Effect of Cold Treatment on Enzymic and Nonenzymic Antioxidant Activities in Leaves of Chilling-Tolerant and Chilling-Sensitive Cucumber (Cucumis sativus L.) Cultivars

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Summary

Low activity of oxygen radical generation in leaves during and after chilling is implicated in the high chilling tolerance of cucumber (Cucumis sativus L.) cultivars (Shen et al., 1999). In this study, the activity or content of enzymic and nonenzymic antioxidants in leaves were compared between chilling-tolerant 'Jinchun No. 3' and chilling-sensitive 'Suyo', during a 24-hr chilling period at 3 °C in the dark and subsequent 24 hr at 28/22 °C (day/night) under a 12 hr-light, 12 hr-dark photoperiod (rewarming). During chilling, activities of catalase (CAT) decreased, while those of superoxide dismutase (SOD), guaiacol peroxidase (POX) and ascorbate peroxidase (APX) were equal to those in control leaves exposed to 15 °C in the dark for 24 hr in both cultivars. Ascorbic acid (AsA), dehydroascorbic acid (DHA), and reduced glutathione (GSH) increased during chilling in 'Jinchun No. 3' but not in 'Suyo'. During rewarming, however, most antioxidant enzymes increased in their activities in chilled leaves. SOD activity increased more greatly in 'Jinchun No. 3' than it did in 'Suyo'. APX activity also increased greatly in 'Jinchun No. 3' but it did not increase in 'Suyo'. The increases in CAT and POX activities were similar in the two cultivars. AsA, GSH, and oxidized glutathione (GSSG) also increased during rewarming in 'Jinchun No. 3' leaves, whereas in 'Suyo' leaves, DHA increased during early rewarming, but GSH and GSSG did so only toward the end of the rewarming period. The results are discussed in relation to the difference in chilling tolerance between the two cucumber cultivars.

Key Words: antioxidant, ascorbate peroxidase, chilling tolerance, cucumber, superoxide dismutase.

Introduction

Oxygen radical generation is often stimulated in plant tissues exposed to chilling temperature (Wise and Naylor, 1987; Hodgson and Raison, 1991; Shen et al., 1995). This increase in oxygen radical generation may cause enhanced lipid peroxidation, which leads to loss of membrane integrity and eventually to tissue damage.

Previously, it was suggested that chilling injury of cucumber leaves was caused mainly by increased membrane lipid peroxidation, based on the increase in malondialdehyde formation in chilled leaves (Shen et al., 1999). In leaves of chilling-sensitive 'Suyo', NADPH-dependent superoxide-generating activity was initially enhanced markedly, during chilling at 3 °C in the dark for 24 hr, a decline during early rewarming, and a subsequent increase during the late 24-hr rewarming period at 28/22 °C (day/night). NADPH oxidase activity changed similarly to NADPH-dependent superoxide-generating activity. However, these parameters were not enhanced significantly in chilling-tolerant 'Jinchun No. 3' leaves during both chilling and rewarming. These results indicate that chilling tolerance of cucumber cultivars may be determined primarily by the ability of leaves to avoid or prevent the activation of NADPH oxidase under chilling and subsequent rewarming conditions.

Plants can protect themselves against damaging effects of oxygen radicals through the operation of sophisticated defence systems (Salin, 1987). The defence systems include antioxidant enzymes such as SOD, CAT, POX, and APX, and nonenzymic radical scavengers such as AsA, GSH, and tocopherol (Halliwell, 1987). The activity of these oxygen radical scavenging systems has been implicated in the genetic variations in plant tolerance to chilling (Jahnke et al., 1991; Walker and McKersie, 1993; Dipierro and Leonardis, 1997) and other stresses (Leprince et al., 1990; Malan et al., 1990). Therefore, we compared the activity or content of enzymic and nonenzymic oxygen radical scavengers in leaves of 'Suyo' and 'Jinchun No. 3' during chilling and subsequent rewarming periods.

Materials and Methods

Plant material

Cucumber (Cucumis sativus L.) cvs. Suyo (a Japanese cultivar) and Jinchun No. 3 (a Chinese cultivar) were used in this study. 'Jinchun No. 3', bred in Tianjin
Cucumber Research Institute in China, is more chilling-tolerant than is ‘Suyo’ (Shen et al., 1999). The seeds were germinated in vermiculite in the glasshouse, and the seedlings were transplanted at the cotyledonary stage to clay pots containing commercial nursery soil. They were grown in a growth chamber kept at 28/22 °C (day/night). Light was provided for 12 hr daily by metalhalide lamps with 250 μmol m⁻² s⁻¹ PPFD on plant canopy. Air humidity was not controlled; it fluctuated between 60 and 75% RH.

**Chilling treatment and sampling**

One lot of plants with the first expanded leaves was placed in a dark incubator held at 3 °C. The atmospheric relative humidity was kept near 100 % by continuously introducing humidified air into the incubator. Another lot of plants which was placed in another dark incubator held at 15 °C and 60 to 75% RH, served as the control. After 24 hr, the potted plants from both treatments were taken out of the incubators and dipped in a hot water bath for 20 min to raise soil temperature, and then returned to the initial growth chamber.

The first leaves were sampled in triplicate periodically during chilling and rewarming periods. They were stored at -80 °C in tightly sealed plastic vials until analysis.

**Enzyme assay**

Enzymes were extracted from leaves according to Puntarulo et al. (1988); leaves were homogenized at 0 °C in an ice-cold mortar with 50 mM potassium phosphate buffer (pH 7.0) containing 0.5% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 120,000 x g for 20 min in a refrigerated centrifuge. The supernatant was used for assay of antioxidant enzymes.

SOD was assayed with the SOD-Test-Wako kit (Wako Pure Chemical Ind., Japan) according to the manufacturer’s protocol. CAT was assayed on the basis of oxygen evolution from hydrogen peroxide in a Clark type oxygen electrode (Warn and Laties, 1982). Assay of POX was based on formation of tetraguaiacol by peroxidation of guaiacol in the presence of hydrogen peroxide (Kumar and Knowles, 1993). APX was assayed by following the decrease in ascorbic acid in the reaction mixture containing hydrogen peroxide; non-enzymic oxidation of ascorbic acid was subtracted (Nakano and Asada, 1981). All these enzyme activities were assayed at 30 °C. The results are the means of at least 3 analyses.

**Chemical analysis**

AsA and DHA were quantified by the method of Walker and McKersie (1993). The assay was based on the reduction of Fe³⁺ to Fe²⁺ by AsA in acidic solution. Total ascorbate (AsA + DHA) extracted in 10% trichloroacetic acid (TCA) was determined through a reduction of DHA to AsA by ethylmaleimide. DHA content was obtained by subtracting AsA from total ascorbate. GSH and GSSG were extracted in 5% TCA and determined fluorometrically with use of o-phthalaldehyde as a fluorescent reagent (Hissin and Hilf, 1976). The results are the means of at least 3 analyses.

**Results**

**Activities of antioxidant enzymes in leaves**

SOD activity in ‘Jinchun No. 3’ leaves under chilling conditions was not different from that in unchilled control leaves (Fig. 1). However, it increased gradually during rewarming and became twice as high as the control activity after 18 hr of rewarming. SOD activity in chilled ‘Suyo’ leaves was the same as that in control leaves during chilling, with minor exceptions. It increased gradually after rewarming, but did not show the

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Fig. 1. Activities of superoxide dismutase (SOD) in cucumber leaves during chilling and subsequent rewarming periods. Temperature and light conditions ( (): light, (): dark) are shown on the top of the panels. Vertical bars indicate ± S.E. (): 3 °C, (): 15 °C.
sharp increase during later rewarming as observed in 'Jinchun No. 3' leaves.

A significant decrease in CAT activity took place during chilling; 'Suyo' was affected less than was 'Jinchun No.3' (Fig. 2). Following rewarming, however, chilled leaves of both cultivars showed similar increases in CAT activity over their respective controls. POX activity in chilled leaves was low during the chilling treatment in both cultivars (Fig. 3). Following rewarming, it increased exponentially with time in both cultivars; after 18 hr, 'Jinchun No. 3' showed slightly higher activity than did 'Suyo'.

Chilled leaves of both cultivars exhibited similar APX activities to their control leaves during chilling (Fig. 4). However, after the plants were transferred to rewarming conditions, APX activity in chilled 'Jinchun No. 3' leaves increased gradually in the first 12 hr and rapidly during the next 6 hr of rewarming. This characteristic increase in APX activity was not observed in 'Suyo' leaves.

**Content of nonenzymic radical scavengers in leaves**

During chilling, AsA in 'Jinchun No. 3' leaves increased significantly over control leaves, with a larger increase in DHA (Fig. 5). AsA increased further during the first 12 hr of rewarming at a rate higher than that during chilling but decreased to control levels at the end of the next 12 hr. DHA fluctuated in the reverse direction to AsA during rewarming. In 'Suyo' leaves, AsA content was unaffected by chilling, whereas DHA decreased during chilling similarly to control leaves. DHA increased rapidly during the first 6 hr of rewarming, followed by a decline to near control levels during the next 6 hr.
Fig. 4. Activities of ascorbate peroxidase (APX) in cucumber leaves during chilling and subsequent rewarming periods. Temperature and light conditions (□: light, ■: dark) are shown on the top of the panels. Vertical bars indicate ± S.E. ○: 3°C, ●: 15°C.

Fig. 5. Content of ascorbic acid (AsA) and dehydroascorbic acid (DHA) in cucumber leaves during chilling and subsequent rewarming periods. Temperature and light conditions (□: light, ■: dark) are shown on the top of the panels. Vertical bars indicate ± S.E. ○: 3°C, ●: 15°C.

GSH in chilled ‘Jinchun No. 3’ leaves was higher than that in control leaves at the end of chilling (Fig. 6). It increased further during the first 6 hr of rewarming, and stayed high thereafter. GSH in control leaves also increased steadily during the first 12 hr of rewarming, but at a lower rate than did GSH in chilled leaves. In ‘Suyo’ leaves, differences in GSH and GSSG levels between chilled and control leaves were small and inconsistent, but their levels were higher in chilled leaves toward the end of rewarming.

Discussion

Our previous study demonstrated that NADPH-dependent superoxide and hydrogen peroxide generation increased markedly during chilling in chilling-sensitive ‘Suyo’ but not in chilling-tolerant ‘Jinchun No. 3’ leaves (Shen et al., 1999). In the present study, however, antioxidant enzyme activities in leaves under chilling conditions did not differ greatly from those of control leaves in both cultivars, except for CAT activity that was lower in chilled leaves. It seems, thus, that the antioxidant enzyme activities are not involved in the different NADPH-dependent superoxide and hydrogen peroxide generation during chilling in the two cultivars.

Compared to the control, chilled leaves had significantly lower CAT activities assayed at 30°C, indicating the cold lability of this enzyme, which has been demonstrated in many plant species including cucumber (Omran, 1980; Feierabend et al., 1992). Saruyama and Tanida (1995) found with rice plants that
CAT in a chilling-sensitive cultivar was more cold-labile than that in a chilling-tolerant cultivar. Our results disagree with theirs.

On the other hand, AsA increased in 'Jinchun No. 3' leaves with a larger increase in DHA during chilling, and indicated that ascorbate synthesis was stimulated in response to chilling and hastened the conversion of AsA to DHA by a nonenzymic reaction of AsA with a superoxide (Halliwell, 1987). Thus, it seems likely that the chill-induced activation of the AsA/DHA radical detoxifying system contributed in part to the low NADPH-dependent superoxide generation in 'Jinchun No. 3' leaves during chilling.

When the chilled plants were transferred to rewarming conditions, most antioxidant enzymes increased in their activities. It has been suggested that plants suffer from post-chilling oxidative stress when they are transferred suddenly from chilling to warm conditions (Saruyama and Tanida, 1995). Previously, NADPH-dependent superoxide generation was enhanced during a later rewarming period in chilled 'Suyo' but was not in 'Jinchun No. 3' leaves (Shen et al., 1999). Thus, the increase of these enzyme activities could be attributable to a reduction reaction to post chilling oxidative stress to leaf cells.

According to Halliwell (1987) and Salin (1987), superoxide anions produced in chloroplasts and mitochondria are catabolized to hydrogen peroxide by SOD, which in turn is reduced to water by AsA as the electron donor. During this process, AsA is peroxidized to DHA, which is then reduced to AsA mainly by GSH. Reduction of the resultant GSSG to GSH is achieved by an NADPH glutathione reductase (GR). It is known that APX loses its activity in the absence of the electron donor, AsA (Asada, 1992). Therefore, it is important to note that high activities or contents of all components of these radical scavenging systems (the SOD and APX-GR systems) are required for efficient detoxification to occur. If the APX-GR system activity is low, hydrogen peroxide will be converted to highly reactive hydroxyl radicals by a Fenton-type reaction (Halliwell, 1987).

During the early rewarming period, SOD and APX activities in 'Suyo' leaves increased at a rate similar to 'Jinchun No. 3' leaves. However, AsA increased only slightly; GSH did not increase, indicating insufficient operation of the APX-GR system in 'Suyo' leaves to cope with the increased hydrogen peroxide production. Contrarily, chilled 'Jinchun No. 3' leaves exhibited significant increases in all these parameters over control leaves. This is indicative of the efficient interaction between the SOD and APX-GR systems. It was shown previously that the activity of methane sulfonic acid formation, as an indicator of hydroxyl radical generation, increased markedly in chilled leaves of 'Suyo' but did not those of 'Jinchun No. 3' during rewarming (Shen et al., 1999).

If the APX-GR system is operative, DHA should increase at the expense of AsA (Halliwell, 1987). However, DHA in chilled 'Jinchun No. 3' leaves decreased during the first 12 hr of rewarming. That AsA is converted preferentially to monodehydroascorbic acid (MDA) in illuminated leaves, and MDA is reduced back to AsA by MDA reductase was reported by Heber et al. (1996). It is possible that in chilled 'Jinchun No. 3' leaves, the AsA/DHA cycle is replaced with the AsA/MDA cycle during the light period, causing a decrease in DHA. However, this possibility needs further investigation, because DHA increased greatly in
chilled ‘Suyo’ leaves during the same period.

During the later rewarming period, ‘Jinchun No. 3’ leaves increased substantially in both SOD and APX activities but a decrease in AsA and an increase in DHA, GSH and GSSG. Hence, an efficient interaction between the SOD and APX–GR radical scavenging systems may have taken place in chilled ‘Jinchun No. 3’ leaves. According to Saruyama and Tanida (1995), a chilling-tolerant rice cultivar exhibited a larger increase in leaf SOD activity than did a chilling-sensitive cultivar during rewarming of plants chilled at 5 °C in darkness for 7 days. Our results are consistent with theirs.

Activities of CAT and POX also increased in chilled leaves during rewarming. However, their contribution to the difference in chilling tolerance of ‘Jinchun No. 3’ and ‘Suyo’ appears small, because the two cultivars had similar increases in these enzyme activities. Our results for POX do not agree with those of Omran (1980), who found no significant increases in POX activity in chilled cucumber leaves during both chilling and rewarming. The reason for the marked increase of POX activity in our study is not known.

In summary, our study suggests that high chilling tolerance of ‘Jinchun No. 3’ is related to its ability to increase the activity of the SOD and APX–GR radical scavenging systems in chilled leaves after being transferred to rewarming conditions, presumably to cope with post-chilling oxidative stress. Further research is needed to understand the biochemical and molecular mechanisms that regulate the activities of antioxidant defence systems in chilled leaves of cucumber cultivars with varying tolerance to chilling temperature.

Literature Cited


低温耐性の異なるキュウリ品種の葉の酵素的および非酵素的抗酸化活動に及ぼす低温の影響

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摘　要

低温耐性の大きいキュウリ品種‘津春三号’と小さい品種‘四葉’を供試して、葉の酵素的および非酵素的抗酸化活性に及ぼす低温処理（3℃・暗黒・24時間）の影響を調べた。低温処理中は、葉のスーパーオキシドディスムターゼ（SOD）、バーキシターゼ（POX）、アスコルビン酸バーキシターゼ（APX）の活性は、両品種とも15℃・暗黒下での活性と大きな差異がなく、カタラーゼ（CAT）活性は両品種ともに顕著に低下した。しかし、低温遭遇個体を常温（28/22℃・12時間日長）に戻すと（常温回復）、‘津春三号’ではSODとAPXの活性が顕著に増大した。SODとAPXの活性の増大は‘四葉’でもみられたが、増大程度はいずれも‘津春三号’より小さかった。POX活性は両品種とも顕著に増大した。

非酵素的抗酸化活性については、‘津春三号’では、常温回復前半にアスコルビン酸（AsA）の増加とデヒドロアスコルビン酸（DHA）の減少が起こったが、後半にはAsAが減少してDHAが顕著に増加した。また、還元型グルタチオン（GSH）と酸化型グルタチオン（GSSG）も常温復帰前半に増加した。

‘四葉’では、常温回復前半にDHAが顕著に増大したが、AsAはほとんど変化しなかった。また、GSHとGSSGは、常温回復後18時間以降に低温遭遇で高くなった以外は対照区と差がなかった。

これらの結果から、低温遭遇個体が常温に戻されたときに、葉のSOD、APX、AsA、GSHからなるラジカル消去系の活性を高める能力の大きいことが、‘津春三号’の高い低温耐性に関係していると推察される。