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Chilling Acclimation Induced Changes in the Distribution of H_2O_2 and Antioxidant System of Strawberry Leaves

Yong Zhang, Ya Luo, Yan-Xia Hou, Hao Jiang, Qing Chen and Hao-Ru Tang
Forestry and Horticultural College, Sichuan Agricultural University,
Yaan 625014, People's Republic of China

Abstract: Changes in the contents of H_2O_2, malondialdehyde (MDA) and endogenous antioxidants, the activities of enzymes involving cell defense in leaves of strawberry (*Fragaria×Ananassa* Duch.) plantlets under chilling acclimation and de-acclimation were studied. With the increase of chilling acclimation times, the contents of H_2O_2 and MDA increased to a certain degree and then decreased. The activities of cell defence enzymes, such as Superoxide Dismutase (SOD), catalase (CAT), peroxidase (POD), Dehydroascorbate Reductase (DHAR), Monodehydroascorbate Reductase (MDAR), Ascorbate Peroxidase (APX) and glutathione reductase (GR) were gradually increased to a certain degree and then kept at a high level. And it increased the contents of reduced ascorbate (ASA), dehydroascorbate (DHA) and reduced glutathione (GSH). Our results clearly suggested that cold-hardening triggers an increase of reactive oxygen species (ROS) and the early accumulation of ROS in plants might lead to the production of antioxidant defense system. Based on the obtained results, it can be concluded that the ascorbate-glutathione (ASA-GSH) cycle plays an impotant role in enhancement of chilling resistance of strawberry during chilling acclimation.

Key words: *Fragaria×Ananassa* Duch, chilling acclimation, antioxidant system, ASA-GSH cycle

INTRODUCTION

Low temperature is one of the most important environmental factors that regulate plant growth and development and limit plant production (Cao *et al*., 2002). Plants can respond and adapt to low temperature by several physiological, biochemical and molecular responses (Foyer *et al*., 1998). Many attempts have been made to improve cold tolerance in plants. One of the methods tested is cold acclimation. Over the past several years, much attention has been focused on the studies of physiological and metabolic changes during freezing acclimation, including alterations in lipid composition and soluble sugar concentration, changes in enzyme activities, etc. Although, these studies have been more or less concerned with the increase of chilling resistance and various biochemical responses of plants to low temperature have been widely documented and reviewed, the mechanism of cold acclimation has not been fully understood (Lin and Zhang, 2000; Lin *et al*., 2005).

Many studies showed that low temperature stress could lead to the occurrence of oxidative oxygen species (ROS) and then result in severe stress. This could increase the level of reactive oxidative injury, give rise to lipid peroxidation, membrane deterioration, protein degradation, nucleic acid damage, chlorophyll bleaching and metabolic function disruption (Luo *et al*., 2007). It has been reported that most plants have evolved several cellular mechanisms to prevent or alleviate the damage from ROS during the periods of normal growth and development. The degree of damage depends on the balance between the formation of ROS and their detoxification by the antioxidative scavenging system. Thus, a high level of protective enzymes and antioxidants is essential for the maintenance of the concentration of ROS at a relatively low level, which is required for the survival of plants under low temperature stress (Scebba *et al*., 1998). Many studies showed that cold acclimation caused an increase in the activity of cell antioxidant enzymes, such as Superoxide Dismutase (SOD), peroxidase (POD) and catalase (CAT), which resulted in the enhancement of chilling resistance (Zhou *et al*., 2005). Recently, it has been reported that the increased activities of Ascorbate Peroxidase (APX), Monodehydroascorbate Reductase (MDAR), Dehydroascorbate Reductase (DHAR) and Glutathione Reductase (GR) involved in the ascorbate-glutathione cycle (ASA-GSH cycle) induced by cold acclimation may

Corresponding Author: Hao-Ru Tang, Forestry and Horticultural College, Sichuan Agricultural University, Yaan 625014, People's Republic of China

be essential to protect plants from low temperature stress (Tao *et al*., 1998; Jin *et al*., 2003). Though there are many reports in the literatures detailing changes in the activities of enzymes involved in antioxidant metabolism in response to low temperature stress (Lin *et al*., 2000; Wu *et al*., 2004; Zhang, 2004), moreover little is known about the correlation between the change in the H_2O_2 and malonaldehyde (MDA) contents, the increased level of defense enzymes such as SOD, POD, CAT, APX, MDAR, DHAR and GR as well as endogenous antioxidants (ASA, DHA and GSH), the elevated capacity of the ASA-GSH cycle and the enhanced degree of chilling resistance induced by cold acclimation.

Strawberry (*Fragaria×ananassa* Duch.) is one of nutritious fruits in the world. Chilling injury of strawberry is a serious problem in subtropical cultivated areas. In the present study, we report a more detail study of the effect of short-term cold-hardening on the changes in the contents of H_2O_2, MDA and endogenous antioxidants, the activities of protective enzymes as well as some main enzymes involving in the ASA-GSH cycle. We tried to explore the role of the antioxidant enzymes and ASA-GSH cycle in the enhancement of chilling resistance of fruit trees and to elucidate the physiological mechanism of chilling resistance induced by cold acclimation.

MATERIALS AND METHODS

Plant materials and treatments: The leaves of strawberry cultivar (*Fragaria ananassa* cv. Toyonaka) were obtained from 4-week-old in vitro culture. The culture was divided into three groups, the first group placed at 25°C was referred to as non-acclimated (NA or control) plants. The second group was transferred to the artificial intelligent growth chamber at 0°C for 0, 6, 12, 24, 48 and 72 h during chilling acclimation and was referred to as Chilling-Acclimated (CA) plants and the CA exposed to 25°C for 2 days were referred to as De-Acclimated (DA) plants. Plants of all treatments were grown under a light intensity of 4800lx provided by cool-white fluorescent lamps.

Assay of H_2O_2 and MDA contents: H_2O_2 was extracted and its content was measured by monitoring the absorbance of the titanium-peroxide complex at 405 nm according to the method of Nanjing Jiancheng Bioengineering Institute (NJBI) determination kit. The contents of H_2O_2 was demonstrated with mmol L^{-1}.

The contents of MDA were extracted according to the method of Hendry (1993). Four milliliter of a reaction mixture containing 3 mL of 0.5% TBA and 1 mL of extract, boiling for 10 min and quickly cooled to room temperature. The contents of MDA were expressed as μmol g^{-1} FW.

Assay of SOD, CAT and POD activities: Total SOD (EC 1.15.1.1) activity was assayed by determination kit (Nanjing Jiancheng Bioengineering Institute NJBI). One unit of SOD activity was defined as the amount of enzyme required for 1 mg tissue protein in 1 mL of a reaction mixture SOD inhibition rate to 50% as monitored at 550 nm. The activities of SOD were demonstrated with U mg^{-1} protein.

CAT (EC 1.11.1.6) activity was assayed by determination kit (Nanjing Jiancheng Bioengineering Institute NJBI). One unit of CAT activity was defined as 1mg tissue protein consumed 1 μmol H_2O_2 at 405 nm for 1 sec. The activities of CAT were demonstrated with U mg^{-1} protein.

POD (EC 1.11.1.7) activity was determined specifically with guaiacol at 470 nm following the method of Amako (1994). One milliliter of the enzymes extract were added to the reaction mixture containing 0.855 μL guaiacol solution and 1.355 μL hydrogen peroxide solution in 3 mL phosphate buffer (pH 7.0). One unit of POD activity was defined as 1 mg tissue proteins catalysised 0.01 μmol H_2O_2 for 1 min. The activities of POD were demonstrated with U mg^{-1} protein.

Assay of DHAR, MDAR, APX and GR activities: DHAR (EC 1.8.5.1) activity was assayed directly by following the regeneration of ASA at 265 nm for 1 min in 2 mL of a reaction mixture containing 1.4 mL phosphate buffer (pH 7.0), 0.2 mL of 20 mmol L^{-1} reduced glutathione (GSH) in phosphate buffer (pH 7.0), 0.2 mL of 2 mmol L^{-1} DHA and 0.2 mL enzyme extract (Hossain and Asada, 1984). DHA was prepared freshly and kept at 4°C to avoid oxidation. One unit of DHAR activity was defined as 1 mg tissue proteins catalysised 1 μmol GSH at 265 nm for 1 min. The activities of DHAR were demonstrated with U mg^{-1} protein.

MDAR (EC 1.6.5.4) activity was determined by following the method of Krivosheeva *et al*. (1996). A reaction mixture containing 0.9 mL of 2 mmol L^{-1} ASA in phosphate buffer (pH 7.0), 0.04 mL of ascorbate oxidase (2 units) in phosphate buffer (pH 5.6), 0.03 mL of 2 mmol L^{-1} NADPH in buffer (pH 7.6) and 0.03 mL crude enzyme was used. The consumption of NADPH was monitored by the reduction of absorbance at 340 nm taking 6.2 (mmol $L)^{-1}$ cm^{-1} as the absorbance coefficients. The activities of MDAR were demonstrated with U mg^{-1} protein.

APX (EC 1.11.1.11) activity was determined by following the method of Nakano and Asada (1994). Two milliliter of a reaction mixture containing 1.66 mL of 0.5 mmol L^{-1} ascorbate in phosphate buffer (pH 7.0), 0.26 mL of 2 mmol L^{-1} H_2O_2, both of which were freshly

prepared and 0.08 mL of enzyme extract. One unit of APX activity was defined as 1mg tissue proteins catalysised 1 μmoL ascorbate at 290 nm for 1 min. The activities of APX were demonstrated with U mg^{-1} protein.

GR (EC 1.6.4.2) activity was determined by following the rate of NADPH oxidation as measured decrease in absorbance at 340 nm. The determination of GR activity was determined with the assays kit (Nanjing Jiancheng Bioengineering Institute). One unit of GR activity was defined as 1 g tissue proteins consumed 1mmol NADPH at 340 nm for 1 min. The activities of GR were demonstrated with U mg^{-1} protein.

Assay of ASA, DHA and GSH contents: ASA and DHA contents were determined according to the method of Takahama and Oniki (1992). ASA content was determined by the measurement of the decrease in the absorbance at 265 nm by ascorbate oxidase (1.25 unit mL^{-1}). The DHA content was determined by measuring an increase of 265 nm absorbance by dithiothreitol. The determination of GSH was measured by using the GSH determination kit (Nanjing Jiancheng Bioengineering Institute NJBI).

Statistical analysis: The experiments were repeated at least 3 times with 3 replicates for each. All the data in this study were expressed as means±SD. The data were analysed using one-way analysis of variance and Duncan's multiple range test at the 5% level of significance from the DPS 7.05 package for windows.

RESULTS

Changes in the contents of H_2O_2 and MDA: The effects of chilling acclimation on the contents of H_2O_2 and MDA in strawberry leaves were shown in Fig. 1. Chilling accliamtion led to a continuous increase in the contents of H_2O_2 and MDA within the whole treatment. A significant increase in the contents of H_2O_2 occurred within the first 24 h of treatment. After 48 h of treatment, the contents of H_2O_2 reached the maximum values, which increased by 78%, respectively, compared with the control values. After 48 h of treatment, the contents of H_2O_2 slowly decreased at 72 h, which was significantly higher than that of control (Fig. 1A). After 2 days of de-acclimation at 25°C, a significant decrease was found in the contents of H_2O_2, which were close to the control level (Table 1). The MDA content in all cases had a behavior similar to that found for H_2O_2 (Fig. 1B).

Changes in the activities of H_2O_2- metabolizing enzymes: Activities of H_2O_2-metabolizing enzymes, such as SOD, CAT, POD, DHAR, MDAR, APX and GR in strawberry leaves were shown in Fig. 2 and 3. The time course of 7 defence enzymes activities showed a gradual enhancement with the time during chilling acclimation at 0°C. Activities of SOD, CAT, POD, DHAR, MDAR, APX and GR firstly increased and then had a tendency to decrease when compared to the activity peak. But these were obvious higher than that of control ones (Fig. 2, 3).

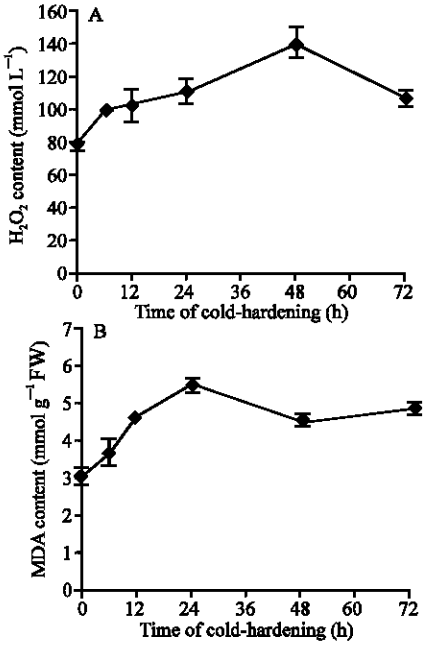

Fig. 1: Changes in the contents of H_2O_2 (A) and MDA (B) of strawberry leaves treated with 0□ for different times. All values are means±SD of three replicates

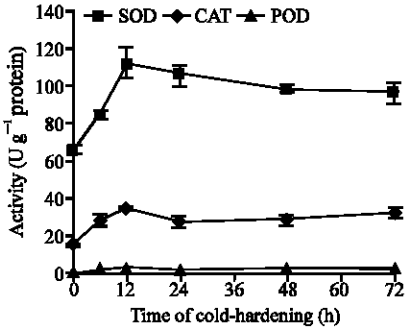

Fig. 2: Changes in SOD, CAT and POD activities of strawberry leaves treated with 0°C for different times. All values are means±SD of three replicates

Table 1: Changes in H2O2 and MDA content, the activities of SOD, CAT and POD in strawberry leaves

Treatments	H_2O_2 content (mmol L^{-1})	MDA content (μ mol g^{-1} FW	SOD activity (U mg^{-1} protein)	CAT activity (U mg^{-1} protein)	POD activity (U mg^{-1} protein)
Control	79.61±3.74	3.12±0.16	69.13±2.67	16.78±0.41	1.04±0.08
CA	105.18±4.63	4.87±0.11	96.39±5.32	32.97±2.98	2.83±0.13
DA	92.63±3.76	3.86±0.10	94.88±4.32	19.18±3.13	2.54±0.05

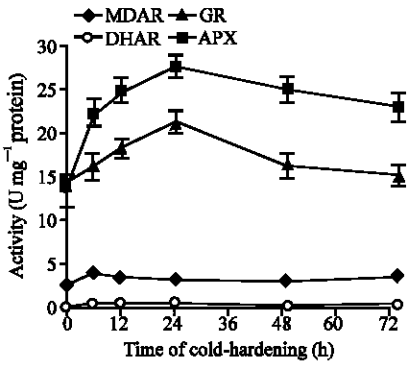

Fig. 3: Changes in DHAR, MDAR, APX and GR activities of strawberry leaves treated with 0°C for different times. All values are means±SD of three replicates

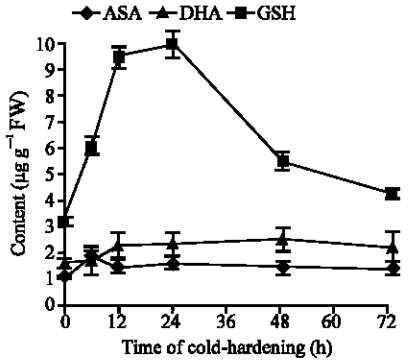

Fig. 4: Changes in the contents of ASA, DHA and GSH of strawberry leaves treated with 0°C for different times. All values are means±SD of 3 replicates

After 72 h of chilling acclimation, 7 enzymes activities in CA plants increased by about 39.4, 96.5, 172.1, 206.7%, 40.2, 55.8 and 6.5%, respectively, compared with those of the control ones. It indicated that different defense enzymes exhibited differently in the increased degree of their activities caused by chilling acclimation at 0°C. However, after 2 days of de-acclimation at 25°C, a significant decrease was found for the activities of all defense enzymes listed above (Table 1, 2).

Changes in the contents of ASA, DHA and GSH: During chilling acclimation at 0°C, there was a significant increase in GSH content of CA plants. After 24 h of treatment, the contents of GSH reached the maximum values, which increased by 220%, respectively, compared with the control values. However, after 72 h of chilling acclimation, the contents of GSH had a tendency to decrease when compared to 24 h treatment, which was significantly higher than that of control (Fig. 4). After 2 days of de-acclimation at 25°C, the contents of GSH returned to about the control level (Table 3). ASA and DHA contents in CA plants gradually increased with time increasing of chilling acclimation during a period of 72 h (Fig. 4). Two days of de-acclimation at 25°C resulted in a decrease in the contents of ASA and DHA, but their contents were close to the control level (Table 3).

Table 2: Changes in the activities of DHAR, MDAR, APX and GR in strawberry leaves

Treatments	DHAR activity (U mg^{-1} protein)	MDAR activity (U mg^{-1} protein)	APX activity (U mg^{-1} protein)	GR activity (U mg^{-1} protein
Control	0.15±0.03	2.54±0.07	14.43±2.56	13.98±0.96
CA	0.46±0.04	3.56±0.17	22.48±1.49	14.89±1.34
DA	0.40±0.21	3.26±0.14	20.33±1.52	14.06±1.45

Table 3: Changes in the contents of ASA, DHA and GSH in strawberry leaves

Treatments	ASA content (μg g^{-1} FW	DHA content (μg g^{-1} FW	GSH content (μg g^{-1} FW
Control	1.07±0.09	1.63±0.27	3.09±0.17
CA	1.29±0.18	2.16±0.61	4.16±0.23
DA	1.14±0.11	1.97±0.35	3.78±0.22

DISCUSSION

Low temperature stress is inevitably associated with increased oxidative stress due to enhanced accumulation of ROS, particularly O_2^- and H_2O_2 in chloroplasts, mitochondria and peroxisomes (Foyer and Noctor, 2005). In the present study, higher H_2O_2 and lipid peroxidation in the leaves of Chilling-Acclimated (CA) plants as compared to that of Non-Acclimated (NA) plants reflected of oxidative stress during the low temperature treatment (Fig. 1). This is in concurrence with our previous study showing higher O_2^- production and membrane damage in CA plants as compared with control ones (Luo *et al*., 2007). High levels of H_2O_2, as observed in the Chilling-Acclimated (CA) plants, led to toxicity and may constitute a signal activating programmed cell death (Vacca *et al*., 2004; Soumen, 2005). The chief toxicity of O_2^- and H_2O_2 is thought to reside in their ability to initiate cascade reactions that result in the production of the hydroxyl radical and other destructive species such as lipid peroxides (Mittler, 2002). Peroxidation of lipids (primarily the phospholipids of cell membranes) was mechanistically important from free-radical production perspective as it was one of the few examples of carbon centered radical production in plant cells (Soumen, 2005). Low temperature stress directly leads to accumulation of ROS and lipids peroxidation in plant cells appears to be initiated by a number of ROS (Zhou *et al*., 2005). Under the consecutive cold-hardening, the contents of MDA were increasing

(Fig. 1B). In parallel, however, relatively lower H_2O_2 levels, as observed during plant stress might be playing a secondary role in stress signaling network by inducing defense pathways (Soumen, 2005). However, the interrelationship between chilling acclimation, ROS and antioxidant defenses in chilling stress signal transduction cascades is still not clear. The effects have been examined systematically of chilling acclimation on the generation of ROS and the activities of several main antioxidant enzymes such as SOD, CAT, POD, APX, DHAR, MDAR and GR in strawberry leaves and the interrelationship among them under a low temperature stress.

Induction of antioxidant defenses differs significantly in the leaves of chilling-acclimated and non-acclimated plants. Higher SOD activity in CA than NA plants during cold-hardening might be one of the reasons for higher H_2O_2 levels in the former than in the latter (Fig. 1A, 2). The ability of plants to overcome oxidative stress partly relies on the induction of SOD activity and subsequently on the up regulation of other downstream antioxidant enzymes and maintenance of ASA-GSH cycle (Alscher *et al*., 2002). Recently dynamic changes in the SOD, CAT and POD enzyme activities in wheat genotypes have been attributed as important antioxidant mechanisms to cope with oxidative stress during water deficit and cold stress (Shao *et al*., 2005). In the present study, the SOD, CAT and POD activities resulted in marked elevation in the leaves of CA plants and also maintained higher activity of these enzymes after de-acclimated (Fig. 2, Table 1). It suggested that enhanced activities of H_2O_2-degrading enzymes caused by chilling acclimation could result in an increased capacity of scavenging H_2O_2, giving rise to the reduction of lipids peroxidation and MDA content, which could be responsible for the elevation of membrane stability and chilling resistance of strawberry. This appears to confirm previous findings that tolerance of plants to low temperature could be related to the activity of the antioxidant system (Massacci *et al*., 1995; Li *et al*., 2000; Wu *et al*., 2004; Luo *et al*., 2007).

A large amount of researches have demonstrated that ASA and APX play critical roles in the ASA-GSH cycle. In this cycle, the detoxification of H_2O_2 is initiated by APX oxidizing ASA to MDHA and then MDHA can be spontaneously disproportionate to DHA. For efficient elimination of H_2O_2, MDHA must be reduced by MDAR with NADPH as a reductant and DHA must be reduced by DHAR with GSH to regenerate to ASA and then GSSH is reduced back to GSH by GR utilizing NADPH as a reductant (Meneguzzo *et al*., 1999; Noctor and Foyer, 1998). It has been suggested that in order to prevent H_2O_2 accumulation to toxic levels during chilling acclimation, an increase in the ASA, DHA and GSH content is necessary to maintain a high capacity of the ASA-GSH cycle (Kocsy *et al*., 2001). In our experiments, it was observed that the contents of ASA, DHA and GSH in CA plants exhibited a gradual increase with the time of treatment. Moreover, the increase in the ASA, DHA and GSH contents caused by chilling acclimation and the decrease in these contents in the recovery periods were in accordance with those of the enzymes (APX, DHAR, MDAR and GR) involved in the ASA-GSH cycle (Fig. 3 , 4, Table 2, 3). It is well known that in most plants subject to various environmental stress, the produced ROS are generally eliminated through a cooperative mediation of protective enzymes (SOD, CAT and POD) and endogenous antioxidants (ASA and GSH) as well as the enzymes (APX, DHAR, MDAR and GR) involved in the ASA-GSH cycle.

CONCLUSION

Chilling acclimation at 0°C could result in the enhancement of SOD, CAT, POD, DHAR, MDAR, APX and GR activities and the increase of ASA, DHA and GSH contents. SOD is an essential component of these defense systems. CAT and POD appeared to play an essential protective role in the scavenging H_2O_2 when coordinated with SOD activity. It seems that the significant increase in the contents of ASA, DHA and GSH and the activities of DHAR, MDAR, APX and GR of strawberry caused by chilling acclimation could result in the increased capacity of the turn-over of the ASA-GSH cycle. These can effectively enhance the capacity of scavenging H_2O_2 and then further give rise to the elevation of membrane stability and chilling resistance of strawberry. Thus, it can be concluded that the ASA-GSH cycle plays an important role in the detoxification of H_2O_2, which is associated with the enhancement of chilling resistance in strawberry.

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