procedure for negative stains, rabbit anti-IgG polyclonal antibody was used instead of rabbit anti-APx antibody. After incubation in the immunoreagents, the sections were rinsed 3 times with TBS buffer for 5min at the each step. The APx was then visualized by reaction with substrate solution containing 0.02% diaminobenzidine (DAB) and 0.001% H_2O_2.

VII-2-5 Electron microscopic observation of H_2O_2-generated site.

Hydrogen peroxide was able to be detected histochemically by its reaction with cerium chloride to produce electron-dense deposits of cerium perhydroxides as described in detail by Bestwick

et al. (1997). Samples were prepared as described by Bestwick *et al.* (1995). Tissue pieces (1 mm^3) were excised from potato tubers with razor blades and incubated for 1 h in 5 mM $CeCl_3$ reaction medium in 50 mM 3-(*N*-morpholino) propaneselfonic acid (Mops) at pH 7.2. Tissues were then fixed in 1.25% glutaraldehyde in 50 mM sodium cacodylate buffer (CAB), pH 7.2, for 1hour. After the pre-fixation, tissues were washed twice for 10 min in CAB buffer and postfixed for 45 min in 1% osmium tetroxide in CAB buffer for 1 hour. Tissues were then washed again in CAB buffer (twice for 10min) and dehydrated in a graded ethanol series. Samples were transferred to two changes of propylene oxide of 20 min each in duration and progressively embedded in Spurr resin. Tissues were polymerized at 60°C for 12 hours. Ultrathin sections were sectioned from resin blocks on an Ultra-microtome MT-1 (Ivan, Sorvall, Inc.), using a diamond knife (Diatome, Bienne, Switzerland), and mounted on uncoated copper grids (200 mesh). In order to distinguish the identical deposits of cerium perhydroxides from non-specific electron-dense materials, energy-filtering transmission electron microscopy (EFTEM, LEO 912AB Omega) was performed as described by Hirai *et al.* (1999).

After identifying the element in electron-dense deposits by using EFTEM (data not shown), sections were examined with 7100 type of electron microscope of Hitachi at an accelerating voltage of 75kV. The frequency of appearance of H_2O_2 generated site was examined in 'Kitaakari' exposed to 1°C and 20°C respectively. The examination was carried out in 50 different cell types of potato tubers, phloem, vascular bundle sheath and parenchymal cells with the magnification of x 2,500.

VII-3 RESULTS

VII-3-1 Appearance of potato tubers during various storage periods.

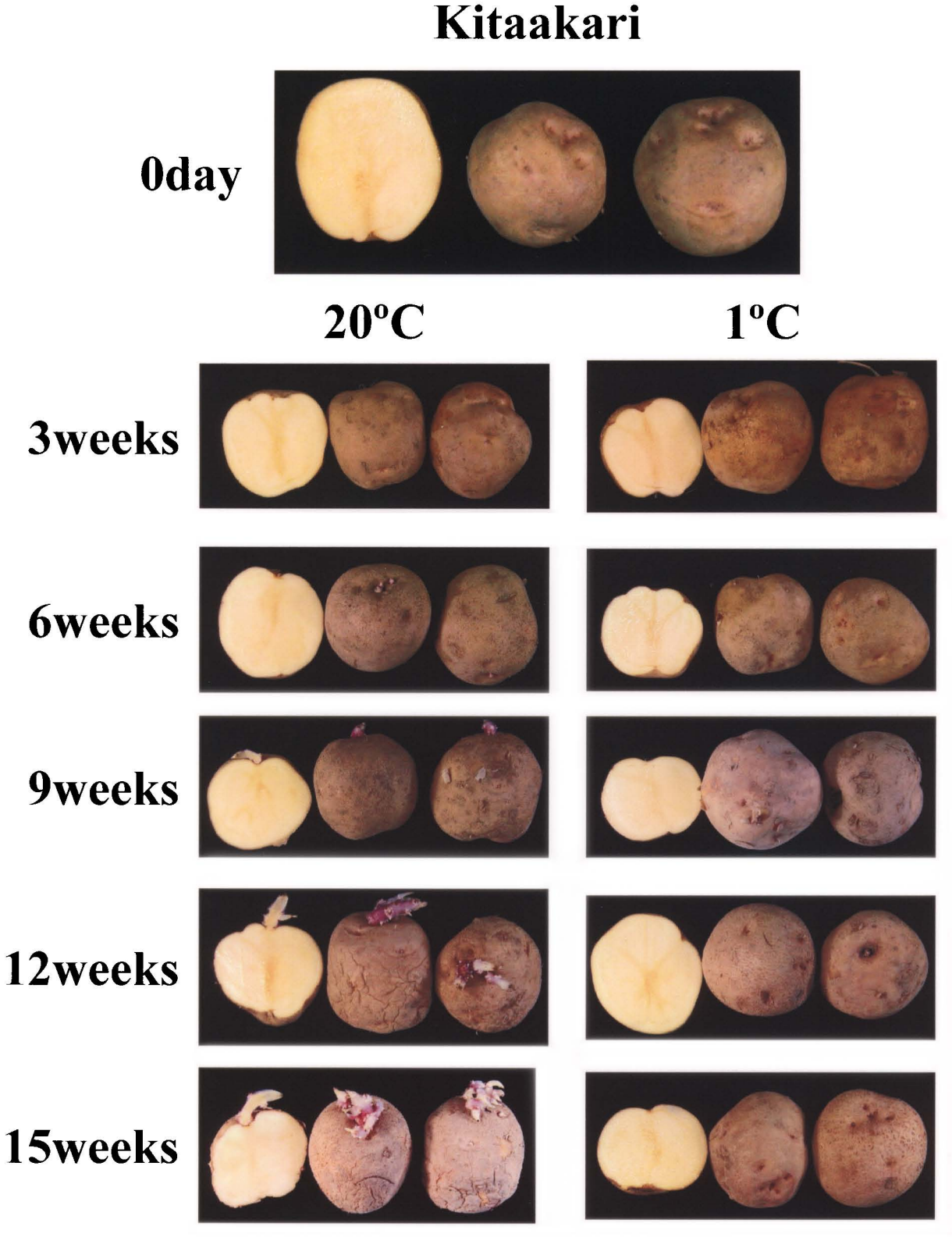
Appearance of potato tubers during various storage periods was compared between 'Kitaakari' and 'Danshaku'. Both potato cultivars never germinated at 1°C through the storage period (**FigureVII-1 and 2**). Different germination patterns were observed between 'Kitaakari' and 'Danshaku' during the storage at 20°C. In the case of 'Kitaakari' (**FigureVII-1**), the germination was earlier than that of 'Danshaku' (**FigureVII-2**). However, the surfaces of cross-sections showed no changes in both cultivars at different storage temperatures through the storage periods.

VII-3-2 Tissue prints of H_2O_2 using cross-sections of potato.

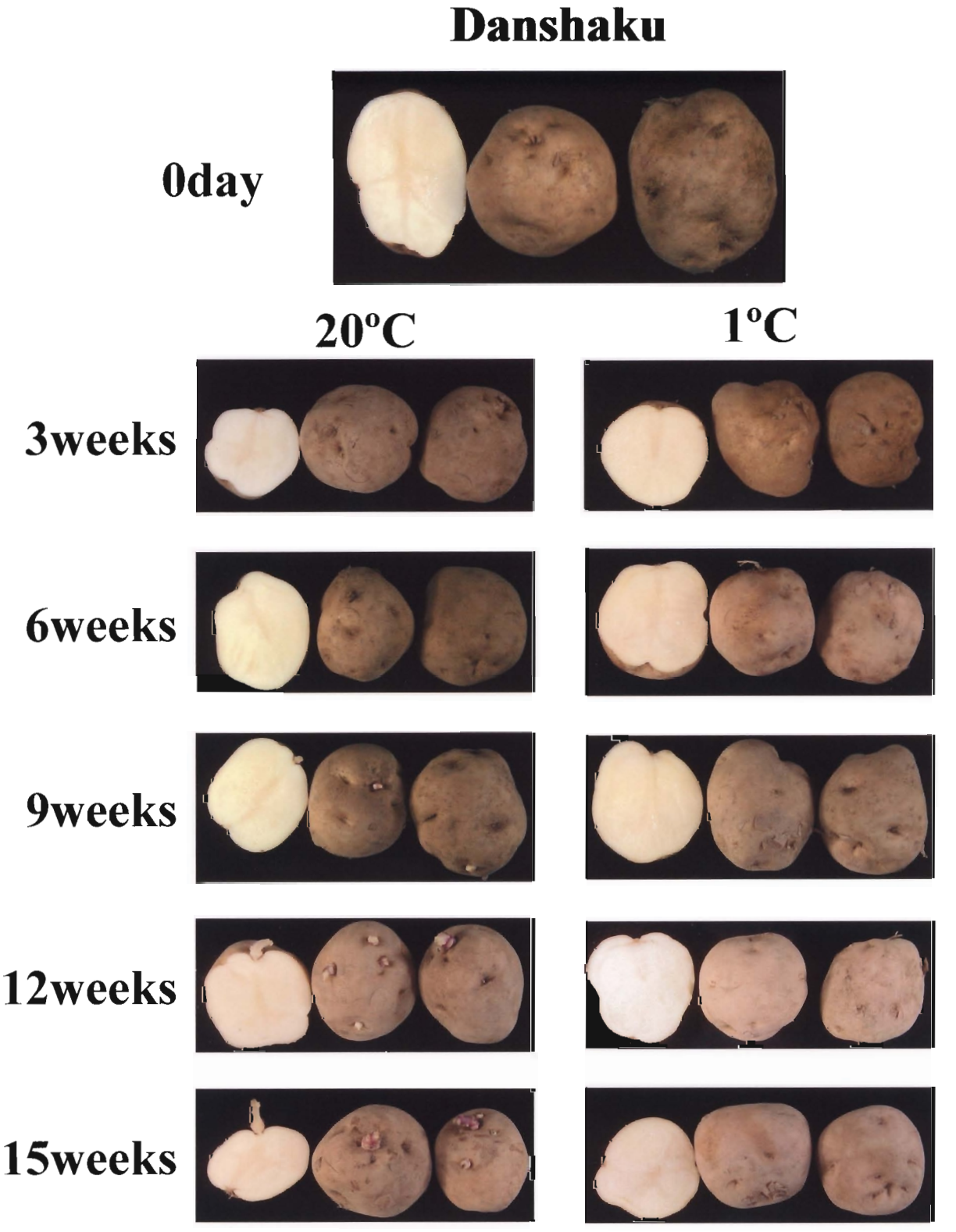
A test with a H_2O_2 reaction medium (**FigureVII-3-A**) showed that the detection limit of this assay was in the range of 0.1 to 2 mM H_2O_2 (0.25-0.5 nmol/dot). The result of tissue printing assay during storage period is shown in **Figure VII-3-B.** The characteristic specific patterns of H_2O_2 distribution in ring vascular bundle and epidermis of 'Kitaakari' were found more drastically at 1°C than at 20°C. Though the contents of H_2O_2 were not highly significant at the 0 week storage in vascular bundles, it was higher at 1°C than at 20°C in 6 and 12 weeks storage. On the contrary, in epidermis, the higher contents of H_2O_2 were detected through the storage period at both storage temperatures.

VII-3-3 Immunostaining prints of APx using tissue-sections of potato.

To elucidate the locating sites of APx in the potato tuber tissue, the author performed a tissue prints assay by immunohistochemical analysis using anti-APx antibodies. As shown in **Figure VII-4,** tissue prints of potato tubers cross-sections displayed some different patterns during storage periods. Although APx was localized only in epidermis at 0 week storage, accumulated APx was also detected in vascular bundles in addition to epidermis in which the tendency was also maintain at the same level, when potato tubers were stored for 6 and 12 weeks. Moreover, APx contents were markedly high in vascular bundle at 1°C during 12 weeks storage.



FigureVII-1. Appearance of potato tuber (c.v.Kitaakari) during storage periods.



FigureVII-2. Appearance of potato tuber (c.v.Danshaku) during storage periods.

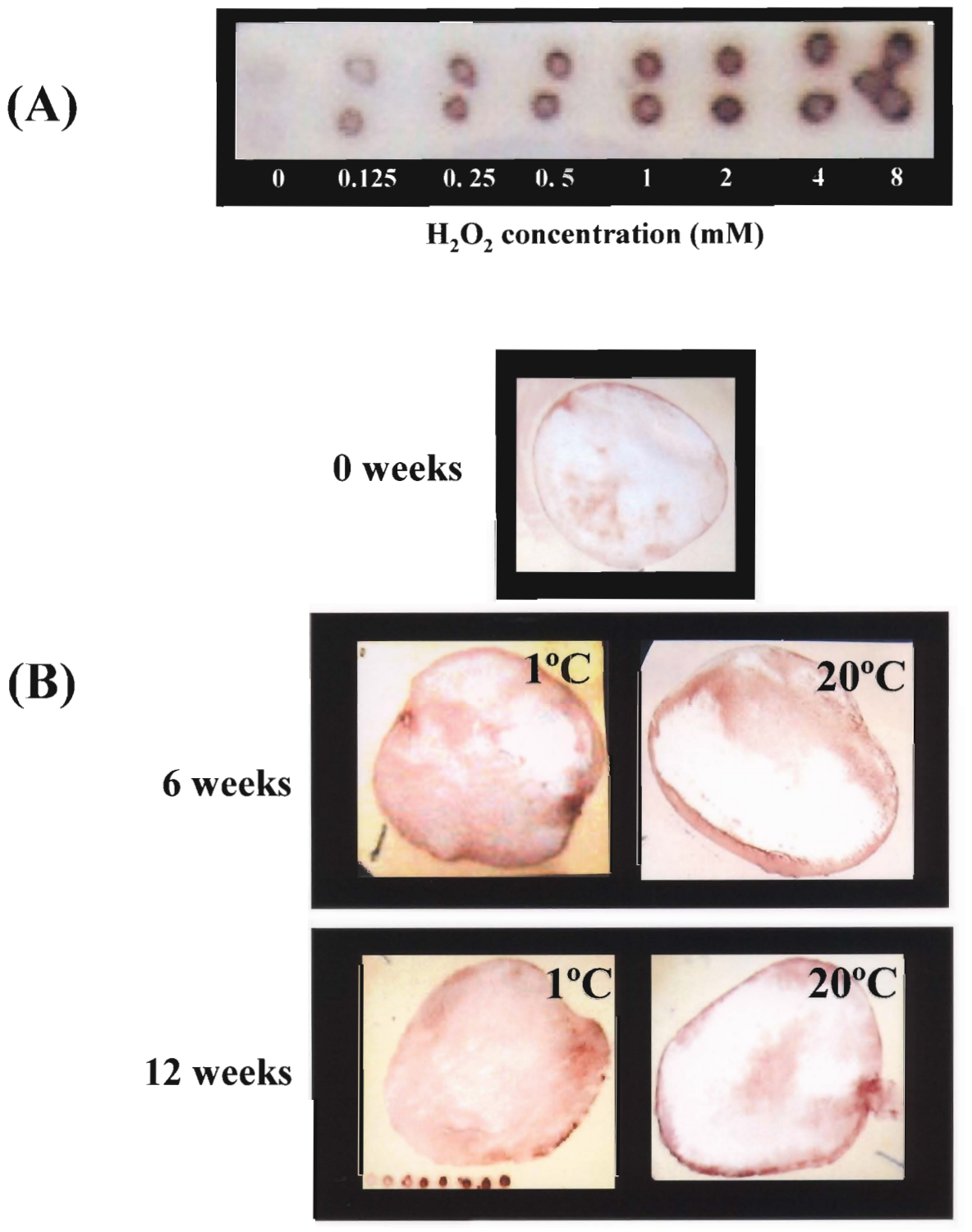


Figure VII-3. The detection of H_2O_2 in 'Kitaakari' potato tubers by tissue printing assay.

(A) Calibration of the H_2O_2 printing assay.

(B) The comparison of the spatial resolution of the H_2O_2 printing assay during storage periods.

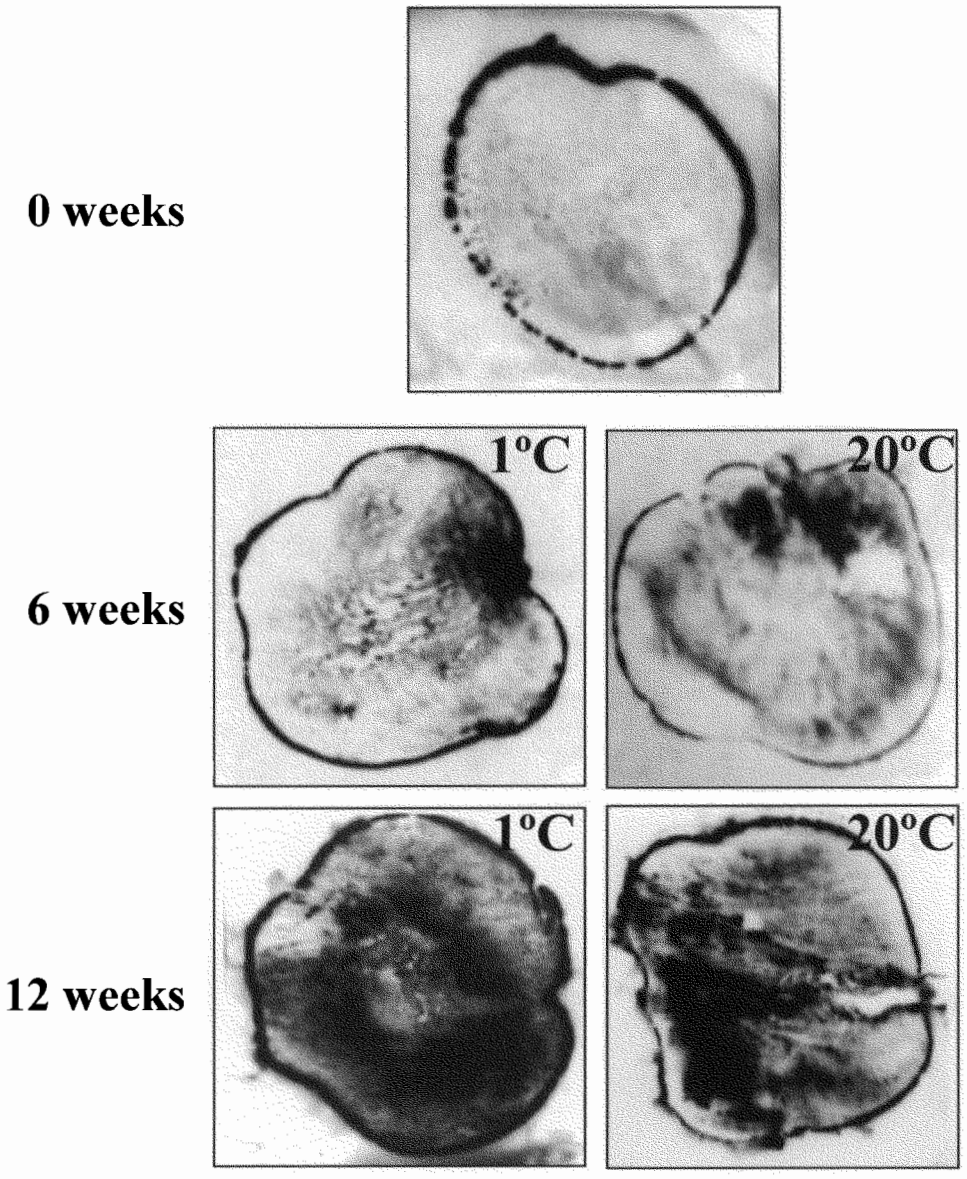


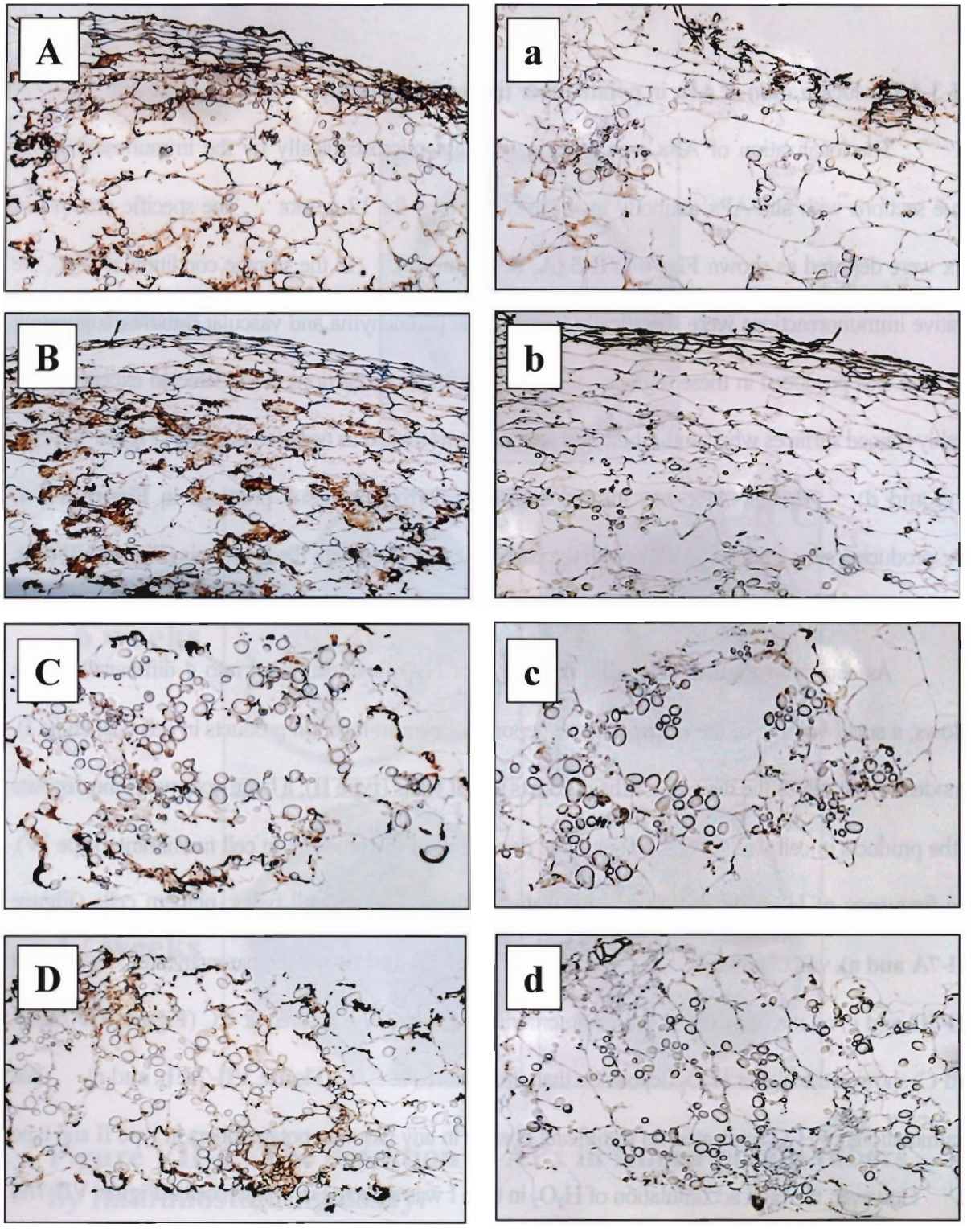
Figure VII-4. The detection of APx in stored potato tubers by immunostaining assay.

VII-3-4 The localization of APx in potato tuber tissues for light microscopy.

The localization of APx was investigated light microscopically by the immunostaining of tissue sections with anti-APx antibody in 'Kitaakari' stored for 12 weeks. The specific sections of APx were detected as shown **Figure VII-5 (A, B, C, and D).** In the storage condition at 20℃, the positive immunoreactions were specifically found in the parenchyma and vascular bundles, suggesting that APx was produced in these regions. On the contrary, no reactions were detected except for the weakly stained surfaces when anti-rabbit IgG antibody was treated as negative controls (**Figure VII-5 a, b, c, and d**). Though APx was located around epidermis by tissue printings in **Figure VII-4**, APx-producing sites were compatible with the parenchyma but not with the epidermis (**Figure VII-5B**).

VII-3-5 Electron microscopic observation of H_2O_2-generated site.

As shown in **Figure VII-6**, the deposition of H_2O_2 was classified into 4 different types as follows; a small volume of the electron-dense deposits of cerium-reaction products in cell wall (**type I**), a moderate volume of the deposits of the products in cell walls (**type II**), a large volume of the deposits of the products in cell walls (**type III**), and the deposition of the products in cell membrane (**type IV**). The frequency of H_2O_2 deposition was estimated at three different cell types: phloem cells (**Figure VII-7A and a),** vascular bundle sheath cells (**Figure VII-7B and b**) and the parenchymal cells (**Figure VII-7C and c**) on the basis of the foregoing criteria. Potato tubers stored at 1℃ (**Figure VII-7A, B, and C**) showed the higher H_2O_2 deposition than those stored at 20℃ (**Figure VII-7a, b, and c**). The accumulations of H_2O_2 indicated no significant change in any tissue in potato tubers in type II and type III. However, the high accumulation of H_2O_2 in type I was detected in phloem cells (**Figure VII-7A**) and vascular bundle sheath cells (**Figure VII-7B**). In type IV, the high accumulations of H_2O_2 were recognized in phloem cells (**Figure VII-7A**) at the late period during storage. As shown in **Figure VII-7A and B**, H_2O_2 accumulation in type I reached a peak at 6 weeks and thereafter decreased gradually to the basal levels.



FigureVII-5. The localization of APx in 12 week-stored potato tubers.

(A) The parenchymal cells under epidermis stored potato tissues at 20°C.

(B) The parenchymal cells under epidermis stored potato tissues at 1°C.

(C) Around vascular bundle cells stored potato tissues at 20°C.

(D) Around vascular bundle cells stored potato tissues at 1°C.

(A), (B), (C), and (D) were positive staining using anti-APx antibody.

(a), (b), (c), and (d) were negative staining using anti- rabbit IgG antibody.

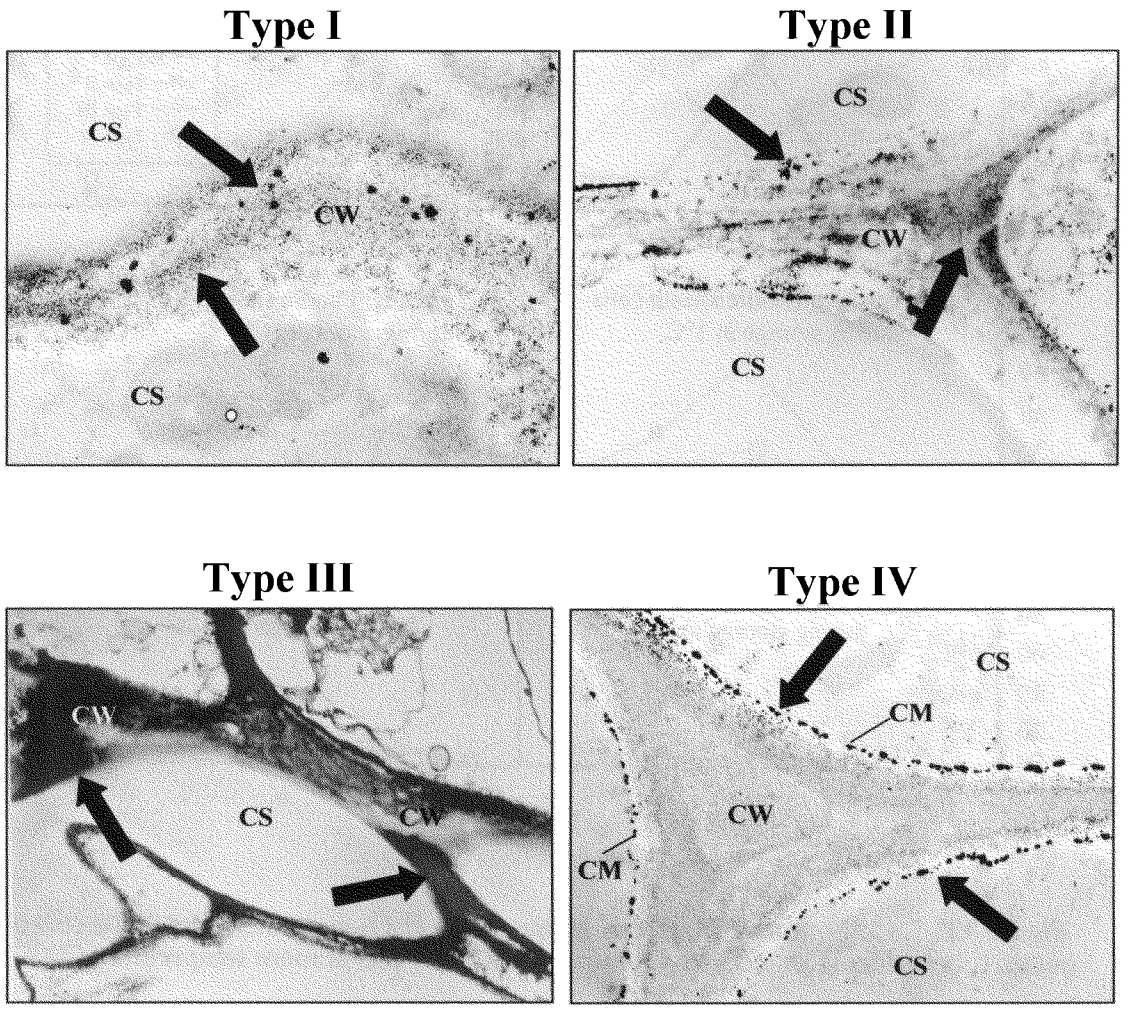


Figure VII-6. The classification of H_2O_2 deposition types.

The arrow indicates the electron-dense deposits of cerium perhydroxides.

Type I, a small volume of electron-dense deposits in cell wall;
Type II, a moderate volume of electron-dense deposits in cell wall;
Type III, a large volume of electron-dense deposits in cell wall;
Type IV, on cell membrane.
CW, cell wall; CS, cytosol; CM, cell membrane.

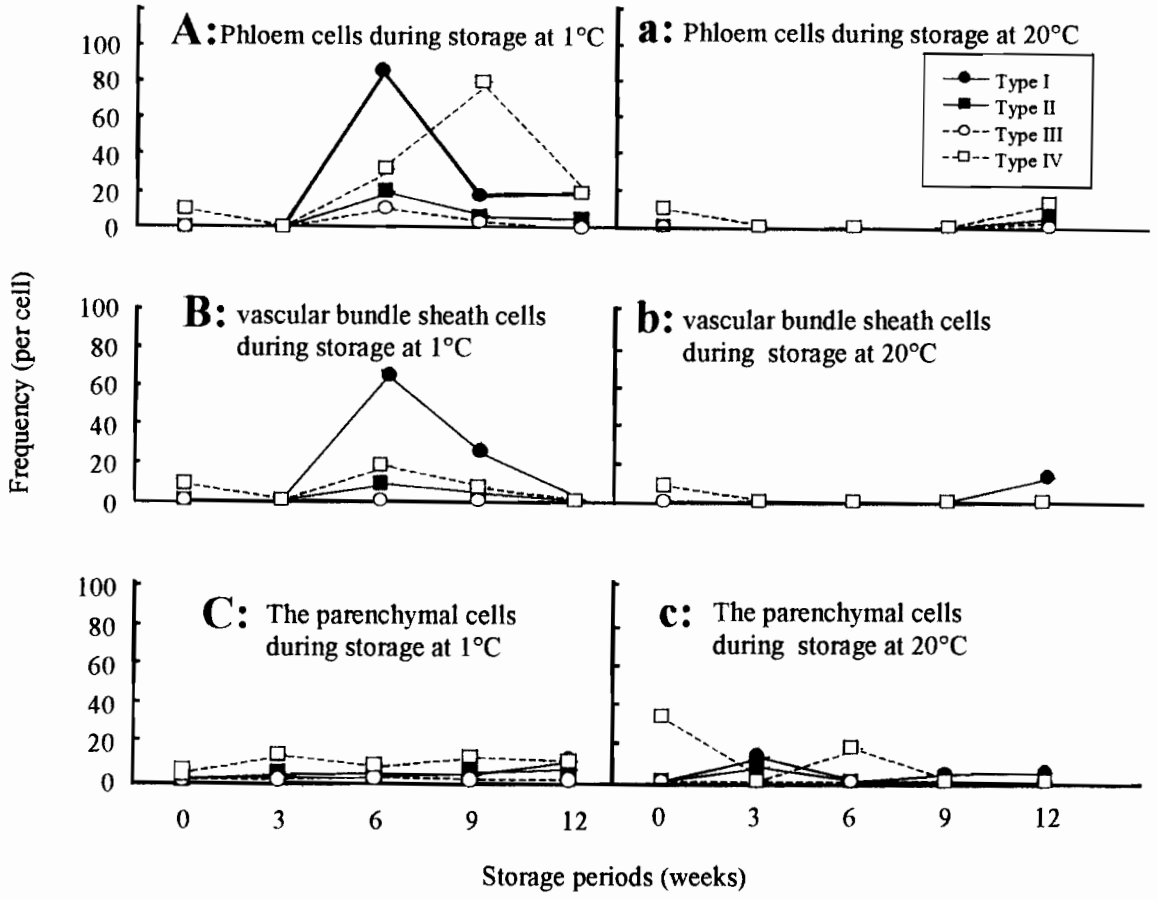


Figure VII-7. Comparative frequency of appearing H_2O_2-generated sites in the cells of potato tubers during stored at low-temperature (1°C) and at room temperature (20°C).

VII-4 DISCUSSION

The aim of this chapter was to examine the cytochemical localization of H_2O_2 and APx in potato tubers during low-temperature storage. **Figure VII-3** showed typical prints prepared from cross-sections of a potato tuber under low-temperature storage. This result was similar to the results obtained by Liu *et al.* (1999). The contents of H_2O_2 were higher in tissue sections prepared from older plants. In addition, phloem, epidermis, and parenchymal cells were compatible with the regions where H_2O_2 markedly accumulated in longer stored potato tubers and the H_2O_2 accumulation was higher in the storage of 1°C than in that of 20°C. However, as it was presumed that significant amounts of H_2O_2 were spontaneously produced in the cell walls at the epidermis for lignin biosynthesis (Schopfer,

1994; Liu *et al.*, 1999), the accumulation caused by low-temperature stress probably occurred in vascular bundle cells. The localization of H_2O_2 was found in cell walls and cell membranes of phloem cells of potato tubers by using the cerium chloride method (**Figure VII-6 and 7**). The contents of H_2O_2 in phloem and vascular bundle sheath cells were increased at 6 weeks and thereafter decreased gradually to basal levels for protracted 6 weeks storage (**Figure VII-7**).

APx, which is an important enzyme to detoxify H_2O_2, was located in the similar region where H_2O_2 was accumulated (**Figure VII-4**). APx expression was at a very high level in vascular bundles at 1°C during 12 weeks storage (**Figure VII-4**) and was remarkably induced by low temperature stress in vascular bundles (**Figure VII-5**). These results are in harmony with the report by Pastori *et al.* (2000). They described the bundle sheathe cells suffered from oxidative damage arising from H_2O_2 accumulation at low-temperature and detoxification of H_2O_2 in virtues of induced APx at low-temperature (Pastori *et al.*, 2000). To be effective, antioxidants must be located at the sites close to the sites of oxidant production and close to sites at high risk of oxidative damage. Therefore, it was presumed that the remarkable expression of APx was induced in the vascular bundle system (including phloem and bundle sheathe cells) by H_2O_2 accumulation.

It has been reported that reactive oxygen intermediates can be formed in the mitochondrial inner membrane by the direct oxidation of the semi-ubiquinone radical by O_2, and that the rate of this reaction increases exponentially as the ubiquinone pool is reduced (Milar and Day, 1997). This seems to suggest that there is the possibility that the site of H_2O_2 generation is the mitochondrial respiration chain. Indeed, measurement of the amount of Q and reduced Q (**Chapter V**) showed that the redox poise of the Q pool tends to be under reduction at a low temperature. Moreover, the activity of Mn-SOD localized in mitochondria was effectively increased by low-temperature (**Chapter VI**). Taking into consideration of these findings, $O_2^{\bullet-}$ will be the first product generated inside mitochondrial inner membrane and then will be converted to O_2 and H_2O_2 by Mn-SOD when potato tubers are stored

at low temperatures. And the generated H_2O_2 whose life time was longer than $O_2^{\bullet-}$ might be defused into cytosol. The evidence that anti-APx antibody is specifically recognized a cytosolic APx leads to the hypothesis that H_2O_2 might be finally detoxified by APx in cytosol of the vascular bundle (**Figure VII-8**).

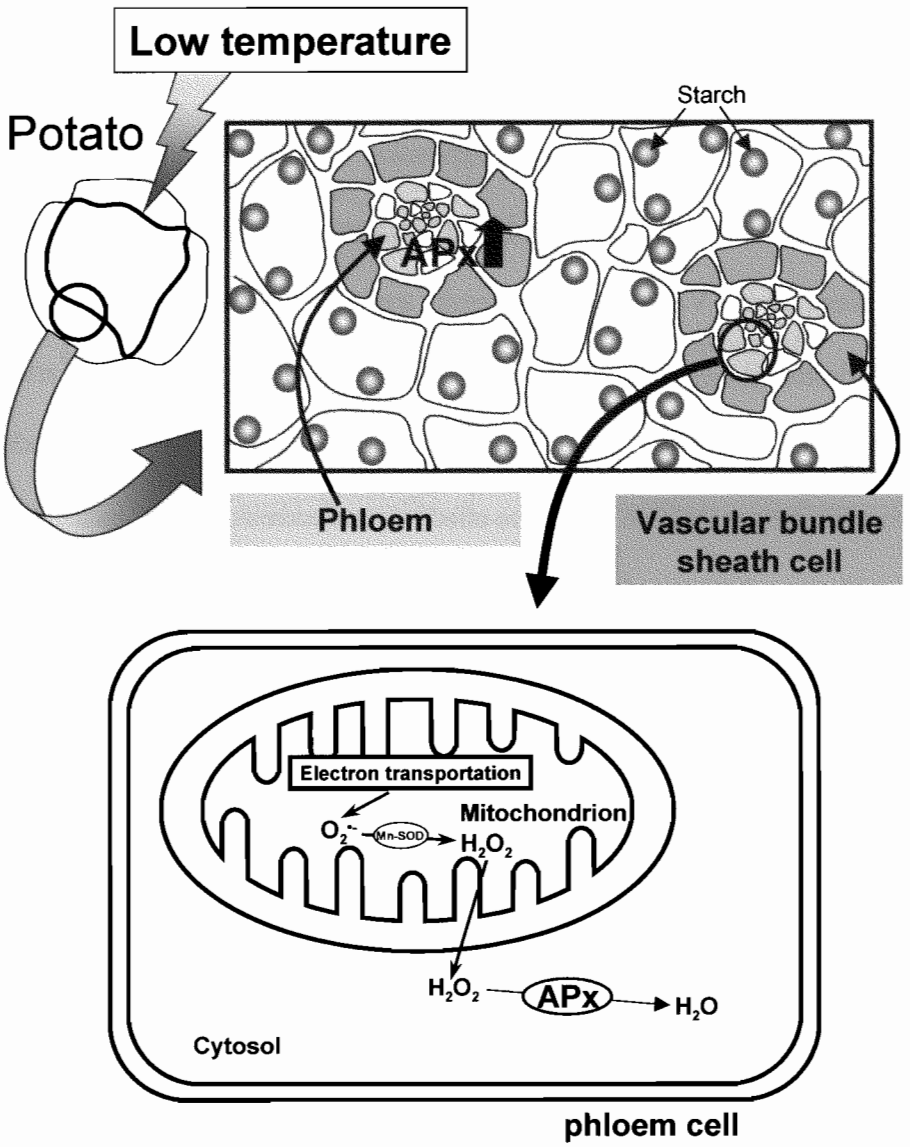


Figure VII-8. Proposal defense mechanism against low temperature stress during storage in potato tubers.

CHAPTER VIII

CONCLUSION

Potato (*Solanum tuberosum* L.) tubers are an important source of vitamin C for many people because much AsA remains in potato tubers even after a long storage time. Low temperature storage of potato tubers can have the beneficial results of lowered respiration rates, slowed physiological aging, inhibition of sprouting, reduced evaporative water loss, and minimized microbial pathogenesis. However, the contents of AsA decreases more rapidly during storage at low temperature than at high temperature. It is well known that reactive oxygen species were generated under low temperature stress, particularly as a chilling injury, and plants have defense mechanisms including antioxidant enzymes against reactive oxygen species. Although potato tubers are said to be insensitive against the chilling injury, the antioxidant enzymes are presumed to play the role in scavenging active oxygen generated during the storage at low temperature.

In **Chapter II** and **V**, the changes in activities of antioxidant enzymes, APx, SOD, and CAT, were investigated during storage of potato tubers between at low and high temperatures. APx was the most sensitive of the antioxidant enzymes at low temperature storage, suggesting that H_2O_2 was generated more at a low temperature (1°C) than at a high temperature (20°C) in potato tubers (**Chapter II**). As it has been reported that APx was inactivated when the concentration of endogenous AsA reached a level less than 30 μM, two potato tuber cultivars with different AsA contents were used to compare the influence of endogenous AsA contents on antioxidant enzymes exposed to low temperature. The 'Kitaakari' and 'Danshaku' cultivars contained high and normal AsA concentration at original levels, respectively. However, The APx activity continued to increase in 'Kitaakari' cultivar throughout the entire low temperature storage period, while it started to decrease after 9 weeks in 'Danshaku' cultivar when endogenous AsA concentration were less than 30 μM at which APx is

inactivated (**Chapter V**). The decrease in the contents of endogenous AsA during storage is considered to be the reason why APx activity showed the tendency of increasing throughout the storage period in 'Kitaakari' and the tendency of decrease after 9 weeks in 'Danshaku'. It is generally believed that MDHA and DHA produced by APx are reduced to AsA by reactions that are catalyzed by MDHAR, DHAR and GR in a series of AsA recycling known as the Halliwell-Asada pathway. It was demonstrated that MDHAR was most sensitive to low temperature. However, the ratio of increase of MDHAR activity was, at its maximum, equivalent to 2% (**Chapter II**) or 1/45-fold (**Chapter V**) of the rate of increase of APx activity, suggesting that the concentrations of endogenous AsA decreased during storage even when the recycling system of AsA was activated by low temperature. Thus, APx is one of the key enzymes against low temperature stress, and its activity depends on an endogenous concentration of AsA in potato tubers.

To investigate the APx response to low temperature stress in potato tubers, in **Chapter III**, a cDNA clone encoding APx was isolated from a potato tuber cDNA library. The full-length clone contains a cDNA that is 1039 bp in length and has an open reading frame of 750 bp coding for 250 amino acids. The sequence was found to have conserved amino acids of APx, including His 42 (active site), His 163 (binding site), Trp 179 (active site), and Asp 208 (active site). The deduced amino acid sequence of APx had high homology with the cytosolic APx (cAPx) from other plant sources and lacked a transit peptide, implying that it encoded a cAPx.

In **Chapter IV**, mRNA and protein levels of APx were investigated to clarify whether APx is produced with *de novo* protein synthesis. APx mRNA levels during storage at 1°C increased 2.5-fold and 1.4-fold in 3 weeks and 6 weeks, respectively, as compared with the initial levels. Levels appeared to decline further during protracted storage, and decreased almost to the initial levels by 15 weeks. Activity staining of APx was low in tubers at zero time storage, and peaked 12 weeks after harvest in tubers stored at 1°C. It increased approximately 3.4-fold during the first 12 weeks of

storage. Thus, the kinetics of their expression closely paralleled the results in the active staining of APx, with a 9-week delay. APx amounts as determined by Western blot analysis using anti-APx antibody correlated well with activity staining of APx. The accumulation of hydrogen peroxide and the induction of APx mRNA expression were also investigated in suspension-cultured potato tuber cells incubated at 5°C. APx mRNA levels peaked at 24 h, and hydrogen peroxide levels started to increase at 6 h, peaking at 24 h. Moreover, APx mRNA levels in the cells stimulated directly with exogenous hydrogen peroxide (100 μM) rapidly increased within 30 minutes. These findings indicate that APx was produced with *de novo* protein synthesis induced by low temperature stress, and detoxified hydrogen peroxide.

It was demonstrated that APx activity was affected by the concentration of endogenous AsA in **Chapter V**. In **Chapter VI**, the influence of endogenous AsA upon APx expression was investigated using the 'Kitaakari' and 'Danshaku' cultivars. In the case of 'Kitaakari' at the high concentration of AsA, APx mRNA levels continued to increase during storage at low temperature. However, in the case of 'Danshaku' at the same concentration of AsA as Touya, its levels increased transiently until 9 weeks and thereafter declined to the almost same level as those at 3 weeks during storage at low temperature. A similar tendency was observed in the activity staining of APx. Thus, expressed APx levels were positively correlated with activity staining of APx. These results demonstrated that APx was regulated by AsA concentration at the level of mRNA expression when potato tubers were stored at low temperature.

In **Chapter VII**, the cytochemical localization of H_2O_2 and APx in potato tuber during low-temperature storage was investigated. The characteristic specific patterns of H_2O_2 distribution in vascular bundle and epidermis were found more drastically at 1°C than at 20°C using tissue printings. Moreover, the localization of H_2O_2 especially in cell wall and the cytomembrane around vascular bundles cell of potato tuber was also detected by TEM using the cerium chloride at low temperature.

APx, which is an important enzyme to detoxify H_2O_2, was interestingly located in the same region (vascular bundle and parenchyma) where H_2O_2 was accumulated as shown by immunostaining with anti-APx antibody using light microscopy. However, H_2O_2 and APx were detected around epidermis without the storage at low temperature, presuming that the remarkable expression of APx could be induced in vascular bundle (especially phloem) where H_2O_2 accumulated.

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