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Temperature Preconditioning Affects Glutathione Content and Glutathione Reductase Activity in Chilled Zucchini Squash

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Summary

Preconditioning zucchini squash (*Cucurbita pepo* L., cv. Elite) at 15 °C for 2 d prior to storage at 5 °C reduced chilling injury and altered glutathione content and glutathione reductase activity. When squash without temperature preconditioning treatment was placed at 5 °C, the contents of total glutathione (TG), reduced form of glutathione (GSH), total non-protein thiol (NPSH), and glutathione reductase (GR) activity increased intially for a short period (2 to 4 d) then declined thereafter. In preconditioned squash, the contents of TG, GSH, and NPSH increased even more after the preconditioning treatment, and the amounts of these substances remained higher in the treated squash than in the control after transfer to 5 °C. GR activity was also higher in the preconditioned squash than in the untreated squash. The oxidized forms of glutathione (GSSG) and non-glutathione thiol (RSH) contents were low in zucchini squash and changed only slightly throughout the storage. Consequently, a higher ratio of GSH to GSSG was found in the preconditioned squash than in the control squash. These results indicate that the protection of SH-containing enzymes against oxidation and the detoxification of reactive oxygen species may also be a factor contributing to the effectiveness of temperature preconditioning in reducing chilling injury.

Key words: Cucurbita pepo, chilling injury, glutathione, preconditioning, postharvest physiology, storage, temperature.

Abbreviations: GR = glutathione reductase; GSH = reduced form of glutathione; GSSG = oxidized form of glutathione; NEM = N-ethylmaleimide; NPSH = non-protein thiol; PVP = polyvinylpyrrolidone; RSH = non-glutathione thiol; TG = total glutathione.

Introduction

Most fruits and vegetables of tropical or subtropical origin are injured when exposed to low but non-freezing temperatures (Hardenburg et al., 1986). While the basic mechanism causing chilling injury has not been determined, exposure of sensitive crops to chilling stress may induce toxic molecular species such as free radicals (Hodgson and Raison, 1991; Wise and Naylor, 1987). Free radicals are highly reactive chemically and can induce oxidative breakdown of membrane components leading to tissue injury (Slater, 1972). The capability of a plant to reduce the deleterious effects of free radicals determines its tolerance to such injury. Antioxidants and the enzyme systems involved in their synthesis and regeneration have been shown to help protect plants under environmental stress (Malan et al., 1990; Pell and Reddy, 1991).

Glutathione, a tripeptide thiol compound, is widely distributed in plant cells (Rennenberg, 1982). The reduced form of glutathione (GSH) is an important antioxidant, protecting labile macromolecules against free radical attack (Alscher, 1989). High GSH concentration in cells is maintained by glutathione reductase (GR), which catalyzes the reduction of

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oxidized glutathione (GSSG) (Rennenberg, 1982). A high GSH/GSSG ratio seems to be necessary for the detoxification of activated oxygen species and for the adaptation of plants to environmental stresses such as drought and extremes of temperatures (Esterbauer and Grill, 1978; Halliwell, 1984; Jocelyn, 1972).

Preconditioning of chilling-sensitive fruits and vegetables at temperatures slightly above the critical chilling range increases the tolerance of these crops to chilling injury during subsequent low-temperature exposure and delays the development of injury symptoms (Hatton, 1990). Zucchini squash develop surface pitting when injured by chilling temperatures below 10 °C (Hardenburg et al., 1986). The onset of these chilling injury symptoms can be delayed by preconditioning treatment at 15 °C for 2 d prior to storage at 5 °C (Kramer and Wang, 1989). The relationship between this beneficial treatment and glutathione content in zucchini squash was investigated in this study. The changes of glutathione reductase activity and the ratio of GSH/GSSG during preconditioning and chilling treatments also were examined.

Materials and Methods

Preconditioning treatment and chilling injury evaluation

‹Elite› zucchini squash (*Cucurbita pepo* L.) used for this study were freshly harvested from a farm near Beltsville, Maryland, USA. Samples were selected for uniformity of size (18 to 22 cm long) and were randomly divided into two groups. The first group was placed at 5 °C and served as control. The second group was preconditioned at 15 °C for the first two days and then transferred to 5 °C for the remainder of the study. Three squash fruit were taken daily from each group throughout the storage period for evaluation of chilling injury and for chemical analysis. The degree of chilling injury, as judged by the extent of surface pitting, was evaluated one day after transfer of squash from storage chambers to room temperature by rating on a scale of 1 to 5, with 1 = no abnormality, 2 = trace, 3 = slight, 4 = moderate, and 5 = severe chilling injury. After evaluation of injury, a 5.0-g sample of exocarp tissue was removed from various locations on each squash and lyophilized. The freeze-dried samples were stored at −80 °C prior to chemical analysis and enzyme assay. The experiment was replicated three times.

Measurement of glutathione, non-protein thiol (NPSH), and non-glutathione thiol (RSH)

The lyophilized tissue (400 mg) was pulverized with 12 mL ice-cold, degassed 7.57 mM sodium ascorbate solution with chilled mortar and pestle at 0 °C. The homogenate was filtered through 4 layers of Miracloth and the filtrate centrifuged at 30,000 × g for 15 min at 0 °C. The supernatant was incubated in a water bath at 100 °C for 3 min and then centrifuged at 15,000 × g for 15 min at 0 °C to remove the protein fraction. The supernatant was used for NPSH and glutathione determination. Total NPSH was determined following titration with 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTBN) as described by Ellman (1959).

Glutathione was determined by a modification of the method described by Guy and Carter (1982). Total glutathione (TG) was determined by reacting 0.1 mL extract with 0.125 mM NaH_2PO_4, 0.28 mM DTBN in 200 mM Tris-HCl, pH 8.0, 3 units GR, and 0.5 mM NADPH. Reaction was followed as the change in absorbance at 412 nm over 3 min in a spectrophotometer (Shimadzu UV-160), and the total glutathione content was calculated from a standard curve. Oxidized glutathione (GSSG) was determined after removal of reduced glutathione (GSH) from the sample extract. GSH was removed by mixing 0.4 mL extract with 0.5 mL 1.25 M NaH_2PO_4 and 0.1 mL 100 mM N-ethylmaleimide (NEM) in 125 mM NaH_2PO_4. The mixture was allowed to stand at room temperature for 70 min and then the excess NEM was removed by repeated solvent extraction (5 times) with an equal volume of ether. Residual ether was removed by bubbling a nitrogen stream through the solution. A portion (100 µL) of the resultant solution was assayed for GSSG using the same procedures as described above for TG. GSH was determined by subtraction of GSSG from TG. The value obtained from the difference between NPSH and GSH represents non-glutathione thiol (RSH).

Extraction and assay of glutathione reductase (GR)

The lyophilized tissue (400 mg) was immediately pulverized in a cold mortar and pestle with 12 mL cold 0.1 M Tris-HCl, pH 7.5, containing 0.05 mM EDTA, 10 mM isoascorbate, 40 mg insoluble polyvinylpyrrolidone (PVP) and 400 mg sea sand. The homogenate was filtered through 4 layers of Miracloth and centrifuged at 20,000 × g for 20 min at 0 °C. The supernatant was used for GR assay. The assay mixture contained 50 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 0.15 mM NADPH, 10 mM GSSG and 0.1 mL of crude enzyme extract. The total reaction volume was 1.0 mL. The activity of GR was assayed by monitoring glutathione-dependent oxidation of NADPH at 340 nm (Foyer and Halliwell, 1976). Protein was determined according to Bradford (1976), using bovine serum albumin as a standard.

Results

Effect of temperature preconditioning on chilling injury

Fig. 1 shows the difference in the severity of chilling injury between preconditioned squash and control samples during storage at 5 °C. Surface pitting, a symptom of chilling injury, occurred in the control samples by the 4th d of exposure to 5 °C. These symptoms progressed rapidly with the increase in storage duration. By the 10th d, most of the

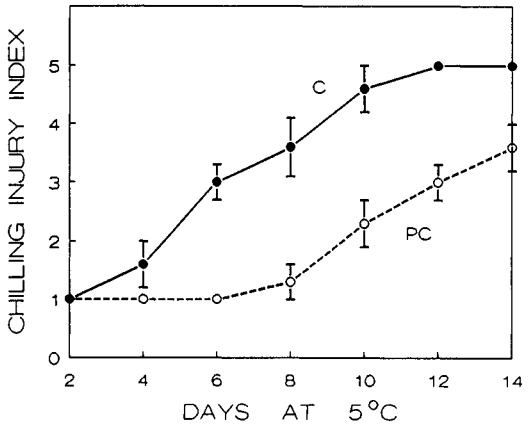

Fig. 1: Effect of temperature preconditioning (PC) on development of chilling injury in zucchini squash. Chilling injury index: 1 = no abnormality, 2 = trace, 3 = slight, 4 = moderate, and 5 = severe. Vertical bars represent SE.

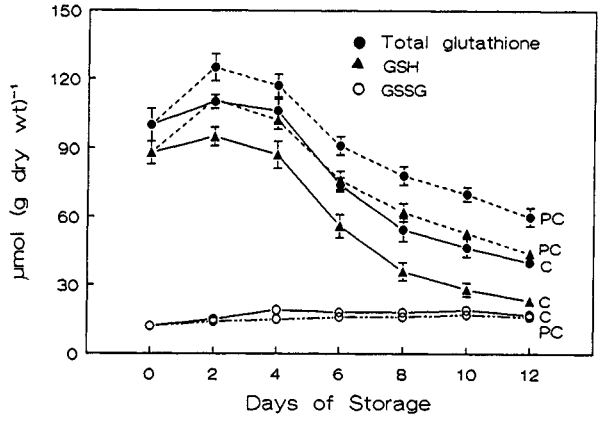

Fig. 2: Effect of temperature preconditioning (PC) on contents of total glutathione and the reduced (GSH) and oxidized (GSSG) forms of glutathione in zucchini squash. The preconditioned squash were stored at 15 °C for the first 2 d and then transferred to 5 °C. The control (C) samples were kept at 5 °C throughout the storage period. Vertical bars represent SE.

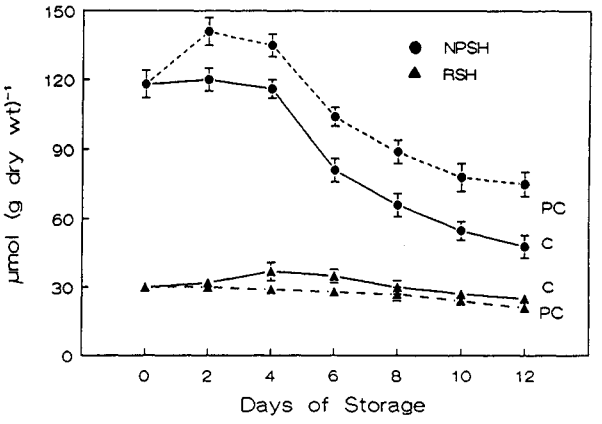

Fig. 3: Effect of temperature preconditioning (PC) on contents of non-protein thiol (NPSH) and non-glutathione thiol (RSH) in zucchini squash. The preconditioned squash were stored at 15 °C for the first 2 d and then transferred to 5 °C. The control (C) samples were kept at 5 °C throughout the storage period. Vertical bars represent SE.

squash had a severe degree of pitting. In comparison, the onset and the development of chilling injury symptoms were delayed by the temperature preconditioning treatment. Surface pitting was not apparent on temperature-preconditioned squash before 8 d at 5 °C, and only slight to moderate pitting was observed on these squash after 14 d of storage at the chilling temperature.

Effect of temperature preconditioning on glutathione content

The major non-protein sulfhydryl compound measured in zucchini squash was GSH, comprising more than 50 % of the total glutathione (Fig. 2). GSSG was also found in zucchini squash, but GSSG content was considerably lower than GSH content in the squash and only changed slightly throughout the storage period (Fig. 2). Total glutathione and GSH contents in the control samples increased slightly during the first 2 d of storage at 5 °C and then declined steadily thereafter. In temperature-preconditioned squash, GSH and total glutathione contents increased more than in control during the preconditioning treatment. They declined after transfer to 5 °C but remained at higher levels than in the control samples throughout the storage period.

Effects of temperature preconditioning on NPSH and RSH

Zucchini squash contain low levels of RSH (Fig. 3), which remained relatively constant during storage. There was no significant difference in RSH content between the control and temperature-conditioned squash. In control samples, NPSH content showed little change at the start of storage and then decreased significantly after 4 d of storage at 5 °C. In preconditioned squash, however, NPSH increased during the first 2 d before starting a gradual decline. A difference between the two treatments was apparent on the second day of storage and this difference persisted throughout the storage period.

Effects of temperature preconditioning on GR and GSH/GSSG ratio

GR activity increased initially upon exposure to 5 °C for 2 d in the control samples, then declined rapidly thereafter (Fig. 4). A similar increase in GR activity was observed after 2 d of preconditioning treatment. After transfer of squash from 15 °C to 5 °C, GR activity in the preconditioned samples showed a further increase for 2 more days before declining. A higher level of GR activity was maintained in the preconditioned squash than in the control samples for the remainder of storage at 5 °C. The ratio of GSH/GSSG was consistently higher in the temperature-preconditioned squash than in the control throughout the storage period.

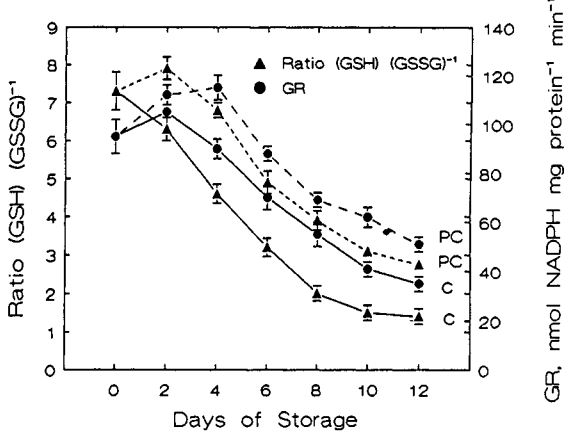

Fig. 4: Effect of temperature preconditioning (PC) on the ratio of GSH/GSSG and glutathione reductase activity (GR) in zucchini squash. The preconditioned squash were stored at 15 °C for the first 2 d and then transferred to 5 °C. The control (C) samples were kept at 5 °C throughout the storage period. Vertical bars represent SE.

Discussion

Glutathione exists in both reduced and oxidized forms in plant tissues (Rennenberg, 1982; Smith et al., 1990). In zucchini squash tissue, most glutathione is maintained in its reduced form (Fig. 2). This is consistent with most other plant tissues. The reduced form of glutathione (GSH) reacts with free radicals including the hydroxyl radical to prevent the oxidation of essential thiol groups (Kosower and Kosower, 1978) and plays an important role as an antioxidant in the stabilization of many enzymes (Halliwell, 1984; Jocelyn, 1972). GSH is also important because of its implication in the enhancement of resistance to environmental stresses (Alscher, 1989). Temperature-preconditioned squash maintained higher GSH levels than the control throughout the storage period (Fig. 2). This indicates that preconditioned squash may have higher capacity for detoxifying destructive O_2 species and therefore having higher tolerance to chilling stress.

In the exocarp tissue of zucchini squash, GSSG content was very low and changed very little after temperature preconditioning treatment and during storage at 5 °C (Fig. 2). This resulted in a higher ratio of GSH/GSSG in the preconditioned squash than in the control. The ratio of GSH/GSSG seems to be even more important than GSH alone in determining resistance to chilling injury. Plants become more susceptible to chilling injury when the GSH/GSSG ratio is low. For example, a dramatic increase in GSSG during exposure to 5 °C predisposed chilling-sensitive cucumber leaf discs to chilling injury, compared with chilling-resistant pea leaf discs in which GSSG did not accumulate at 5 °C (Wise and Naylor, 1987). A high GSH/GSSG ratio could promote protein synthesis through the control of monosome formation (Fahey et al., 1975), activation and inactivation of redox-dependent enzyme systems (Ziegler, 1985), and regeneration of the cellular antioxidant ascorbic acid (Foyer and Halliwell, 1976).

Non-protein thiol (NPSH), which includes GSH and RSH, increased in the first 2 d in preconditioned squash (Fig. 3). Even though NPSH content in preconditioned squash declined during the subsequent storage at 5 °C, the levels remained were higher than in the control. The mechanism of stress injury has been suggested to be related to the thiol group (Levitt, 1980). Stress-induced increased cell permeability may be the result of a loss of membrane thiol. The loss of membrane thiol is due to the oxidation of -SH to -SS groups. Therefore, maintenance of high levels of thiols can effectively protect plants from stress injury (Levitt, 1980). High levels of NPSH in temperature preconditioned zucchini squash may prevent the formation of irreversible sulphur bonds and keep proteins in a viable active state, thereby increasing chilling tolerance.

Temperature preconditioning treatment maintained higher GR activity in zucchini squash than in the control (Fig. 4). GR catalyzes the NADPH-dependent reduction of GSSG to GSH and maintains the high GSH/GSSG ratio in plant tissues (Gamble and Burke, 1984; Jocelyn, 1972). It is apparent that temperature preconditioning treatment increased the antioxidant activity in zucchini squash and produced a protective function against free radical attack. This probably has contributed to the increase of resistance of preconditioned squash to chilling stress.

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