PHYSIOLOGIA PLANTARUM 115: 571–576. 2002
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ISSN 0031-9317

Chilling tolerance of maize, cucumber and rice seedling leaves and roots are differentially affected by salicylic acid

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Received 31 January 2002; revised 11 March 2002

Salicylic acid (SA) is one component of a complex signalling pathway that is induced by a number of biotic and abiotic stresses. Exposing seedling radicles to aqueous solutions of 0.5 m*M* salicylic acid for 24 h before chilling at 2.5°C for 1–4 days reduced the chilling-induced increase in electrolyte leakage from maize and rice leaves, and cucumber hypocotyls, but not from their radicles. The SA treatments that induced chilling tolerance in the aerial portion of the seedlings did not induce chilling tolerance in the radicles, even though the SA treatments were applied to the radicles. A comparison of activity among five antioxidant enzymes showed that SA did not alter enzyme activities in the radicles, but that chilling tolerance induced by SA in the aerial portions of maize and cucumber plants was associated with an increase in the activity of glutathione reductase and guaiacol peroxidase.

Introduction

Salicylic acid (SA) has been reported to induce a number of defense responses and increase chilling tolerance in plants. Conversely, exposure to ozone and ultraviolet light stimulated the accumulation of SA and the subsequent acquisition of acquired resistance to pathogens (Yalpani et al. 1994). Salicylic acid appears to be a signal molecule in the induction of certain pathogenesis-related proteins (Metraux et al. 1990). Application of SA induced the synthesis of a number of pathogenesis-related proteins involved in systemic acquired resistance in tobacco plants (Ward et al. 1991, Yalpani et al. 1994). A 24-h hydroponic treatment of 2-week-old maize plants with 0.5 m*M* SA increased their tolerance to chilling-induced increases in electrolyte leakage and changes in chlorophyll fluorescence (Janda et al. 1999). We have previously shown that a number of abiotic stresses (e.g. heat-shock, cold-shock, salinity, and ethanol) also induced tolerance to chilling-induced increase in electrolyte leakage and decreases radicle growth in cucumber seedlings (Jennings and Saltveit 1994b, Rab and Saltveit 1996b). There may be a connection among the ability of an abiotic stress to induce the synthesis of SA, the presence of SA in the tissue, and the increase in chilling tolerance.

Chilling can lead to increased concentrations of toxic oxygen compounds in susceptible tissue (Wise and Naylor 1987, Hodgson and Raison 1991). A number of enzymes participate in protecting plants from oxidative damage (Asada and Takahashi 1987, Hauptmann and Cadenas 1997). Members of the enzymatic antioxidant defense system include superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), phenolic peroxidases such as guaiacol peroxidase (GPX; EC 1.11.1.7), and the ascorbate/glutathione cycle that includes glutathione reductase (GR; EC 1.6.4.2). The superoxide radicle (O^{-}_{2}) is dismutated to H_2O_2 by SOD, and CAT, APX and GPX metabolize H_2O_2 to H_2O. APX requires reduced ascorbate and GPX requires a phenolic compound like guaiacol to function. GR functions in the regeneration of reduced ascorbate after it is converted to monodehydroascorbate by APX.

There appears to be a relationship between antioxidant enzyme activity and chilling tolerance. In chilling

Abbreviations – APX, ascorbate peroxidase; CAT, catalase; DPPH, α,α-diphenyl-β-picrylhydrazyl; GPX, guaiacol peroxidase; GR, glutathione reductase; ROS, reactive oxygen species; SOD, superoxide dismutase.

tolerant cucumber cultivars, the activity of SOD and APX in leaves was higher than in chilling sensitive cultivars (Shen et al. 1999). The activities of CAT, APX and GR were higher in the rind of chilling tolerant mandarin fruits stored at low temperature than in susceptible fruit (Sala 1998). The chilling tolerance of leaves of rice cultivars was closely linked to the cold stability of CAT and APX (Saruyama and Tanida 1995). Reduced activities of CAT, APX, and MDHAR (mono-dehydroascorbate reductase) may contribute to limiting chilling tolerance at the early stages of development in maize (Hodges et al. 1997). We recently showed that increased activity of APX, CAT, and DPPH-radical scavenging activity was positively correlated with chilling tolerance in cucumber (Kang and Saltveit 2001) and rice (Kang and Saltveit 2002) seedling radicles. Treatments that increase chilling tolerance also increase antioxidant enzyme activity.

In the series of experiments reported in this paper, maize, cucumber and rice seedlings were treated with aqueous solutions of SA to see if their chilling tolerance would be significantly affected. Salicylic acid treatments increased the chilling tolerance of the aerial portion of the plants, but not their radicles. Activity of antioxidant enzymes was assayed to see if they correlated with the differentially induced chilling tolerance in aerial tissue and radicles. The SA-induced chilling tolerance in the aerial portions of maize and cucumber plants appeared to be associated with an increase in the activity of GR and GPX.

Materials and methods

Plant material

Maize (*Zea mays* L., cv. Golden jubilee), cucumber (*Cucumis sativus* L., cv. Poinsett 76) and rice (*Oryza sativa* L., cv. M202) seeds were obtained from local vendors. Five g of seeds were imbibed in 1-l aerated water overnight at 25°C. Imbibed seeds were transferred to moist paper towelling overlying capillary cloth that was sandwiched between two 15 × 30 cm Plexiglas plates (6-mm thick) that were held together with rubber bands. The seeds were orientated normally in the radicle down position and the units were held in a vertical position at 25°C in a humid, ethylene-free atmosphere for about 24 h, or until the radicles were about 10-mm long.

Germinating seeds with 10 ± 1-mm long radicles were removed from the large Plexiglas sandwich and gently transferred to moist paper towelling overlying capillary cloth and sandwiched between two 7 × 13 cm Plexiglas plates (3-mm thick) as before. Each smaller plate held 9 seedlings and was treated as a unit of replication. The plates were positioned vertically in white translucent plastic trays and covered with aluminium foil. The trays were either held at 25°C for the initial measurements of radicle growth, or chilled at 2.5°C before being moved to 25°C for the growth measurements. Imbibed cucumber and maize seeds were germinated in moist vermiculite for 4 days at 25°C. After emergence, the seedlings were periodically watered with Miracle-Gro nutrient solution and grown for 2 weeks under fluorescent lights (16-h/8-h light/dark). These seedlings were used for measurement of ion leakage and enzyme activity.

Measures of chilling injury

The extent of chilling injury was measured as ion leakage and radicle growth. The rate of ion leakage was measured from 6-mm diameter maize and rice leaf discs, 15-mm cucumber hypocotyls segments excised 5 mm below the cotyledons, and 20-mm long apical segments of the radicle. Tissue was put in 50-ml centrifuge tubes with 20 ml of 0.3 *M* mannitol and the conductivity of the solution was periodically measured during 4 h of gentle shaking. The tubes were then capped and subjected to three cycles of freezing (− 20°C) and thawing (25°C) before the total conductivity of the solution was measured after 1 h of shaking. The rate of leakage was calculated and expressed as a percent of total conductivity.

Subsequent radicle growth was measured after chilling (Rab and Saltveit 1996a) by a method modified from that previously described (Jennings and Saltveit 1994a). Radicle length was measured with a clear ruler to the nearest mm before and after treatment, after chilling, and periodically during growth at 25°C. The growth measurements for each seedling were regressed over time and the slope and correlation coefficient were calculated.

Application of salicylic acid

The SA solutions were made up in 0.002 N NaOH to facilitate dissolving the SA. The pH of all treatment solutions was adjusted to 6.8 before use. Seeds were imbibed in aerated solution of, and/or grown on capillary cloth moistened with solutions of 0.0–16 m*M* SA.

Preparation of enzyme extract

Radicle tips (1-cm in length) or leaf discs (6-mm diameter) were excised from the seedlings before any treatment (i.e. controls), or after treatment and/or chilling. The 0.5 g FW was homogenized at 4°C in 1 ml of extraction buffer (0.05 *M* Tris-HCl buffer, pH 7.5, 3 m*M* $MgCl_2$, 1 m*M* EDTA, and 1.5% w/v PVPP) with mortar and pestle. The extraction buffer used for the APX assay contained 0.2 m*M* ascorbate. The homogenate was then centrifuged at 25 000 *g* for 20 min and the supernatant was used as the crude extract for the assays of antioxidant enzyme activity.

Enzyme assay

SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of NBT using the method of Dhindsa et al. (1981). The 3 ml reaction mixture contained 50 m*M* phosphate buffer (pH 7.8), 13 m*M* methionine, 75 μ*M* NBT, 2 μ*M* riboflavin, 0.1 m*M*

EDTA, and 0 or 50 µl-enzyme extract. Riboflavin was added last and the tubes were shaken and placed 30 cm below a light bank consisting of two 15 W fluorescent lamps for 10 min. The absorbance by the reaction mixture was read at 560 nm.

CAT activity was assayed by measuring the rate of disappearance of hydrogen peroxide using the method of Maehly and Chance (1959). The reaction mixture contained 2.5 ml of 50 m*M* phosphate buffer (pH 7.4), 0.1 ml of 1% hydrogen peroxide, and 50 µl enzyme extract diluted to keep measurements within the linear range of the analysis. The decrease in hydrogen peroxide was followed as a decline in absorbance at 240 nm.

APX activity was determined according to the method of Chen and Asada (1989) with minor modification. The 1 ml reaction mixture was composed of 50 m*M* phosphate buffer (pH 7.0) containing 0.1 m*M* EDTA, 0.5 m*M* ascorbate, 1.54 m*M* hydrogen peroxide, and 50 µl enzyme extract. The oxidation of ascorbate was followed by the decrease in the absorbance at 240 nm.

GPX activity was determined according to Upadhyaya et al. (1985). The reaction mixture contained 2.5 ml of 50 m*M* phosphate buffer (pH 6.1), 1 ml of 1% hydrogen peroxide, 1 ml of 1% guaiacol and 20 µl enzyme extract. The increase in absorbance at 420 nm was followed for 1 min.

GR activity was assayed by measuring the decrease in absorbance at 334 nm due to the oxidation of NADPH (Klapheck et al. 1990). The 1 ml reaction mixture contained 0.1 *M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 0.1 m*M* NADPH, 1 m*M* GSSG and 50 µl enzyme extract at 30°C.

Protein content was determined using BSA as a standard, according to the method of Bradford (1976). All enzymes activity was calculated per milligram of protein per minute and expressed as a percentage of the control.

Statistical analysis

All experiments were repeated at least twice with similar results. Each of the three replicate per experiment contained 7–10 seedlings for growth measurements and approximately 150 radicle tips for enzyme assays. Data were subjected to an analysis of variance and means and standard deviations were calculated.

Results and discussion

Chilling increased the rate of ion leakage from maize and rice leaf discs and radicles, and from cucumber hypocotyls segments and radicles (Fig. 1). Compared to seedlings treated with water (controls), application of 0.5 m*M* SA for 24 h before chilling did not significantly alter rates of electrolyte leakage from tissues excised from non-chilled seedlings. Also, SA treatments did not significantly alter electrolyte leakage from radicles excised from any of the chilled seedlings. There was a slight reduction in leakage from chilled maize radicles, but although the differences were consistently observed, they were not statistically significant.

In contrast to the lack of an effect on the chilling tolerance of maize, cucumber and rice radicles, the chilling tolerance of leaves or hypocotyls were significantly increased by application of 0.5 m*M* SA (Fig. 1). Chilling-induced electrolyte leakage from excised maize and rice leaf discs, and from excised cucumber hypocotyls segments was reduced by 47% (from 60% of total to 32%), 45% (from 6.0% of total to 3.3%), and 36% (from 25% of total to 16%), respectively.

Response of the tissue to SA depended on the time of application. Cucumber seedling radicle growth was significantly reduced by 0.5–16 m*M* SA solutions when seedlings were imbibed in aerated water and grown on capillary cloth moistened with water until the radicles were 10 ± 1 mm in length and then transferred to small plates made up with the various SA solutions and irrigated with fresh solution every 24 h. When the rate of radicle growth was plotted over the log of SA concentration, the reductions in radicle growth rate [-0.52 ± 0.03 mm (h log mM)$^{-1}$] were linear ($r^2 = 0.98$) for the initial 36 h of growth, and for the next 48 h for the non-chilled seedlings. Chilling reduced the rate of growth over all the SA concentrations [− 0.21 ± 0.05 mm (h log

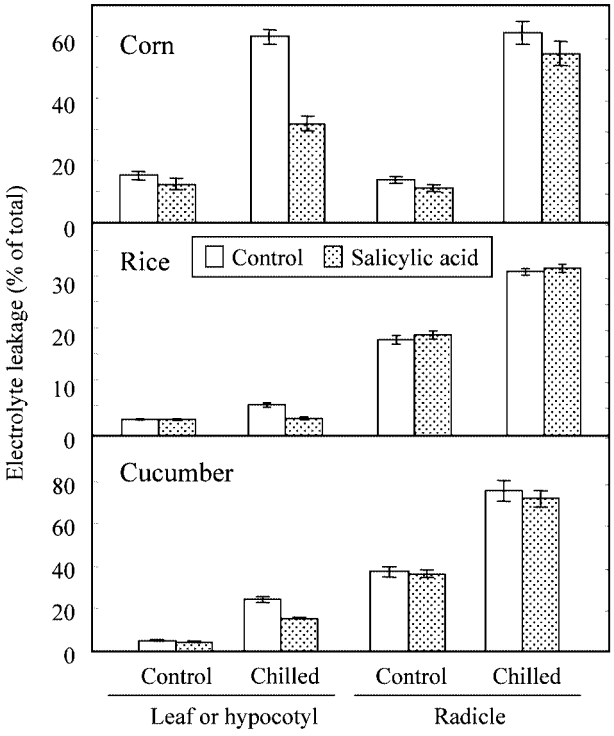

Fig. 1. Electrolyte leakage from excised maize (leaves and radicles), rice (leaves and radicles) and cucumber (hypocotyls and radicles) tissue into an isotonic mannitol solution. Seedlings were treated with 0.0 or 0.5 m*M* salicylic acid 24 h before chilling (maize at 2.5°C for 5 days, rice at 5°C for 1.5 days, and cucumber at 2.5°C for 2 days). Control plants were not chilled. Tissues were excised after 1 day of recovery at 25°C. The vertical line atop each bar is the standard deviation for that mean.

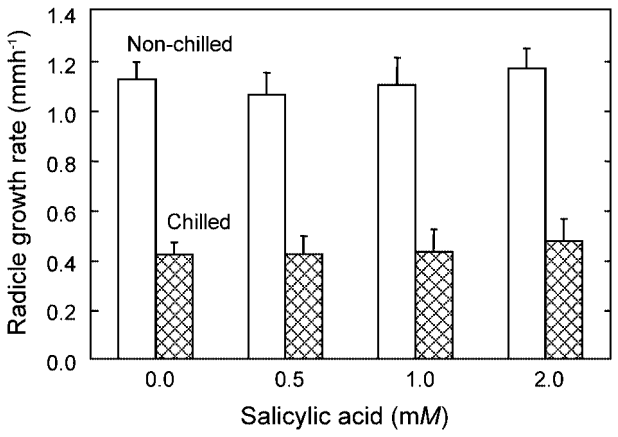

Fig. 2. Rate of cucumber seedling radicle growth for seeds imbibed in, and grown on capillary cloth moistened with 0.0–2.0 m*M* salicylic acid at 25°C, and then chilling at 2.5°C for 0.0 or 4 days followed by growth at 25°C. The radicles were initially 10 ± 1 mm in length. The vertical line atop each bar is the standard deviation for that mean.

m*M*)$^{-1}$] in a linear fashion ($r^2 = 0.94$) for the 48 h after chilling at 2.5°C for 4 days. Obviously, exposure to SA concentrations as low as 0.5 m*M* was a severe shock to the seedlings as indicated by the significant reductions in subsequent radicle growth.

In contrast to this severe inhibitory effect on subsequent radicle growth when the seedlings were exposed to SA solutions after being imbibed in water and grown on capillary cloth moistened with water, was the effect when the seeds were exposed to SA during imbibition and growth. Exposure to aerated aqueous SA solutions during imbibition and growth did not significantly affect the rate of cucumber seedling radicle growth at 25°C (Fig. 2). Seeds imbibed in, and grown on capillary cloth moistened with water had radicles that elongated at a rate of 1.12 ± 0.07 mm h^{-1} during the 72 h of growth at 25°C. Rates of radicle growth were 1.06 ± 0.09, 1.10 ± 0.11, and 1.16 ± 0.08 mm h^{-1} for seeds imbibed in, and grown on capillary cloth moistened with 0.5, 1.0, or 2.0 m*M* SA, respectively. It appears that seedlings exposed to SA from germination may become acclimated to SA and not perceive it as a stress or a stress signal.

Chilling at 2.5°C for 4 days significantly reduced the rate of subsequent radicle elongation 63% from 1.11 ± 0.04 mm h^{-1} for non-chilled seedlings to 0.42 ± 0.05 mm h^{-1} for cucumber seedlings chilled for 4 days at 2.5°C (Fig. 2). Imbibition in, and growth on, capillary cloth moistened with 0.5, 1.0, and 2.0 m*M* SA did not have a significant effect on the chilling-induced reduction in radical growth. The subsequent rate of radicle growth from chilled seedlings was 0.43 ± 0.07 mm h^{-1} across all three SA concentrations.

Results from these experiments indicated that exposure to SA during imbibition and growth did not confer chilling tolerance to cucumber seedling radicles. The reported beneficial effect of SA (Janda et al. 1999) occurred when the roots of 2-week-old hydroponically grown maize plants were immersed in 0.5 m*M* SA for 24 h before chilling. However, our experiments showed that such an exposure to SA by seedlings grown on capillary cloth moistened with water severely reduced their subsequent radicle growth, and that none of the SA concentrations from 0.5 to 16 m*M* conferred any significant level of chilling tolerance to the radicles. The 0.5 m*M* concentration was selected because it gave consistent results and did not cause overt damage to the plants. However, since actively growing tissue is more chilling sensitive than tissue growing less rapidly (Rab and Saltveit 1996a), any treatment that reduced the rate of growth would also confer some level of chilling tolerance. The SA treatment may have been just like any other minor abiotic stress; not great enough to cause visual damage but sufficient to reduce the rate of growth by inducing stress related proteins (e.g. HSPs).

Since the reported beneficial effect of SA (Janda et al. 1999) occurred in a monocot (i.e. *Zea mays*) the experiments were repeated with the monocots maize (a C4 plant) and rice (a C3 plant). No significant effect is observed on the chilling sensitivity of rice radicles from exposures to 0.5 or 1.0 m*M* SA 24 h before chilling (data not shown), from exposure during imbibition (data not shown), or from exposure during imbibition, chilling and growth (Fig. 3). Illustrative of the ineffectiveness of SA is the data presented for the growth of radicles of rice seedlings imbibed in, and grown on, capillary cloth moistened with 0.0, 0.5 or 1.0 m*M* before and after chilling at 25°C (Fig. 3). The non-chilled seedlings had radical growth rates of 0.67 ± 0.05 mm h^{-1} for seedlings imbibed in, and grown on, capillary cloth moistened with 0.0, 0.5, and 1.0 m*M* SA. Chilling for 30 h at 2.5°C reduced subsequent radical growth, and it took almost 48 h before a stable rate of growth was re-established.

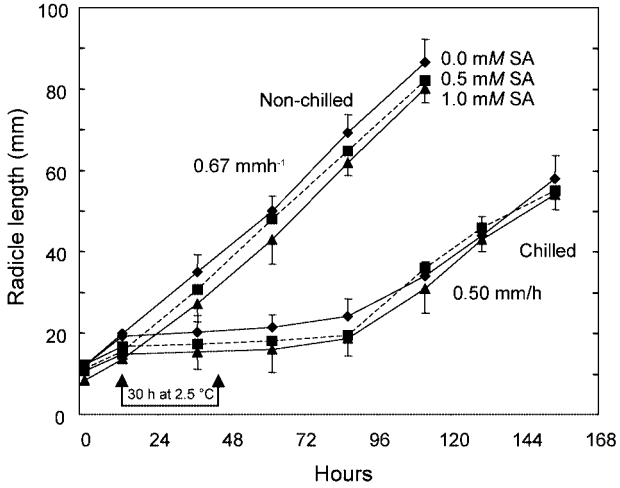

Fig. 3. Rate of rice seedling radicle growth for seeds imbibed in, and grown on capillary cloth moistened with 0.0–1.0 m*M* salicylic acid at 25°C and then chilling at 2.5°C for 0.0 or 30 h followed by growth at 25°C. The radicles were initially 10 ± 1 mm in length. The vertical line associated with each data point is the standard deviation about that mean.

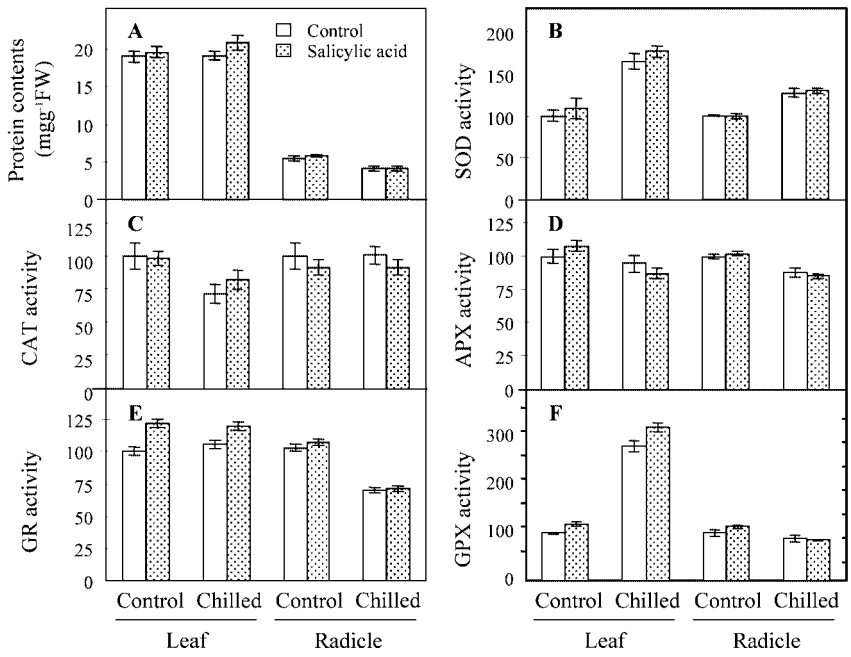

Fig. 4. Effect of chilling at 2.5°C for 5 days and 1 day of recovery at 25°C on protein content and antioxidant enzyme activity of maize seedlings. (A) Protein content; (B) superoxide dismutase activity (SOD); (C) catalase activity (CAT); (D) ascorbate peroxidase activity (APX); (E) glutathione reductase activity (GR); and (F) guaiacol peroxidase activity (GPX). The vertical line atop each bar is the standard deviation for that mean.

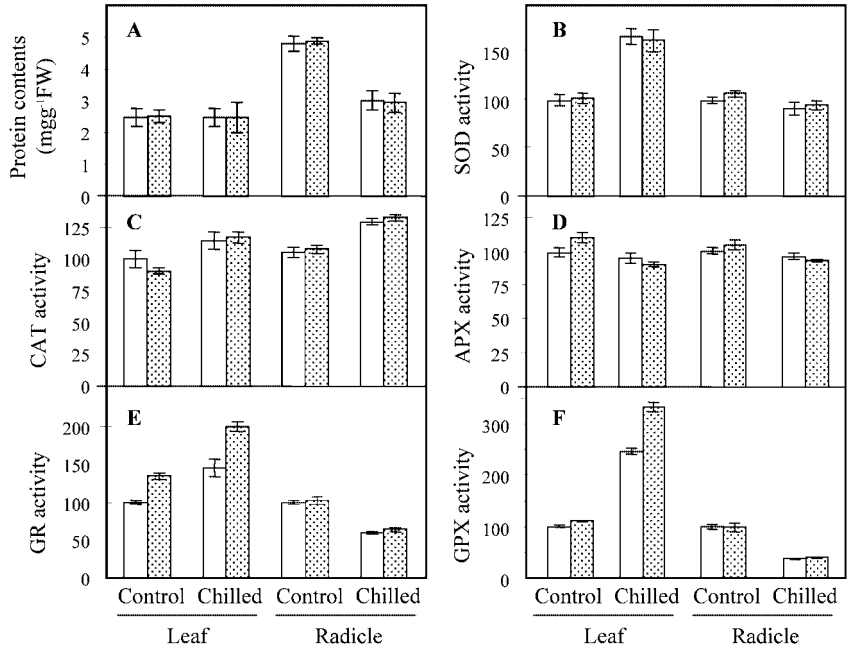

Fig. 5. Effect of chilling at 2.5°C for 2 days and 1 day of recovery at 25°C on protein content and antioxidant enzyme activity of cucumber seedlings. (A) Protein content; (B) superoxide dismutase activity (SOD); (C) catalase activity (CAT); (D) ascorbate peroxidase activity (APX); (E) glutathione reductase activity (GR); and (F) guaiacol peroxidase activity (GPX). The vertical line atop each bar is the standard deviation for that mean.

The stable rate of radicle growth was 0.50 ± 0.06 mm h^{-1} for seedlings growing on capillary cloth moistened with water, 0.5 or 1.0 m*M* SA.

The results with electrolyte leakage (Fig. 1) and radicle growth (Figs 2 and 3) indicated that the ability of SA to induce chilling tolerance is different between aerial and radicle tissue of maize, cucumber and rice: SA induced chilling tolerance in the aerial portion but not in the radicles. Five members of the enzymatic antioxidant defense system (i.e. APX, CAT, GPX, GR, and SOD) were assayed in maize and cucumber aerial tissue and radicles to see if the induced chilling tolerance was correlated with changes in their levels of activity. Chilling had no significant effect on the protein content of maize leaves and cucumber hypocotyls, and maize radicles, but it did cause a significant reduction in cucumber radicles (Figs 4A and 5A).

Chilling had mixed effects on the activity of the antioxidant enzymes. Chilling either had no effect or depressed CAT, APX, and GR activity in maize leaves and

radicles, while it increased the activity of SOD (65%) and GPX (180%) in leaves and SOD (27%) activity in radicles (Fig. 4). In cucumber hypocotyls, chilling increased the activity of SOD (67%), CAT (15%), GR (48%) and GPX (144%), while it increased the activity of CAT (20%) only in radicles (Fig. 5).

Application of 0.5 m*M* SA had no significant effect on the protein content of any of the tissues in any of the treatments (Figs 4A and 5A). Also, SA application did not have a consistent or statistically significant effect on the activity of SOD, CAT, or APX. In maize leaves, SA significantly increased the activity of GR in control (21%) and chilled (14%) tissue, and of GPX in control (21%) and chilled (13%) tissue (Fig. 4E,F). In cucumber hypocotyls, SA significantly increased the activity of GR in control (36%) and chilled (38%) tissue, and of GPX in chilled (36%) tissue (Fig. 5E,F), while having no effect on the activity of these enzymes in radicle tissues (Figs 4 and 5).

In the series of experiments reported in this paper, exposure to 0.5 m*M* SA conferred chilling tolerance to the aerial tissue of maize, cucumber and rice seedling, thereby confirming the report of Janda et al. (1999) who used chlorophyll fluorescence and electrolyte leakage from fully expanded light-grown maize leaves to measure the extent of chilling injury. However, we have additionally shown that the SA treatments that induced chilling tolerance in the aerial portion of the plants did not induce chilling tolerance in the radicles, even though the SA treatments were applied to the radicles. The SA-induced chilling tolerance in the aerial portions of maize and cucumber plants appears to be associated with an increase in the activity of GR and GPX.

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Edited by J. G. Scandalios