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Changes in the Activities of Antioxidant Enzymes during Chilling Stress in Chilling-Tolerant and Chilling-Sensitive Cultivars of *Cucurbita* spp.

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Abstract. To determine whether antioxidant enzyme systems are related to chilling tolerance, changes of antioxidant enzyme activities during the chilling stress were determined in the leaves of a chilling-tolerant cultivar (*Cucurbita ficifolia*, cv. Heukjong) and a chilling-sensitive cultivar (*Cucurbita moschata*, cv. Jaerae 13). Leaves of chilling-tolerant plant have two major isoforms, Fe-SOD and Mn-SOD, at the Rm values of 0.20 and 0.52, respectively. In leaves of chilling-sensitive plant, two major isozymes of SOD was observed, one isoform is Mn-SOD at the Rm value of 0.20, and the other isoform is Cu/Zn-SOD at the Rm value of 0.58. When plants were treated with chilling stress, Cu/Zn-SOD at the Rm value of 0.58 was newly expressed at 10 days after chilling stress in the chilling-tolerant plants, and density of this band increased at five days after chilling stress in the chilling-sensitive plants. One APX isozyme band was observed in unstressed plants of both cultivars. Under the chilling stress one APX isozyme band was newly expressed at 10 days after chilling stress in the chilling-tolerant cultivar. Significant genotype differences were observed for POD isozyme banding patterns such as four main isozyme bands in chilling-tolerant plants, and one band in chilling-sensitive plants. Densities of three POD isozyme bands at the Rm of 0.36, 0.40 and 0.54 increased at 10 days after chilling stress in the chilling-tolerant plants, while two bands at the Rm of 0.36 and 0.54 increased at 10 days and 20 days after chilling stress in the chilling-sensitive plants, respectively. Activities of SOD, APX and POD significantly increased during five days after chilling stress in both cultivars. In the chilling-tolerant cultivar, activities of these enzymes were higher in chilling-stressed plant than in unstressed plants. However, activities of these enzymes in the chilling-sensitive cultivar decreased rapidly after five days of chilling stress, and were lower in chilling stressed plants than in unstressed plants.

Key words : ascorbic peroxidase, chilling, peroxidase, superoxide dismutase

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Introduction

The oxidative stress induced by active oxygen species due to environmental stresses is the major cause of injury in plants (Asada, 1999; Bowler et al., 1992; Heath, 1987). The active oxygen species, such as hydrogen peroxide, superoxide, hydroxyl radical and singlet oxygen, are present in all plants as cytotoxic compounds, and are mediators of stress response (Foyer, 1993; Lee and Lee, 2000). Although active oxygen species are generated at various levels in growth and developmental process, they are overproduced in response to environmental stresses in all plants (Elstner, 1987). The active oxygen species can cause damage to cellular components such as cell membranes, lipids, proteins, nucleic acids, disrupting metabolic function and leading to injury or death under environmental stress (Fridovich, 1991; Hernandez et al., 1995; Monk et al., 1989; Zhang and Kirkham, 1994). As a defense mechanism complex antioxidant systems composed of non-enzymatic and enzymatic agents are produced in plants (Foyer, 1993). Complex antioxidant systems, especially antioxidant enzyme systems, are very important in protection of cellular membranes and organelles against active oxygen species in plants (Davies, 1995).

A major scavenger in antioxidant enzyme systems is superoxide dismutase (SOD, EC. 1.15.1.1), which converts superoxide anion radicals to hydrogen peroxide and oxygen by disproportionation (Asada and Kiso, 1973; Fridovich, 1986). Many SOD isoforms have been identified, and are known to be differently distributed within the plant (Bowler et al., 1994) and to be distinguishable by their different sensitivity to cyanide and hydrogen per-

oxide. The copper/zinc SOD, which is inhibited by cyanide and H_2O_2, is mainly located in cytosol, chloroplasts, mitochondria, peroxisomes and glyoxisomes in higher plants (Bowler et al., 1992; Longa et al., 1994). Mn SOD is mainly located in the mitochondrial matrix of higher plants (van Camp et al., 1990). Fe SOD, which is inhibited by H_2O_2, is located in chloroplasts of several higher plant species (Almansa et al., 1991; Bannister et al., 1987; Bridges and Salin, 1981). Toxic hydrogen peroxide produced by dismutation of SOD is reduced to H_2O by ascorbate peroxidase or catalase which is the key enzyme involved in hydrogen peroxide scavenging (Anderson et al., 1995; Asada, 1994; Asada and Takahashi, 1987; Salin, 1991). However, reduction of hydrogen peroxide by catalase is less efficient than that by ascorbate peroxidase (Graham and Patterson, 1982).

Chilling stress is one of the most serious problems that affects the productivity of fruit and vegetable crops in temperate and subtropical regions of the world. A number of reports show that chilling stress can induce oxidative stress which can be alleviated by antioxidant enzymes (Prasad et al., 1994; Wise and Naylor, 1987). The objective of this study was to determine the response of antioxidant enzymes in chilling-tolerant and chilling-sensitive cucurbit species under chilling stress.

Materials and Methods

Plant materials and growing conditions

Seeds of chilling-tolerant (*Cucurbita ficifolia* cv. Heukjong) and chilling-sensitive (*Cucurbita moschata*; cv. Jaerae 13) plants were sown in vermiculite, and transferred six days after germination in a pot containing commercial soil mix. Seedlings were grown in a controlled environment growth chamber at 18°C, 12 h photoperiod, and a light intensity of 200 $\mu mol^{-2}s^{-1}$. Plants at the second leaf stage were chilling stressed in a cold chamber at 5/10°C (night/day) with 12 h photoperiod. Control plants were treated at 18/25°C (night/day) with 12 h photoperiod. Samples were collected periodically over a 20 days period.

Total protein extractions

Total leaf proteins were extracted with 100 mM Tris-HCl (pH 8.0) containing 0.1 mM EDTA. Samples (2 g) were homogenized with cold mortar and pestle in a 4.0 mL extraction buffer. The homogenate was centrifuged at 1,500×g at 4°C for 15 min, and the supernatant was collected. The supernatant was passed through a PD-10 column previously equilibrated with the extraction buffer. A total of 3.5 mL was collected from the column and this protein fraction was used for enzyme activity and isozyme analysis. Protein contents were measured according to the method of Bradford (1976) with bovine serum albumin as a standard.

Native PAGE and Isozyme Analysis

The isozymes were seperated electrophoretically in 10 % polyacrylamide slab gels in the absence of SDS. Samples (50 mg of proteins) were applied with a micropipette. Electrophoresis was run using Tris-HCl (pH 8.3) electrode buffer at 4°C for 5 h with a constant current of 30 mA. After electrophoresis the gels were stained for the enzyme activities, and relative mobility (Rm) of each isozyme bands were calculated by measuring the distance from the origin.

Gels were stained for superoxide dismutase (SOD, EC.1.15.1.1) isoforms by equilibrating with 50 mM potassium phosphate (pH 7.8) containing 2.5 mM NBT in darkness for 20 min, followed by soaking in 30 mM riboflavin in the darkness for 30 min. Gels were then exposed to light for 30 min. Identification of SOD isoforms was accomplished by treating the gels with 50 mM potassium buffer (pH 7.8) containing 2 mM KCN as an inhibitor of Cu/Zn-SOD activity or 5 mM H_2O_2 as an inhibitor of Cu/Zn-SOD and Fe-SOD activities for 30 min before staining for SOD activity. Staining of peroxidase (POD, EC.1.11.1.7) isoforms was achieved by incubating the gels in sodium citrate buffer (pH 5.0) containing 9.0 mM r-phenylenediamine and 4.0 mM H_2O_2 for 15 min. Ascorbic peroxidase (APX, EC 1.11.111) activity was detected by incubating the gel with 50 mM potassium phosphate (pH 7.0) containing 2.0 mM ascorbate for 30 min. The gels were incubated in the same buffer containing 4.0 mM ascorbate and 2 mM H_2O_2 for 20 min, and then stained in 50 mM potassium phosphate (pH 7.8) containing 30 mM TEMED and 2.5 mM NBT for 15 min.

Enzyme activity

SOD activity was assayed by the Nitro Blue Tetrazo-

lium (NBT) reduction method (Beyer and Fridovich, 1987). The reaction mixtures contained 50 mM Na-carbonate (pH 10.2), 1.3 mM riboflavin, 13 mM methionine, 65 mM NBT and protein extracts. The reaction was initiated by light illumination in chamber at 25°C for 10 min, followed by measurement of absorbance at 560 nm. Unilluminated samples served as control. The SOD activity was expressed as % inhibition of NBT reduction by mg protein.

The APX activity was measured spectrophotometrically by monitoring the decrease in absorbance of the substrate at 290 nm as ascorbate (extinction coefficient $=2.8 mM^{-1}cm^{-1}$) was oxidized (Chen and Asada, 1989). The reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.5), 0.5 mM ascorbate, 0.2 mM hydrogen peroxide, and enzyme extracts. The reaction was initiated by adding 0.2 mM hydrogen peroxide, and the absorbance decrease was recorded for 1 min at 25°C. Activities are defined as mmol ascorbate oxidized/min/mg protein.

The POD activity was determined spectrophotometrically by monitoring the formation of tetraguaiacol from guaiacol (extinction coefficient at 436 nm=6.39 $mM^{-1}cm^{-1}$) in the presence of hydrogen peroxide (Putter, 1974). The reaction mixtures consisted of 10 mM potassium phosphate buffer (pH 7.0), 0.3 mM guaiacol, 0.1 mM hydrogen peroxide, and enzyme extracts. The reaction was initiated by adding hydrogen peroxide, and the absorbance was recorded for 1 min at 25°C. Activities are defined as mmol guaiacol oxidized.min^{-1}.$protein^{-1}$.

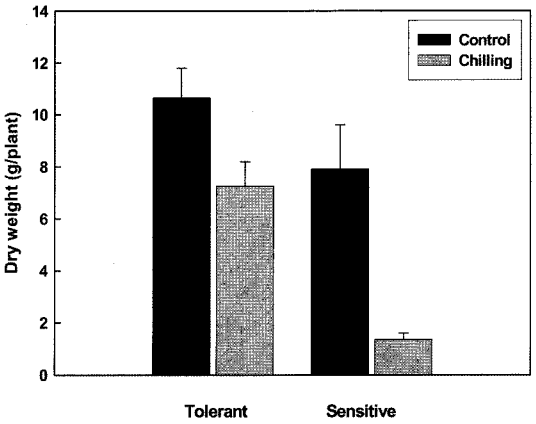

Fig. 1. Dry matter in chilling-tolerant and chilling-sensitive plants. Plants were grown for 20 days at 5/10°C (night/day) and control plants were grown at 18/25°C (night/day). Whole plants were collected 20 days after treatment, and dried at 80°C for 48 h to determine dry weight.

Results

Significant differences in growth and development of chilling-sensitive and -tolerant cucurbit plants were observed in response to chilling. After 20 days of chilling treatment, the growth of chilling-sensitive cucurbit cultivar, Jaerae 13, was severely retarded while that of chilling-tolerant cucurbit cultivar, Heukjong, was not signifi cantly affected (Fig. 1).

Superoxide dismutase (SOD) isozyme banding patterns in the leaves of cv. Heukjong and cv. Jaerae 13 are shown in Fig. 2. In unstressed plants two SOD isoforms were detected with Rm values of 0.20 and 0.52 in chill-

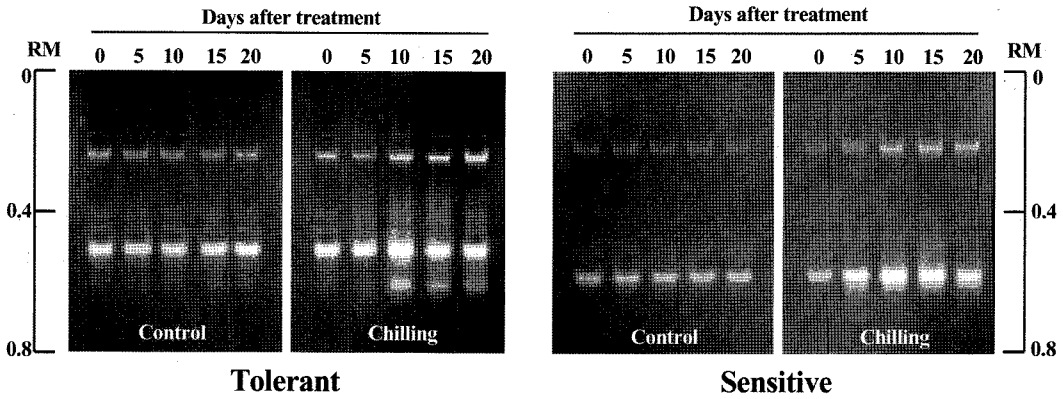

Fig. 2. Superoxide dismutase (SOD) isozymes in leaves of chilling-tolerant cultivar, Heukjong and chilling-sensitive cultivar, Jaerae 13. Proteins were extracted from the unstressed (18°C) as a control and chilling stressed (5°C) leaves. Electrophoresis was conducted using a 10% polyacrylamide slab gel without SDS.

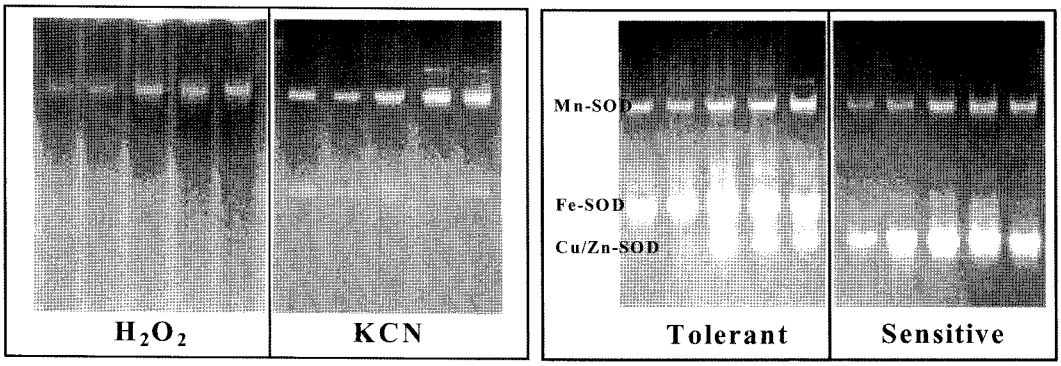

Fig. 3. Identification of SOD isozymes in leaves of cucurbits chilling-tolerant cultivar, Heukjong and chilling-sensitive cultivar, Jaerae 13. Gels were incubated in the SOD staining solutions with 2 mM KCN as inhibitor of Cu/Zn-SOD or 1.5 mM H_2O_2 as inhibitor of Cu/Zn-SOD and Fe-SOD.

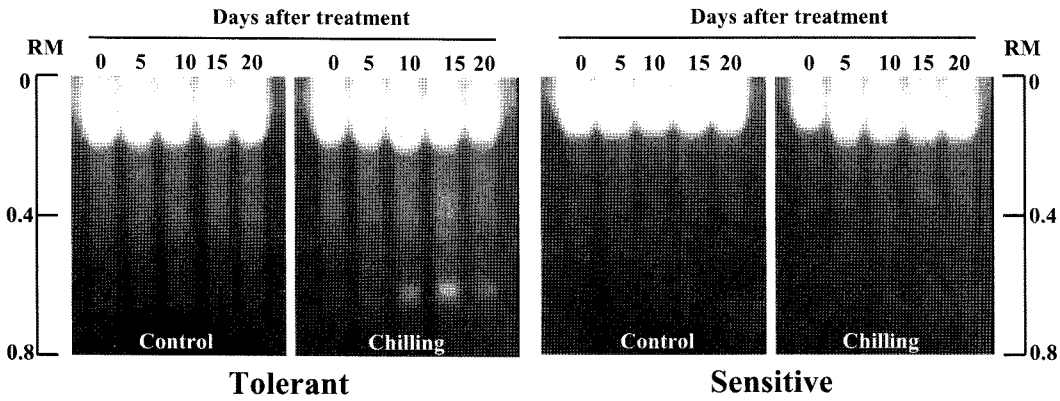

Fig. 4. Ascorbic peroxidase (APX) isozyme activity in leaves of chilling-tolerant cultivar, Heukjong and chilling-sensitive cultivar, Jaerae 13. Proteins were extracted from the unstressed (18°C) as a control and chilling stressed (5°C) leaves. Electrophoresis was conducted using a 10% polyacrylamide slab gel without SDS.

ing tolerant Heukjong and 0.20 and 0.58 in chilling sensitive Jaerae 13.

The SOD isozyme with a Rm of 0.20 was Mn-SOD as it was insensitive to KCN and H_20_2 (Fig. 3). Similarly, enzymes with Rm of 0.52 and 0.58 were identified as Fe-SOD and Cu/Zn-SOD, respectively. Although Mn-SOD was present in both chilling-tolerant and chilling-sensitive cucurbits, Fe-SOD was detected only in chilling-tolerant cucurbit, cv. Heukjong. The Cu/Zn-SOD was not found in unstressed chilling-tolerant cucurbit plants but was activated in response to chilling stress. Similarly, this isozyme showed an increased activity in chilling-sensitive plants as well in response to chilling stress.

There was no significant difference in APX isozyme activity in unstressed chilling-tolerant and chilling-sensitive cultivars (Fig. 4). However, when chilling-tolerant cultivar, Heukjong, was subjected to chilling, a new APX isozyme (Rm-0.60) activity was detected after 10 days of chilling. The chilling-sensitive cultivar, Jaerae 13, did not show any changes in APX isozyme activity in response to chilling.

A number of POD isozymes were identified in both chilling-tolerant and chilling-sensitive cucurbits (Fig. 5). A major difference between these two groups of plants was that three isozymes (Rm-0.36, 0.40 and 0.44) were unique characteristics of chilling-tolerant cultivar. However, the isozyme with Rm of 0.54 was common to both chilling-tolerant and chilling-sensitive cultivars. In chilling-tolerant plants, isozymes with Rm of 0.36, 0.40 and 0.54 dramatically increased their activities after 10 days of chilling treatment, while in chilling-sensitive plants an increase in the isozyme (Rm-0.54) was noted after 10 days of chilling

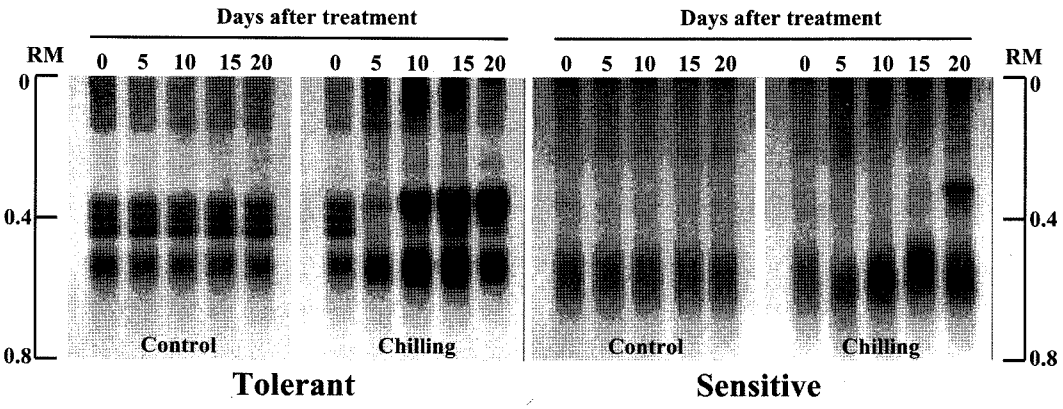

Fig. 5. Peroxidase (POD) isozyme banding patterns in leaves of chilling-tolerant cultivar, Heukjong and chilling-sensitive cultivar, Jaerae 13. Proteins were extracted from the unstressed (18°C) as a control and chilling stressed (5°C) leaves. Electrophoresis was conducted using a 10% polyacrylamide slab gel without SDS.

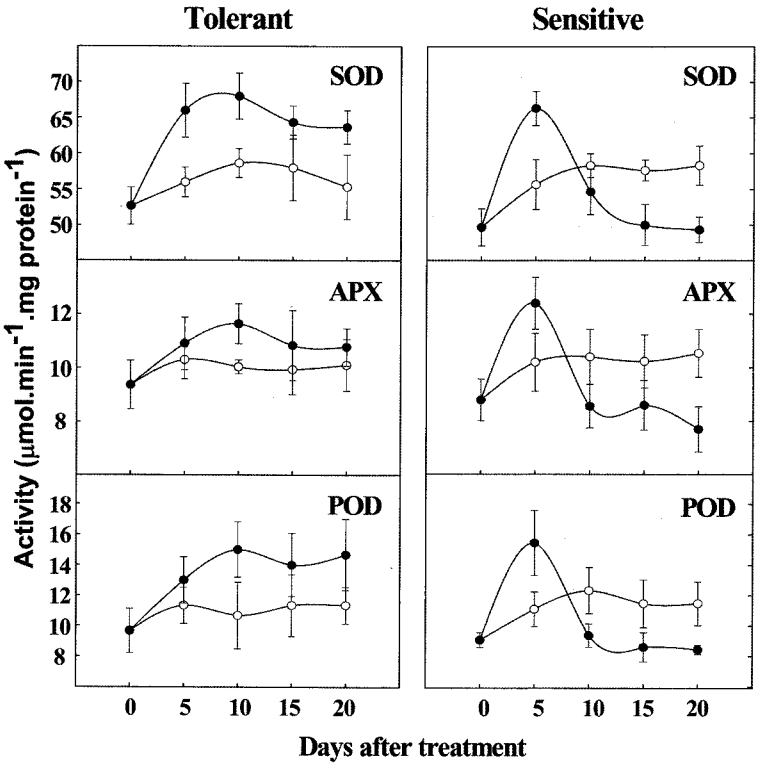

Fig. 6. Changes in leaf-SOD, APX and POD activities of chilling-tolerant cultivar, Heukjong and chilling-sensitive cultivar, Jaerae 13 during chilling stress. Seedlings were chilling stressed (●) in a cold chamber at 5/10°C (night/day) with 12 h photoperiod. Control seedlings were grown (○) at 18/25°C (night/day) with 12 h photoperiod during 20 days. The SOD activities are defined as % inhibition of NBT reduction per mg protein. The APX activities are defined as mmol ascorbate oxidized per mg protein. The POD activities are defined as mmol guaiacol oxidized per min per mg protein.

treatment and also, activity of isozyme (Rm-0.36) was detected after 20 days of chilling treatment.

Activities of antioxidant enzymes in leaves of chilling-tolerant and chilling-sensitive plants were monitored during chilling stress (Fig. 6). The response of SOD, APX and POD was strikingly similar for both groups of plants.

In response to chilling, a significant increase was found in the activities of all the three enzymes in chilling-tolerant cultivar. The activities of these enzymes remained higher than the those in unstressed plants during the 20 days of chilling treatment. However, in chilling-sensitive cultivar there was an initial sharp transient increase in the enzyme activities of all the three enzymes within first 5 days of chilling treatment and thereafter decreased to the levels below those of unstressed plants. Thus, SOD, APX and POD activities in the chilling-sensitive cultivar were actually lower after 8 days of chilling than in the unstressed plants as well as in chilling-tolerant cultivar.

Discussion

Our results show that clear differences in isoforms of antioxidant enzymes and their activities exist between chilling-tolerant and chilling-sensitive cultivars of cucurbit species. The response of SOD isozymes varied in relation to the cultivars and to the chilling treatment. The Mn-SOD activity was found in both chilling-tolerant and chilling-sensitive cultivars while Fe-SOD activity was found only in chilling-tolerant cultivar. Its activity appears to increase slightly in response to chilling. Although it is not clear as to the specific role Fe-SOD in chilling-tolerant cultivar, it was unique to the chilling-tolerant cultivar. On the other hand, Cu/Zn-SOD activity was present in only in chilling-sensitive cultivar under normal growing conditions, and was inducible both in chilling-tolerant cultivar by chilling treatment. In fact, the activity of this isozyme rises in response to chilling even in chilling-sensitive cultivar. In fact, the activity of this isozyme increased with longer duration of chilling, at least in the chilling-sensitive cultivar. This suggests that Cu/Zn-SOD is more responsive to chilling treatment in these cucurbit species and is perhaps associated with susceptibility to chilling injury. However, examination of total SOD activity in the leaves suggests that it increases in response to chilling treatment, albeit in a transient fashion in chilling-sensitive cultivar. In addition, the fact that the SOD activity remains high throughout the chilling treatment in chilling-tolerant cultivar indicates that SOD activity may play a key role in reducing reactive oxygen species in the chilling-tolerant cultivar. A number of studies have shown that chilling tolerance is associated with higher antioxidant activity in many crops including tomato (Walker and Mckersie, 1993), cucumber (Shen et al., 1999), zucchini squash (Wang, 1996), and rice (Saruyama and Tanida, 1995). Fe-SOD activity which was unique to chilling-tolerant cucurbit in our study has been shown to provide protection against oxidative stress in transgenic tobacco plants (van Camp et al., 1996). In addition, other isozymes like Cu/Zn-SOD and Mn-SOD have also been implicated in conferring tolerance against oxidative injury on plants resulting from chilling and freezing stress (Gupta et al., 1993; Clare et al., 1984; McKersie et al., 1993).

With regard to APX, the activity of an isozyme (Rm-0.60) was induced in the chilling-tolerant cultivar after 10 days of chilling treatment. However, in the chilling-sensitive cultivar there was no clear detectable differences in isozyme activity with chilling treatment. Nonetheless, similar to total SOD activity, APX activity increased in response to chilling treatment in both chilling-tolerant and chilling-sensitive cultivars. APX activity in plants has been shown to increase in response to chilling to reduce the harmful active oxygen, specifically, H_2O_2 (Lee and Lee, 2000) and is important to protect plants against chilling stress (Asada, 1992). Three unique POD isozymes were identified in the chilling-tolerant cultivar (Rms-0.36, 0.40, and 0.54) and of these isozymes, the ones with Rm 0.36 and 0.40, and another isozyme (Rm-0.54) were inducible by chilling treatment. Their activities increased significantly after 10 days of chilling in the chilling-tolerant cultivar. However, in the chilling-sensitive cultivar, isozyme with Rm of 0.54 increased its activity rather gradually with chilling treatment. From these results we can conclude that certain POD isozymes may be associated with chilling tolerance and can respond to chilling stress in cucurbit species. However, it is unclear how these specific isozymes contribute to chilling tolerance in cucurbit plants. Nonetheless, the general and sustained increase in activities of SOD, APX and POD suggests that these antioxidants play a key role in reducing injury caused by chilling or conferring tolerance in chilling-tolerant cucurbit species.

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내저온성과 민감성 호박 품종의 저온 스트레스에 대한 항산화효소의 활성 차이

강남준* · 권준국 · 조용섭 · 최영하
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적 요. 저온에 대한 생육 반응이 다른 호박 두 품종 간 항산화효소의 활성 변화를 분석해 본 결과, 저온에 강한 '흑종'의 잎에는 Rm이 0.20인 Mn-SOD와 와 0.52인 Fe-SOD가 주된 밴드였고, 저온에 약한 '재래 13호'의 잎에는 Rm이 0.20인 Mn-SOD와 0.58인 Cu/Zn-SOD가 주된 밴드였다. 저온 처리 후 10일째에 '흑종'의 잎에는 Rm이 0.58인 Cu/Zn-SOD 밴드가 발현되었고, '재래 13호'의 경우 밴드의 밀도가 증가하는 경향을 보였다. APX 밴드 발현 양상은 두 품종 모두 적온 처리에서는 차이가 없었지만, 저온 처리 후 10일경부터 저온에 강한 '흑종'의 잎에서 새로운 APX 밴드가 발현되었다. POD 밴드의 발현 양상은 품종간에 뚜렷한 차이가 있었는데, 적온 하에서 '흑종'의 잎에서는 4개의 주된 밴드가, '재래 13호'의 잎에서는 한 개의 주된 밴드가 발현되었다. 그러나 저온 처리시 '흑종'의 잎에서는 Rm이 0.36, 0.40 및 0.54인 밴드의 밀도가 급격하게 증가한 반면 '재래 13호'의 잎에서는 Rm이 0.36과 0.54인 밴드의 밀도가 증가하였다. 저온에 대한 내성과 관계없이 두 품종 모두 저온 처리 후 초기에는 SOD, APX 및 POD의 활성이 급격하게 증가하는 경향을 보였지만, 저온 처리 후 기간이 경과할수록 품종간 차이가 뚜렷하였다. '흑종'의 잎에서는 이러한 활성이 지속적으로 유지되었지만, '재래 13호'의 잎에서는 저온 처리 후 5일경부터 급격하게 감소하여 적온 처리구보다 낮은 활성을 보여 저온에 대한 품종간 내성 차이를 잘 반영해 주었다.

주제어 : 저온, ascorbic peroxidase, peroxidase, superoxide dismutase