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Responses in Osmolyte Accumulation to Chilling Stress in *Cucurbits* Plants

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Abstract. An accumulation levels of osmolytes in chilling-tolerant and chilling-sensitive cultivar of *Cucurbits* against chilling stress were determined during chilling stress. Total soluble sugar contents in tolerant cultivar did not changes for 10 days after chilling stress, but then slightly increased 20 days after chilling stress. In sensitive cultivar, it was increased rapidly in the beginning of chilling stress, and increased 3.4 times as much 20 days after chilling stress as compared with unstressed plants. Proline contents in tolerant cultivar was rapidly increased by the beginning of chilling stress, and then increased 26.6 times 20 days after chilling stress as compared with unstressed plants. In sensitive cultivar, it was increased 22.0 times 20 days after chilling stress as compared with unstressed plants. A levels of glycine betaine (GB) in tolerant cultivar increased 1.9 times as much during the 20 days of chilling stress. However, concentration of GB in sensitive cultivar did not change during the chilling stress. When plants were treated exogenous GB as a foliar spray, chilling tolerance was significantly enhanced in both cultivars. The foliar application of exogenous GB was induced chilling tolerance by accumulation of GB in the plant organs. However, it does not accumulate endogenous proline.

Key words : chilling stress, glycine betaine, proline

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Introduction

The accumulation of osmolytes during environmental stresses including cold, salinity and drought has been reported in many crops (Hanson and Wyse, 1982; Hitz et al., 1982; Koster and Lynch, 1992). Osmolytes typically considered as osmoprotectant or cryoprotectant consist of soluble sugars that accumulate in response to cold (Koster and Lynch, 1992; Perras and Sarham, 1984), proline and GB that accumulate in response to drought, excess salts, and cold in a number of plants (Aspinall and Paley, 1981; Kishitani et al., 1994; Naidu et al., 1991; Robinson and Jones, 1986). Compatible solutes can regulate cellular osmotic balance, and are also known to stabilize the structure and function of macromolecules such as proteins, enzymes, and organelles (Gorham, 1995; Murata et al., 1992; Rhodes and Hanson, 1993). Thus, they are believed to play a key role in providing protection against these stresses. In fact, exogenous application of GB has been shown to induce tolerance to freezing stress in strawberry (Rajashekar et al., 1999) and *Arabidopsis* (Xing and Rajashekar, 2001) and offer protection against water stress in *Phaseolus vulgaris* (Xing and Rajashekar, 1999). Chilling stress is common in most vegetable and fruit growing regions, especially in temperate and subtropical areas of the world. As with most vegetable crops, the growth and development of *Cucurbits* are highly sensitive to chilling temperatures and are injured by temperatures between 0 and 12°C. Although there have been many studies dealing with the role of osmolytes in a wide variety of stresses including freezing, their possible role in chilling has not been clearly understood. Therefore, in the present study, we have characterized osmolyte accumulation in response to chilling in chilling-sensitive and chilling-tolerant species of *Cucurbit* to elucidate the role of these osmolytes in chilling tolerance. In addition, we have examined the effects of exogenous GB on alleviating chilling injury and on the osmolyte accumulation in chilling-sensitive and chilling-tolerant *Cucurbit* plants.

Materials and Methods

Plant materials and growing conditions

Seeds of chilling-tolerant cultivar (*Cucurbita ficifolia* cv. Heukjong) and chilling-sensitive cultivar (*Cucurbita moschata* cv. Jaerae 13) were sown in vermiculite, and seedlings (6 days old) were transferred to a pot containing commercial soil mix. Seedlings were grown in a growth chamber at 18°C, 12 h photoperiod, and a light intensity of 200 $\mu mol^{-2} \cdot s^{-1}$. At the second leaf stage, plants were chilling stressed in a cold chamber at 5/10°C (night/day) with 12 h photoperiod. Control plants were grown at 18/25°C (night/day) with 12 h photoperiod. Leaf samples were collected periodically for osmolyte analysis over a 20 days period.

Foliar application of glycine betaine (GB)

Plants were sprayed with 2 mM GB solution containing 0.1% Tween-20 at the second leaf stage. Foliar application was made on plants that were incubated at 10 for 6 h to facilitate better absorption of GB by the leaves. Plants were subjected to chilling in a cold chamber at 5 with 12 h photoperiod for 15 days. Control plants were sprayed with distilled water containing 0.1% Tween-20, and then treated chilling stress. Leaf samples were collected after 15 days of treatment and washed in distilled water before analysis for osmolytes.

Analysis of soluble sugars

Total soluble sugars were measured according to the methods of Nelson (1944). Whole leaves were cut into small segment, dried at 80°C for 48 hours, and ground into a fine powder in a mill. Samples (50 mg) were extracted with aliquots of 1 mL of 80% EtOH. The samples were incubated in a water bath at 80°C for 30 minutes, centrifuged and the supernatants collected. This extraction was repeated twice and the supernatants combined. Reaction mixture consisted of 1 mL extract and 2 mL of working solution (1 g anthrone in 500 mL H_2SO_4). Reaction mixtures were incubated at 80°C for 7 minute 30 second, and then cooled under running tap water. The absorbance was read at 620 nm (Beckman, Du-600). The concentration was determined from a standard curve.

Analysis of proline

Proline contents were determined according to the procedure of Bates (1973) with some modifications. Leaf samples (5 g) were homogenized in 10 mL of 3% sulfosalicylic acid and centrifuged at 5,000 g for 10min. The homogenate (2 mL) was mixed with 2 mL of acid ninhydrin and 2 mL of glacial acetic acid in a test tube for 1 h at 100°C, and the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 mL toluene, and the chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance was read at 520 nm using toluene for a blank. The proline contents was determined from a standard curve and calculated on a fresh weight basis.

Analysis of glycine betaine (GB)

Glycine betaine was extracted according to the procedure of Jones et al. (1986). One gram of leaf sample was homogenized in liquid nitrogen, and 5 mL of 2 N H_2SO_4, and incubated with slow shaking at 25°C for 2 h. After incubation, the homogenates were centrifuged at 9,600 rpm at 25°C for 15 min, and the supernatants collected. The pellets were further extracted with 2.5 mL 2 N H_2SO_4, and the supernatants combined. The supernatants were filtered through a paper (Whatman No. 2), and the filterate was incubated with 1.2 mL cold KI-I_2 reagent (40% of total supernatant volume) in an ice bath for 80 min. The filterate was centrifuged with 4,300 rpm at 4°C for 30 min. The pellet was collected and dried at room temperature for over night. The peroidide crystals were dissolved in 0.6 mL of 0.5 mM t-butanol containing CD_3OD (EXPAND), and determined using 500 MHz 1H-NMR spectrometer (Varian).

Results

As expected chilling-sensitive plants was severely affected by chilling, its growth and development were drastically retarded while very few signs of chilling injury were noticeable in chilling-tolerant plants. Dry weight and leaf area were reduced in chilling-sensitive plants by 81.3% and 93.3% compared to those in the unstressed control plants after 20 days of chilling (Fig. 1).

Although soluble sugars accumulated in response to

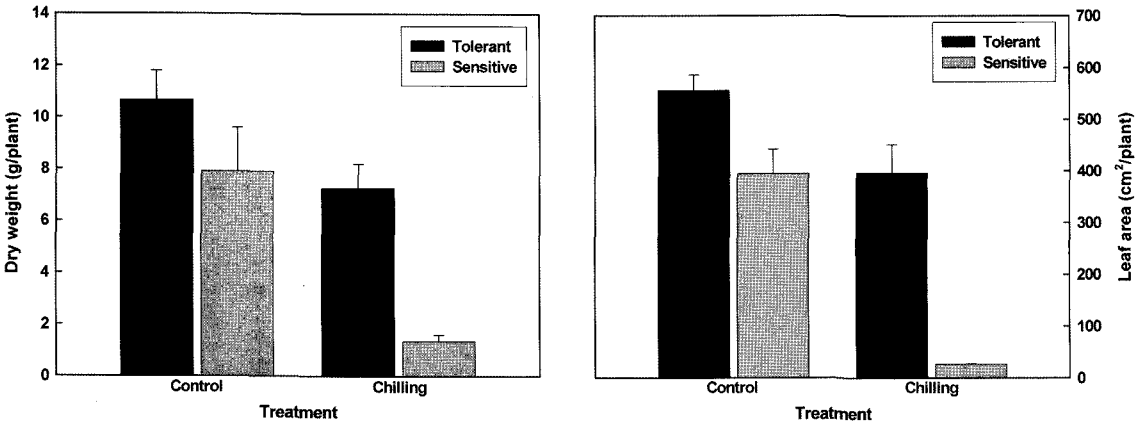

Fig. 1. Comparison of dry matter and leaf area in chilling-tolerant and chilling-sensitive cultivar of *Cucurbits* during chilling stress. Plants were grown for 20 days at 5/10°C (night/day), and control plants were grown at 18/25°C (night/day).

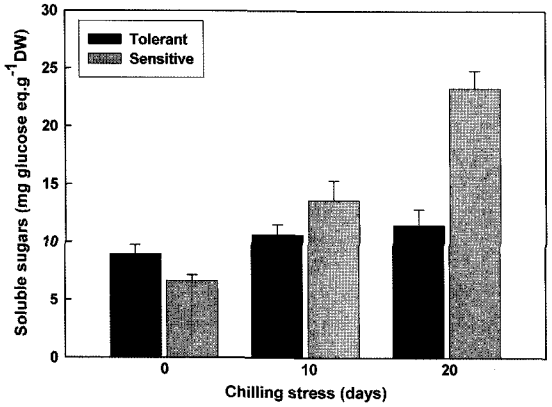

Fig. 2. Changes in soluble sugar accumulation in the leaves of chilling-tolerant and chilling-sensitive cultivar of *Cucurbits* during chilling stress. Plants were chilling stressed at 5/10°C (night/day) with 12 h photoperiod for 20 days.

chilling in both chilling-sensitive and chilling-tolerant plants, the accumulation was much higher in chilling-sensitive than in chilling-tolerant after 20 days of chilling. The level of soluble sugars in chilling-sensitive plants was more than twice as much as in chilling-tolerant (Fig. 2).

The increase in proline levels in both groups of plants was dramatically increased during chilling stress (Fig. 3). However, a levels of in chilling-tolerant plants were more responsive than the chilling-sensitive plants. The chilling-tolerant plants had higher levels of proline, and they accumulated more than twice as much as the their chilling-sensitive counterparts in response to 20 days of chilling,

There was a clear difference in GB response to chilling between chilling-sensitive and chilling-tolerant plants (Fig.

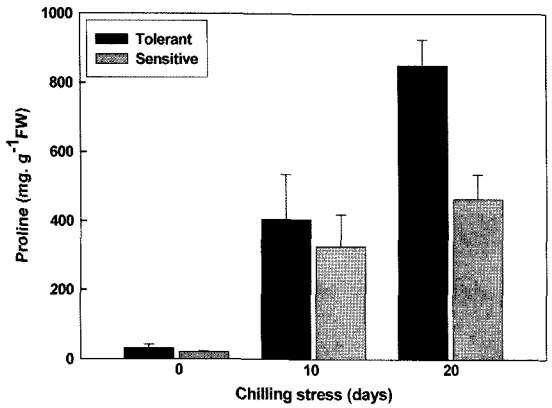

Fig. 3. Changes in proline accumulation in the leaves of chilling-tolerant and chilling-sensitive cultivar of *Cucurbits* during chilling stress. plants were chilling stressed at 5/10°C (night/day) with 12 h photoperiod for 20 days.

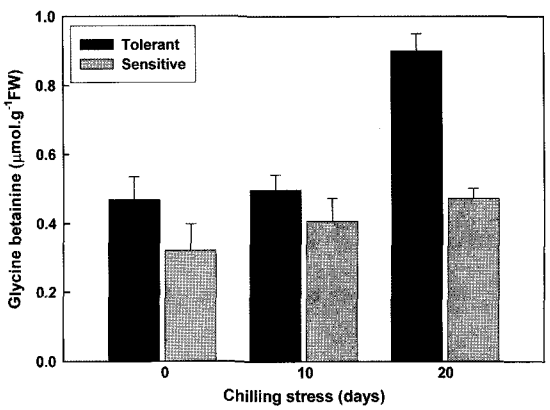

Fig. 4. Changes in GB accumulation in the leaves of chilling-tolerant and chilling-sensitive cultivar of *Cucurbits* during chilling stress. Plants were chilling stressed at 5/10°C (night/day) with 12 h photoperiod for 20 days.

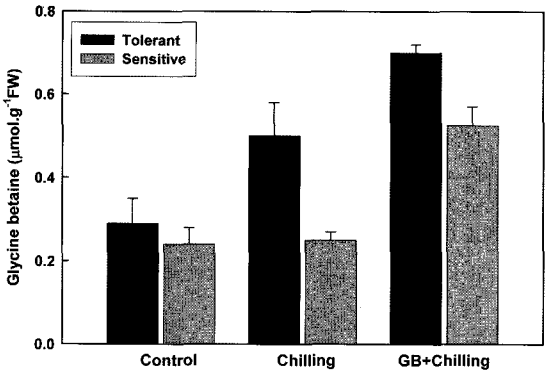

Fig. 5. Effect of exogenous GB on the GB accumulation during chilling stress in chilling-tolerant and chilling-sensitive cultivar of *Cucurbits*. Levels of GB were measured in the leaves of unstressed (control) and chilling stressed without GB (chilling) or with GB application (GB+chilling) to plants after 15 days of chilling stress.

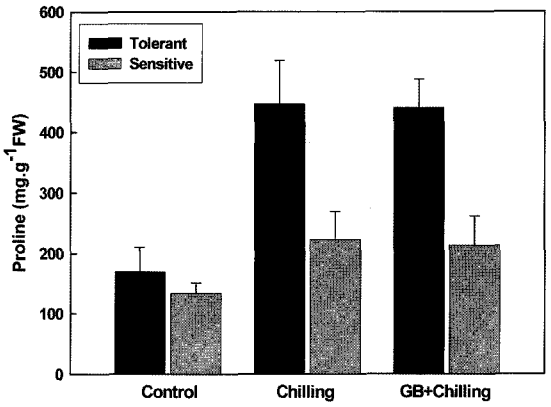

Fig. 6. Effect of exogenous GB on the proline accumulation during chilling stress in chilling-tolerant and chilling-sensitive cultivar of *Cucurbits*. Proline was measured in the leaves of unstressed (control) and chilling stressed without gb (chilling) or with GB (GB+chilling) to plants after 15 days of chilling stress.

4). In response to chilling, GB levels in leaves of chilling-tolerant plants increased more than two-fold while no significant accumulation of GB took place in the chilling-sensitive plants. Most of the increase in GB in chilling-tolerant plants appears to occur after 10 days of chilling treatment.

To determine if applied GB taken up by the plants, its levels were monitored in the leaves. A significant accumulation of GB was observed both in chilling-tolerant and chilling-sensitive plants. In chilling-sensitive plants no accumulation of GB was noted in response to chilling, however, a two-fold increase in GB level was observed after exogenous application (Fig. 5). Similarly, an increase in GB level in chilling-tolerant plants occurred as well, indicating that GB is readily absorbed by cucurbit plants. Since chilling-sensitive plants did not accumulate during chilling stress, they were treated with exogenous GB to assess its possible role in inducing chilling tolerance. The typical chilling injury symptom in chilling-sensitive plants was severe wilting in addition to poor growth. When the chilling-sensitive plants were pre-treated with 2 mM GB before the chilling treatment, the chilling injury symptoms were not observed. In fact, GB application appears to reduce chilling injury in chilling-tolerant plants as well.

As proline accumulation in cucurbit plants appear to correlate with chilling tolerance, its endogenous levels in leaves were determined after exogenous application of GB. The results show that GB did not affect the accumulation of endogenous proline either in the chilling-sensitive or chilling-tolerant plants (Fig. 6).

Discussion

The study shows that accumulation of osmolytes including soluble sugars, proline and GB occur in response to chilling in both chilling-tolerant and chilling-sensitive plants. Such a response has been documented in relation to other environmental stresses including freezing (Koster and Lynch, 1992; Perras and Sarham, 1984). The increase in soluble sugars in response to chilling was more dramatic in chilling-sensitive plants than in chilling-tolerant plants. Unlike soluble sugars, both proline and GB accumulate to higher levels in response to chilling in chilling-tolerant plants than in chilling-sensitive plants. In chilling-tolerant plants, the leaf proline content was approximately twice as much as in chilling-sensitive plants after chilling treatment. Similarly, more than two-fold increase in GB was observed in chilling-tolerant plants while no accumulation of GB occurred in chilling-sensitive plants after the chilling treatment. The results indicate that greater accumulation of proline and GB may play a role in chilling tolerance in *Cucurbits* plants. Beneficial role of proline in relation to cold, drought and salt stresses has been well documented (Aspinall and Paley, 1981; Rudolph and Crowe, 1985; Storey and Wyn Jones, 1977). Similar results have been reported with respect to

GB as well (Robinson and Jones, 1986; Wyn Jones and Storey, 1981). In addition to greater responsiveness of osmolyte accumulation of chilling-tolerant plants to chilling, these plants have inherently higher proline and GB levels. It is interesting to note that the chilling-tolerant plants perform well under chilling, their growth and development does not appear to be affected by low temperatures. Studies show that GB protect many organelles and photosynthetic proteins, specifically, PSII complex (Papageorgiou et al., 1991) and ATP synthesis under environmental stress (Mamedow et al., 1991). To determine if osmolyte accumulation can induce chilling tolerance in *Cucurbits* plants, exogenous GB was applied. Of all the osmolytes GB was chosen as the chilling-sensitive plants did not accumulate in response to chilling. The applied GB was readily taken up by the plants. The leaf GB level was increased by two-fold by exogenous application. The exogenous application increased the chilling tolerance in chilling-sensitive plants as indicated by reduced chilling injury symptoms in these plants. A number of studies have shown that exogenous application of GB can alleviate the environmental stresses, and can confer tolerance (Allard et al., 1998; Rajashekar et al., 1999; Xing and Rajashekar, 1999), and improve crop production (Agboma et al., 1997; Makela et al., 1997). The results show that osmolyte accumulation is an important factor in inducing chilling tolerance and suggest that it is possible to increase chilling tolerance in *Cucurbits* plants by exogenous application of GB.

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저온 스트레스에 의한 호박 식물체내 삼투조절물질의 축적

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적 요 저온 스트레스에 대한 호박의 생육과 식물체 내 삼투조절 물질의 반응을 분석한 결과, 삼투조절물질의 축적은 저온에 대한 내성 증가에 큰 영향을 미쳤다. 가용성 당은 저온에 강한 품종보다는 저온에 약한 품종에서 축적량이 많았다. 저온 처리 시 proline은 대조구에 비해 저온에 강한 품종과 약한 품종에서 모두 증가하는 경향을 보였다. 그러나 저온에 대한 내성에 따라 축적량에는 차이가 뚜렷하였는데, 저온 처리 후 20일째의 proline 축적량은 대조구에 비해 저온에 약한 품종에서는 22배 증가하였고, 저온에 강한 품종에서는 26.6배 증가하였다. 또한 저온에 약한 품종에서는 glycine betaine이 축적되지 않았지만, 강한 품종에서는 대조구에 비해 1.9배의 증가를 보였다. Glycine betaine을 엽면 처리하면 두 품종 모두 저온에 대한 내성이 증가하였는데, 이는 체내에 glycine betaine의 축적과 밀접한 관계가 있었다.

주제어 : glycine betaine, proline, 저온 스트레스