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Inhibition of glutathione synthesis reduces chilling tolerance in maize

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Abstract. The role of glutathione (GSH) in protecting plants from chilling injury was analyzed in seedlings of a chilling-tolerant maize (*Zea mays* L.) genotype using buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine (γEC) synthetase, the first enzyme of GSH synthesis. At 25 °C, 1 mM BSO significantly increased cysteine and reduced GSH content and GSH reductase (GR: EC 1.6.4.2) activity, but interestingly affected neither fresh weight nor dry weight nor relative injury. Application of BSO up to 1 mM during chilling at 5 °C reduced the fresh and dry weights of shoots and roots and increased relative injury from 10 to almost 40%. Buthionine sulfoximine also induced a decrease in GR activity of 90 and 40% in roots and shoots, respectively. Addition of GSH or γEC together with BSO to the nutrient solution protected the seedlings from the BSO effect by increasing the levels of GSH and GR activity in roots and shoots. During chilling, the level of abscisic acid increased both in controls and BSO-treated seedlings and decreased after chilling in roots and shoots of the controls and in the roots of BSO-treated seedlings, but increased in their shoots. Taken together, our results show that BSO did not reduce chilling tolerance of the maize genotype analyzed by inhibiting abscisic acid accumulation but by establishing a low level of GSH, which also induced a decrease in GR activity.

Key words: Abscisic acid – Buthionine sulfoximine – Cysteine – Glutathione – Glutathione reductase – *Zea*

Introduction

Glutathione (GSH) forms part of the ascorbate-GSH pathway which is involved in decomposing and thus detoxifying the excess of hydrogen peroxide generated during oxidative stress (Foyer and Halliwell 1976; Rennenberg and Brunold 1994; Alscher et al. 1997; May et al. 1998; Noctor and Foyer 1998; Noctor et al. 1998). If this pathway does not function sufficiently, hydrogen peroxide may accumulate to damaging levels. Such conditions, established by chilling temperature, have been described (Prasad et al. 1994), and the possible protective role of GSH at chilling temperatures in various system has been discussed (Guy et al. 1984; Vierheller and Smith 1990; Anderson et al. 1992; Stuiver et al. 1992; Badiani et al. 1993; Walker and McKersie 1993; Fadzillah et al. 1996; Kocsy et al. 1996, 1997, 1999; O'Kane et al. 1996; El-Saht 1998; Zhao and Blumwald 1998). Results corroborating a role for GSH in chilling tolerance were obtained using a chilling-tolerant species of *Lycopersicon* (Walker and McKersie 1993) and a series of maize genotypes with different chilling tolerances (Kocsy et al. 1996, 1997). More information about the role of GSH during chilling resulted from genetic studies with wheat which indicated a contribution of GSH to an improved low-temperature tolerance (Kocsy et al. 1999). Transgenic plants overexpressing enzymes of GSH synthesis provide additional evidence for the involvement of GSH in protecting from oxidative stress, as shown in paraquat-treated transgenic poplar overexpressing γ-glutamylcysteine (γEC) synthetase (Noctor et al. 1998), an enzyme with high flux control on GSH synthesis (Rüegsegger and Brunold 1992). Interestingly, GSH also increased in chilling-sensitive *Glycine max* (L.) Merr. (Vierheller and Smith 1990), clearly indicating that GSH is not the only parameter relevant for chilling tolerance. Indeed, participation of fatty acid desaturation (Sommerville and Browse 1991), protein metabolism (Guy 1990), abscisic acid (ABA; Janowiak and Dörffling 1996), xanthophyll cycle pigments (Verhoeven et al. 1999) and osmotic adjustment together with antioxidants (Noctor and Foyer 1998) were postulated as being involved in chilling tolerance, making it difficult to estimate the relative contribution of each of these protecting mechanisms.

Abbreviations: ABA = abscisic acid; BSO = buthionine sulfoximine; γEC = γ-glutamylcysteine; GR = glutathione reductase; GSH = glutathione; GSSG = oxidized GSH

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During detoxification of hydrogen peroxide in the ascorbate-GSH pathway, GSH is oxidized to GSSG and subsequently regenerated to GSH by glutathione reductase (GR; Foyer and Halliwell 1976; Foyer et al. 1997; Noctor et al. 1998). Correspondingly, GR activity increased after 3 d at 4 °C in *Arabidopsis* callus (O'Kane et al. 1998) and after 4 weeks at 5 °C in roots of jack pine (Zhao and Blumwald 1998). However, cultivation for 3 weeks at 5 °C had no effect on GR activity in leaves of soybean (Vierheller and Smith 1990), and GR activity even decreased in shoot cultures of rice (Fadzillah et al. 1996). In tolerant genotypes of maize and tomato, GR activity was higher than in sensitive ones cultivated under normal temperatures (Walker and McKersie 1993; Kocsy et al. 1997), and its increase during chilling was greater in a tolerant tomato species compared to a sensitive one (Bruggemann et al. 1999). Overexpression of GR in chloroplasts increased low-temperature tolerance in poplar (Foyer et al. 1997), but not in tomato (Bruggemann et al. 1999).

In all, the available information about the role of GSH and GR in protecting plants from chilling-induced injury results in an equivocal picture. With the aim of contributing to the clarification of this situation, we applied buthionine sulfoximine (BSO) to a chilling-tolerant maize variety grown on nutrient solution. Buthionine sulfoximine is a specific inhibitor of γEC synthesis (Griffith and Meister 1979), which makes it possible to gradually decrease γEC and GSH levels in maize (Farago and Brunold 1994). The system has the additional benefit that the GSH level reduced by BSO can be gradually increased by simultaneous addition of γEC or GSH (Farago and Brunold 1994).

Materials and methods

Plant material and growth conditions. Kernels of the highly chilling-tolerant maize (*Zea mays* L.) inbred line Z 7 (Stamp et al. 1983; Kocsy et al. 1996) were obtained from P. Stamp (IPW, ETH, Zürich, Switzerland). They were germinated between two layers of damp paper in a photoperiod of 12 h at 25 °C for 3 d. Twelve seedlings were placed into pots filled with 850 mL Henschel nutrient solution (Henschel 1970) modified according to Nussbaum et al. (1988), containing 10-fold iron. The plants were cultivated in a photoperiod of 12 h at 300 μmol m^{-2} s^{-1}, 25 °C and 60% relative humidity for 4 d. The experimental procedure routinely consisted of a precultivation of 4 d at 25 °C, subsequent chilling at 5 °C for 7 d and a recovery phase at 25 °C for an additional 7 d. Buthionine sulfoximine alone or BSO in combination with γEC or GSH was added at the beginning of precultivation. The culture medium was renewed at the beginning of the recovery phase. Controls were cultivated at 25 °C during the whole experiment, with or without additions.

Relative injury and relative protection. The relative injury of the plants was routinely estimated according to the followings scale: 0, no necrosis; 1, less than 25%; 2, 25–50%; 3, 50–75%; 4, 75–90%; 5, 90–100% necrosis of the shoot at the end of the recovery phase. The mean values of these estimations were calculated as a percentage of completely necrotic shoots. Relative protection was calculated by subtracting relative injury from 100%.

Assay for GR. The plant material was homogenized in 0.1 M Na-K-phosphate buffer, pH 7.5 (1:5, w/v), containing 0.2 mM diethylenetriamine pentaacetic acid and 4% (w/v) polyvinylpolypyrrolidone in an ice-cooled glass homogenizer and centrifuged at 30 000 *g* for 10 min at 4 °C. The supernatant was used for measuring GR activity. Activity of GR was measured according to Smith et al. (1988). The assay mixture contained, in a total volume of 1 mL, 100 mM Na-K-phosphate (pH 7.5), 0.2 mM diethylenetriamine pentaacetic acid, 0.75 mM 5,5′dithio-bis-(2-nitrobenzoic acid), 0.1 mM NADPH, 10 mM dithioerythritol, 0.5 mM GSSG and 50 μL plant extract.

Protein determination. Proteins were determined according to Bradford (1976) using BSA as standard. The reaction mixture contained in a total volume of 1 mL 200 μL protein assay reagent (Bio Rad) and 5 μL extract.

Determination of cysteine, γEC and GSH. The plant material was extracted 1:10 (w/v) in 0.1 M HCl, containing 1 mM Na_2EDTA, in an ice-cooled glass homogenizer. The extracts were filtrated through viscose fleece, centrifuged for 30 min at 30 000 *g* and 4 °C. Then 400 μL of supernatant was added to 600 μL of 0.2 M Ches [2-(cyclohexylamino)ethane sulfonic acid; pH 9.3] and reduced with 100 μL of a freshly prepared 400 mM $NaBH_4$ solution. The mixture was kept on ice for 20 min. For derivatization, 330 μL of this mixture was added to 15 μL of 15 mM monobromobimane and kept in the dark at room temperature for 15 min. The reaction was stopped with 250 μL of 5% (v/v) acetic acid. The samples were analyzed as described by Schupp and Rennenberg (1988), as modified by Rüegsegger and Brunold (1992), by reverse-phase HPLC and fluorescence detection.

A mixture of the three thiols in different concentrations (0, 1, 2, 3, 4, 5 μM) was used for qualitative and quantitative identification, as well as for recovery experiments. For determination of the recovery, the individual thiols were measured in the plant sample (T_p), in the internal standards (T_s) and in a mixture of plant sample and internal standards (T_{p+s}). The percentage recovery was calculated according to: $100(T_{p-s} - T_p) \cdot T_s^{-1}$. The recovery of the individual thiols varied between 88 and 96% in shoots and roots, respectively. To check a possible effect of BSO on derivatization of the thiols with monobromobimane, BSO at 0, 0.2, 2, 10, 20, 200 μM was added to the 2 μM standard mixture of the three thiols, but no effect was detected.

Abscisic acid. Abscisic acid (ABA) was measured immunologically according to Gergs et al. (1993) using a monoclonal antibody obtained from E. Weiler (Ruhr University, Bochum, Germany).

Statistics. Data of six (growth parameters 12) measurements from three independent experiments were compared using two-component analysis of variance (Microsoft Excel 97). The significance of differences was calculated with the *t*-test, and the mean differences were compared pairwise with the Tukey test (Systat for Windows, Version 5).

Chemicals. Monobromobimane was obtained from Calbiochem, γEC from Nacalai Tesque (Kyoto, Japan) and BSO from Sigma. All other chemicals were purchased from Fluka.

Results

Inhibition of GSH synthesis by BSO. At 25 °C, the highest BSO concentration applied (1 mM) had no significant effect on fresh and dry weights of shoots and roots, or on relative injury. During the 7-d chilling period there was no visible difference between the BSO-treated and control plants; at the end of the recovery phase, however, the fresh and dry weights of shoots and

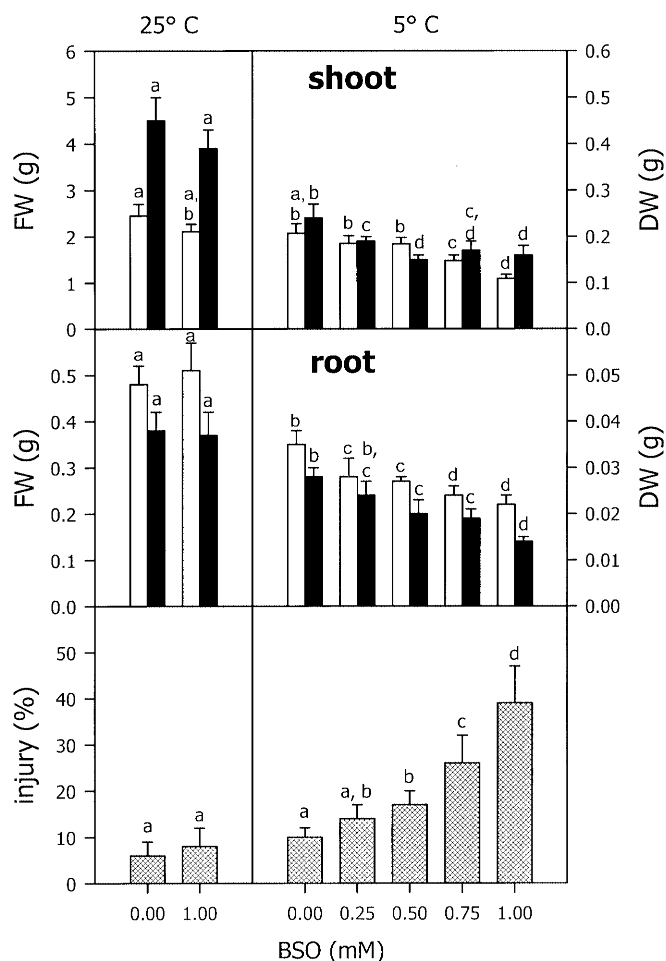

Fig. 1. Effect of different BSO concentrations on fresh weight (*white bars*) and dry weight (*black bars*) of shoots and roots and relative injury (*hatched bars*) of maize seedlings cultivated at 25 °C for 4 d, then at 5 °C for 7 d, and at 25 °C for an additional 7 d. Controls were cultivated with 0 or 1 mM BSO at 25 °C during the whole experimental period. Mean values of 12 measurements ± SD from 3 independent experiments are presented. Values carrying different letters are significantly different at $P \leq 0.05$

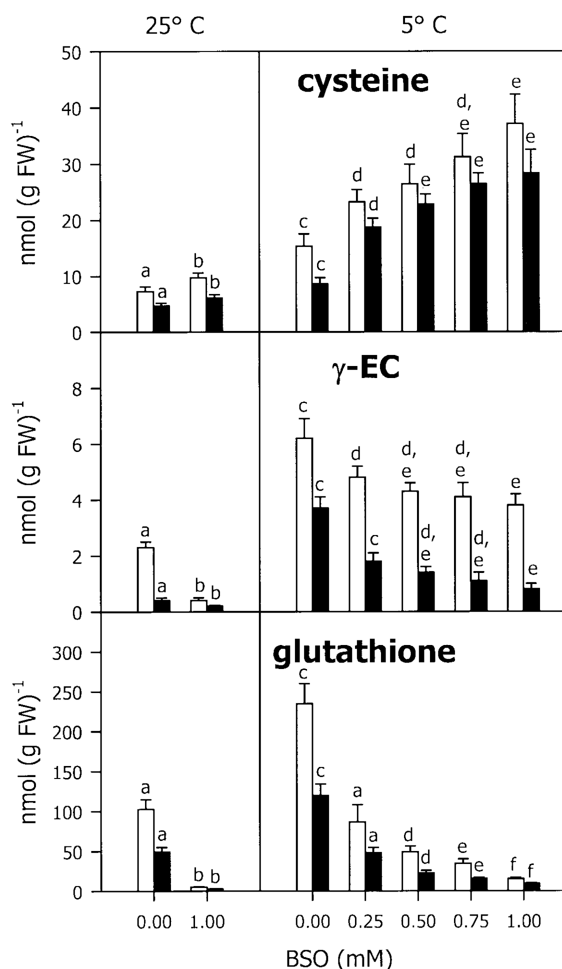

Fig. 2. Effect of different BSO concentrations on cysteine, γEC and GSH contents of roots (*black bars*) and shoots (*white bars*) of maize seedlings cultivated at 25 °C for 4 d, then at 5 °C for 7 d. Controls were cultivated with 0 or 1 mM BSO at 25 °C during the whole experimental period. Mean values of six measurements ± SD from three independent experiments are presented. Values carrying different letters are significantly different at $P \leq 0.05$

roots of BSO-treated seedlings were lower compared to controls. This effect increased with increasing BSO concentrations (Fig. 1). The chilling-induced relative injury of the seedlings also increased, 1 mM BSO resulting in a 4-fold increase in injury compared to controls (Fig. 1).

At the end of the chilling phase, cysteine levels had increased parallel to BSO concentrations added, whereas γEC and GSH had gradually decreased (Fig. 2). Corresponding effects on the thiol levels were detected in control seedlings treated with 1 mM BSO at 25 °C, both in roots and shoots (Fig. 2), with GSH decreasing to very low levels. This decrease had no negative effect on fresh and dry weights of the seedlings (Fig. 1), demonstrating that low levels of GSH do not affect these parameters during cultivation at 25 °C.

Time courses of the changes in thiol levels during the routinely applied temperature changes are presented in Fig. 3. In the control plants cultivated without BSO, the level of all thiols measured increased during the chilling phase both in roots and shoots. During the recovery phase, the thiol levels decreased, reaching concentrations comparable to those at the beginning of the chilling period. Addition of 1 mM BSO to the culture medium induced a much more pronounced increase in cysteine than in the control cultures, both in shoots and roots. Interestingly, γEC also increased in the shoots during chilling with BSO, whereas in roots no significant change could be detected. Addition of BSO reduced the amount of GSH during cultivation at 25 °C to a very low level which was maintained both during chilling and the recovery phase (Fig. 3).

Treatment with BSO also affected GR activity, both in roots and shoots at 25 °C and at 5 °C (Fig. 4). At the end of the chilling period, the GR level in shoots had decreased concomitant with increasing BSO concentrations. In roots, however, 0.25 mM BSO had already reduced GR activity to a very low level which was not further reduced by the higher BSO concentrations (Fig. 4). The effect of 1 mM BSO on GR activity

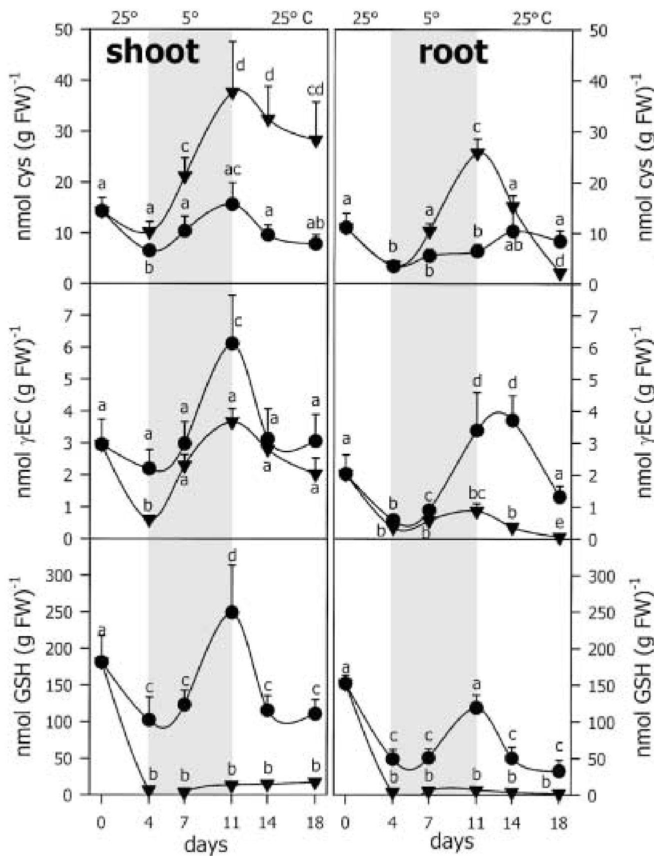

Fig. 3. Changes in cysteine, γEC and GSH contents in roots and shoots of maize seedlings cultivated with 0 (●) or 1 (▼) mM BSO at 25 °C for 4 d, then at 5 °C for 7 d, and at 25 °C for an additional 7 d. Mean values of six measurements ± SD from three independent experiments are presented. Values carrying different letters are significantly different at $P \leq 0.05$

in roots and shoots during the routinely applied temperature changes is shown in Fig. 5. In the controls, this enzyme activity increased in shoots during the chilling period, whereas in roots there was an initial increase and a subsequent decrease (Fig. 5). After the chilling period the enzyme level decreased to the initial one in shoots, but remained at a high level in roots of control plants. In the presence of BSO, GR activity decreased during the chilling period, reaching levels which were 30% and 25% of the controls in roots and shoots, respectively (Fig. 5). In shoots there was an additional decrease during the recovery phase, whereas in roots the enzyme activity increased to the level of control plants (Fig. 5), even though the GSH concentration remained low.

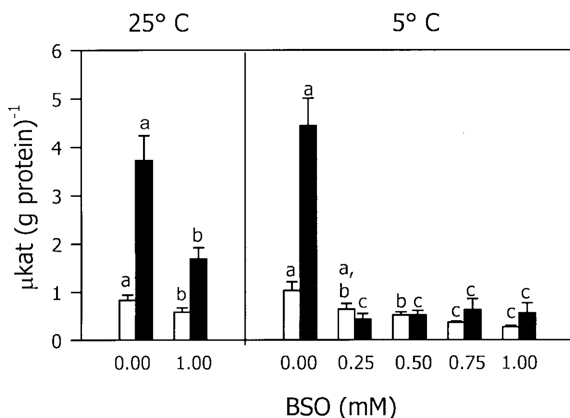

Fig. 4. Effect of different BSO concentrations on GR activity in roots (*black bars*) and shoots (*white bars*) of maize seedlings cultivated at 25 °C for 4 d, then at 5 °C for 7 d. Controls were cultivated at 25 °C during the whole experimental period. Mean values of six measurements ± SD from three independent experiments are presented. Values carrying different letters are significantly different at $P \leq 0.05$

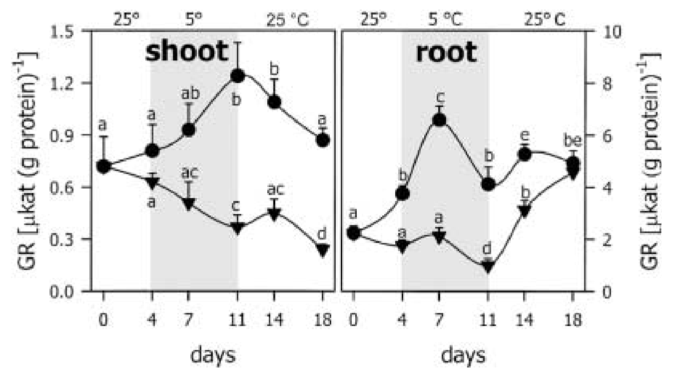

Fig. 5. Changes in GR activity in roots and shoots of maize seedlings cultivated with 0 (●) or 1 (▼) mM BSO at 25 °C for 4 d, then at 5 °C for 7 d, and at 25 °C for an additional 7 d. Mean values of six measurements ± SD from three independent experiments are presented. Values carrying different letters are significantly different at $P \leq 0.05$

Compensation of the inhibitory effect of BSO with exogenous γEC and GSH. Simultaneous addition of 1 mM BSO and concentrations of γEC up to 1 mM increased fresh weight and dry weight of both roots and shoots and reduced the relative injury compared to controls which were treated with 1 mM BSO alone (Fig. 6). Figure 7 demonstrates that the addition of γEC together with 1 mM BSO reduced the levels of cysteine both at 25 and 5 °C in shoots and roots but had already increased γEC and GSH significantly at the lowest γEC concentration applied (0.25 mM). The highest GSH levels obtained by this treatment were only half of those detected in shoots of seedlings cultivated without BSO, but at comparable levels in roots (Figs. 2, 7).

A protective effect during chilling could also be obtained by adding 1 mM GSH to the culture medium together with BSO, which reduced the relative injury by 50% (Table 1). The fresh and dry weight both of roots and shoots was 50–100% higher than in controls cultivated at 5 °C with BSO alone and was comparable to the values measured at 25 °C, demonstrating a good protection from chilling by GSH. Table 2 presents the increased levels of thiols in the seedlings treated with a combination of BSO and GSH instead of BSO alone. The GSH content in shoots of seedlings treated with a combination of BSO and GSH was comparable to that of seedlings cultivated without BSO. The roots of these

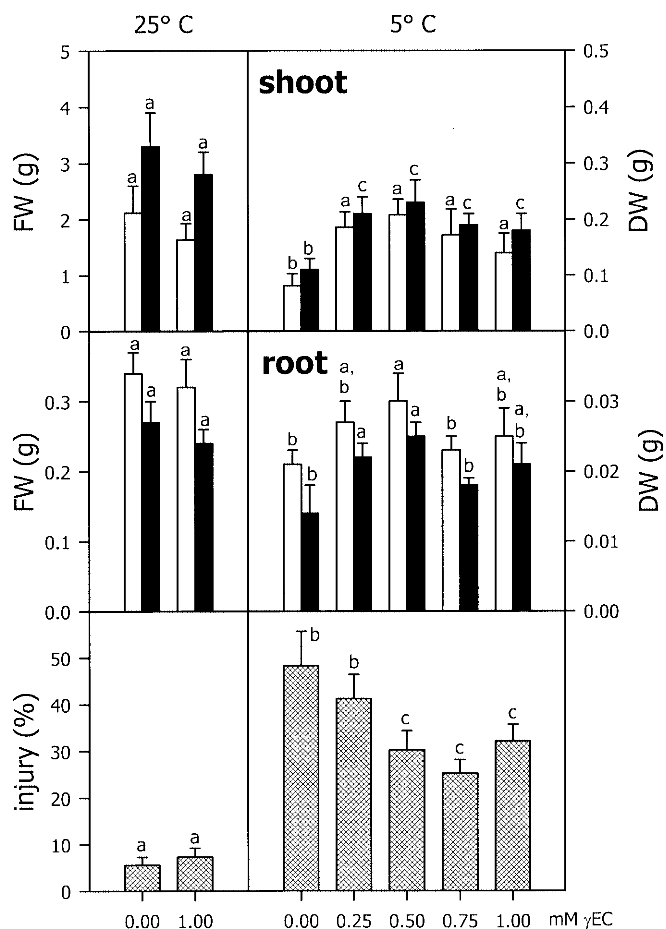

Fig. 6. Effect of different γEC concentrations on fresh weight (*white bars*) and dry weight (*black bars*) of roots and shoots and relative injury (*hatched bars*) of maize seedlings cultivated in the presence of 1 mM BSO at 25 °C for 4 d, then at 5 °C for 7 d, and at 25 °C for an additional 7 d. Controls were cultivated with 0 or 1 mM γEC at 25 °C during the whole experimental period. Mean values of six measurements ± SD from three independent experiments are presented. Values carrying different letters are significantly different at $P \leq 0.05$

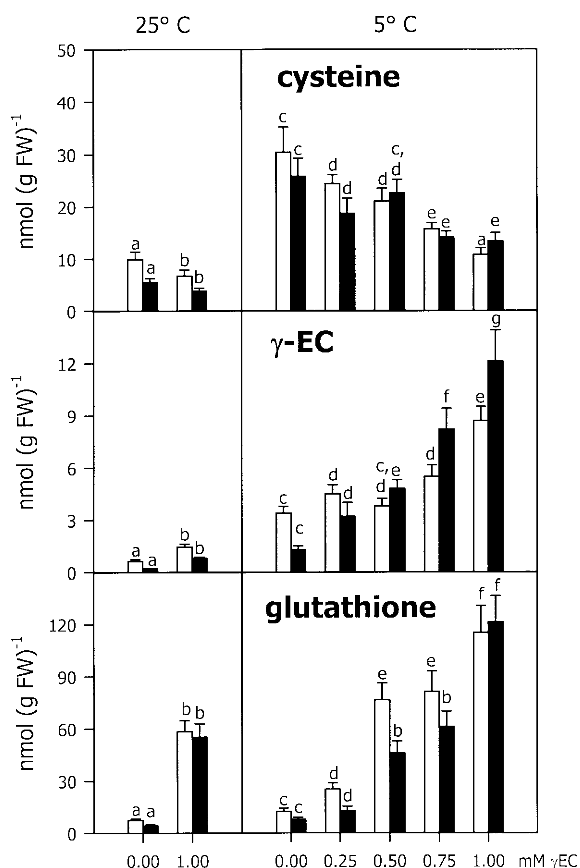

Fig. 7. Effect of different γEC concentrations on cysteine, γEC, and GSH contents of roots (*black bars*) and shoots (*white bars*) of maize seedlings cultivated with 1 mM BSO at 25 °C for 4 d, then at 5 °C for 7 d. Controls were cultivated at 25 °C during the whole experimental period. Mean values of six measurements ± SD from three independent experiments are presented. Values carrying different letters are significantly different at $P \leq 0.05$

plants contained even higher GSH concentrations than plants cultivated without BSO. Interestingly, the combined treatment with BSO and GSH at 25 °C reduced the GSH content to levels which were much below those obtained at 5 °C (Table 2). Table 2 also shows that the treatment with BSO and GSH at 5 °C induced a 10-fold increase in GR in the shoots and a doubling in roots. The enzyme activity obtained in the shoots by this treatment was higher than that detected at 25 °C and that induced during chilling at 5 °C. Consistent with these results, γEC also increased GR activity

Table 1. Injury, and shoot and root fresh and dry weight of maize seedlings cultivated with 1 mM BSO in the absence or presence of 1 mM GSH at 25 °C for 4 d, then at 5 °C for 7 d and at 25 °C for an additional 7 d (=chilling treatment). Controls were cultivated at 25 °C during the whole experimental period. Mean values of 12 measurements ± SD from three independent experiments are presented. Values carrying different letters are significantly different at $P \leq 0.05$

Treatment	Injury (%)	Fresh weight (g)		Dry weight (mg)	
		Shoot	Root	Shoot	Root
Chilling treatment					
BSO	39 ± 8^{b}	1.09 ± 0.09^{b}	0.21 ± 0.03^{b}	16 ± 2^{b}	14 ± 1^{b}
BSO + GSH	18 ± 7^{c}	2.45 ± 0.32^{a}	0.34 ± 0.04^{c}	25 ± 4^{c}	29 ± 5^{a}
Control					
BSO	8 ± 4^{a}	2.11 ± 0.16^{a}	0.51 ± 0.09^{a}	39 ± 5^{a}	37 ± 6^{a}
BSO + GSH	7 ± 3^{a}	1.90 ± 0.31^{a}	0.42 ± 0.06^{a}	$31 \pm 4^{a,c}$	28 ± 3^{a}

Table 2. Cysteine, γEC, GSH contents and GR activity of maize seedlings cultivated with 1 mM BSO in the absence or presence of 1 mM GSH at 25 °C for 4 d, then at 5 °C for 7 d (=chilling treatment). Controls were cultivated at 25 °C during the whole experimental period. Mean values of six measurements ± SD from three independent experiments are presented. Values carrying different letters are significantly different at $P \leq 0.05$

Treatment	Cysteine [nmol (g FW)$^{-1}$]		γEC [nmol (g FW)$^{-1}$]		GSH [nmol (g FW)$^{-1}$]		GR [μkat (g protein)$^{-1}$]	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Chilling treatment								
BSO	30.1 ± 5.2^{c}	25.8 ± 3.5^{c}	3.4 ± 0.4^{c}	1.6 ± 0.4^{c}	12.5 ± 1.9^{c}	7.9 ± 1.1^{c}	0.29 ± 0.08^{b}	0.47 ± 0.08^{c}
BSO+GSH	10.7 ± 1.3^{a}	18.9 ± 1.2^{b}	9.9 ± 1.9^{d}	12.4 ± 1.7^{d}	185.2 ± 13.5^{d}	221.7 ± 16.5^{d}	3.08 ± 0.52^{c}	0.79 ± 0.12^{d}
Control								
BSO	9.9 ± 1.5^{a}	5.5 ± 1.2^{a}	0.6 ± 0.1^{a}	0.2 ± 0.1^{a}	7.4 ± 0.9^{a}	4.2 ± 0.6^{a}	0.74 ± 0.12^{a}	1.65 ± 0.18^{a}
BSO + GSH	15.7 ± 1.5^{b}	17.1 ± 1.2^{b}	1.2 ± 0.3^{b}	4.6 ± 0.3^{b}	72.9 ± 8.5^{b}	29.6 ± 3.4^{b}	0.55 ± 0.04^{a}	3.42 ± 0.55^{b}

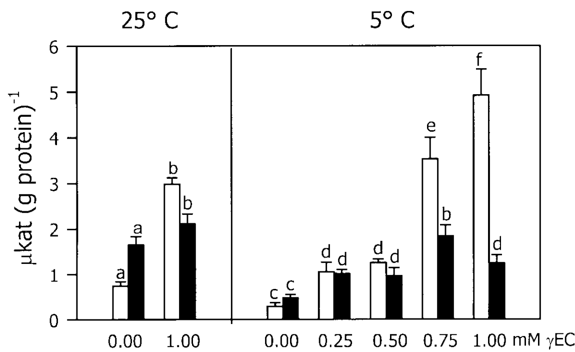

Fig. 8. Effect of different γEC concentrations on GR activity in roots (*black bars*) and shoots (*white bars*) of maize seedlings cultivated with 1 mM BSO at 25 °C for 4 d, then at 5 °C for 7 d. Controls were cultivated at 25 °C during the whole experimental period. Mean values of 6 measurements ± SD from three independent experiments are presented. Values carrying different letters are significantly different at $P \leq 0.05$

both at 25 and 5 °C in roots and shoots (Fig. 8). At 5 °C, the level obtained in shoots with 0.75 and 1 mM γEC was higher than that obtained during chilling without BSO (Fig. 4).

The involvement of rapidly increased and maintained high levels of ABA was suggested to be an important prerequisite for chilling tolerance. With the aim of examining an effect of BSO via ABA, we analyzed the ABA level both in shoots and roots (Fig. 9). Abscisic acid increased during the chilling period both in roots and shoots; there was no difference, however, between BSO-treated and control plants. During the recovery phase, the ABA level decreased correspondingly in shoots and roots of controls and roots of BSO-treated seedlings, but increased to even higher levels in shoots of BSO-treated seedlings (Fig. 9).

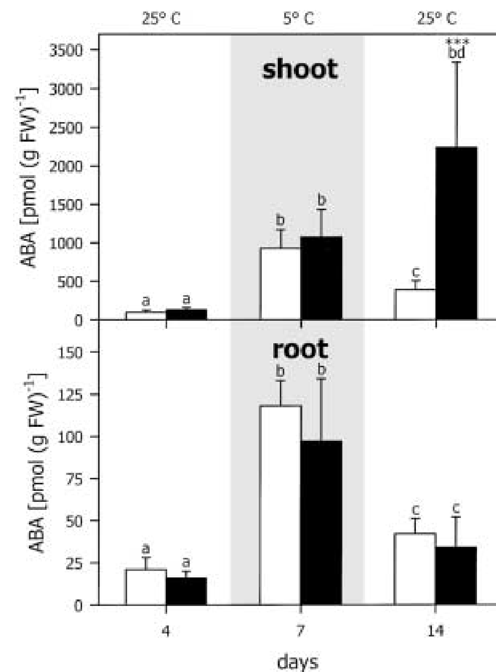

Fig. 9. Levels of ABA in roots and shoots of maize seedlings cultivated with 0 (*open bars*) or 1 mM BSO (*black bars*) at 25 °C for 4 d (*4*), at 5 °C for additional 3 d (*7*), and at 25 °C for 3 d after chilling for 7 d (*14*). Values from 6 measurements ± SD from three independent experiments are presented. Values carrying different letters are significantly different at $P \leq 0.05$. The value carrying *** is significantly different at $P \leq 0.01$

Discussion

Even though a possible function of GSH in protecting plants against chilling-induced oxidative stress has been postulated in a series of publications (Badiani et al. 1993; Walker and McKersie 1993; Fadzillah et al. 1996; Kocsy et al. 1996, 1997; Alscher et al. 1997; Foyer et al. 1997; Noctor and Foyer 1998), the present work demonstrates unequivocally for the first time the relative contribution of GSH to chilling protection of maize (Fig. 10). This became possible because the GSH level could be gradually decreased to very low levels using various concentrations of BSO or increased to almost normal levels by simultaneous addition of BSO and γEC or GSH (Farago and Brunold 1994). Our results demonstrate that the relative protection of the shoots was gradually decreased by gradual reduction of the GSH level (Fig. 10). Since GSH functions as a substrate in enzyme-catalyzed detoxification reactions it does not seem surprising that the relationship between GSH and relative protection corresponds to Michaelis-Menten

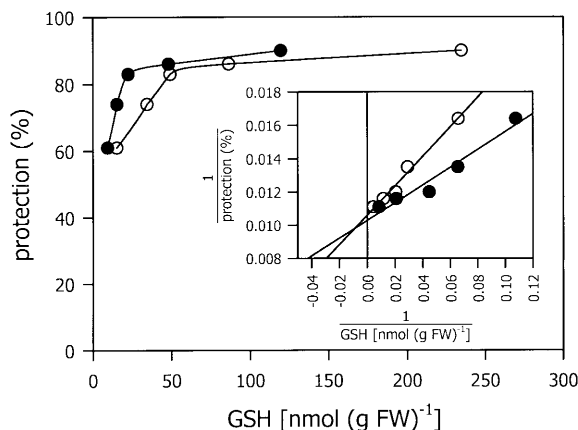

Fig. 10. Effect of GSH levels in shoots or roots on the relative protection of maize seedlings from chilling. The relative protection was calculated from Fig. 1, the GSH levels were taken from Fig. 2. *Insert*, Lineweaver-Burk plot, with $r^2 = 0.98$ for shoot values and $r^2 = 0.95$ for root values

and Lineweaver-Burk plots (Fig. 10). Vice versa, increasing additions of γEC to the nutrient solution in the presence of BSO, gradually increased the GSH content and decreased relative injury (Fig. 6). These results are in agreement with our previous ones obtained with various maize genotypes, which showed that GSH was significantly increased in the chilling-tolerant genotypes during growth at chilling temperatures (Kocsy et al. 1996). The present results are also consistent with those obtained with transgenic poplars (Noctor et al. 1998) overexpressing γEC synthetase since these transformants contained increased levels of GSH and were better protected from paraquat-induced oxidative stress. In contrast, Creissen et al. (1999) very recently reported that tobacco plants overexpressing a chloroplast targeted γEC synthetase and containing increased foliar pools of both GSH and γEC suffered from greatly enhanced oxidative stress, which was manifested as light-intensity-dependent chlorosis or necrosis. These authors did not detect increased GR activity in these transformants whereas it was clearly demonstrated here that the levels of GR activity changed with GSH contents. This indicates a signalling process present in maize as reported here but missing in the transformed tobacco plants.

It is interesting to note that cultivation at 25 °C for 18 d in the presence of 1 mM BSO, which reduced the amount of GSH to very low levels neither affected the increase in fresh and dry weight nor the phenotype of the seedlings, indicating that in maize higher levels of GSH are not necessary for sustaining normal growth at 25 °C. It should be pointed out, however, that experiments with BSO-treated *Arabidopsis* revealed morphological changes (Sanchez-Fernandez et al. 1997). When the plants were subjected to chilling at 5 °C, decreased GSH levels correlated with decreased fresh and dry weight and increased relative injury, clearly demonstrating the putative protective function of GSH (Kocsy et al. 1996, 1997; Foyer et al. 1997; Noctor and Foyer 1998).

Together with previously published results, the present findings can be used for establishing a picture of GSH functions during chilling stress. In the ascorbate-GSH pathway (Foyer and Halliwell 1976) GSH is involved in H_2O_2 detoxification, thus contributing to the prevention of oxidative injury. In addition GSH can be involved as a reductant in assimilatory sulfate reduction (Suter et al. 2000). The key enzyme of this pathway, adenosine 5′-phosphosulfate reductase reduces adenosine 5′-phosphosulfate using electrons from GSH and forming SO_3^{2-}, 5′-AMP and GSSG. Sulfite is subsequently reduced to sulfide, which is incorporated into *O*-acetyl-L-serine, thus forming cysteine. This amino acid can subsequently be used for GSH synthesis (Brunold and Rennenberg 1997). Finally, GSH is involved in the detoxification of toxic lipid peroxidation products via GSH-S-transferases (Mullineaux et al. 1998). Before a comprehensive picture of GSH functions during chilling stress can be presented, however, it will be necessary to assay the redox state of GSH in all conditions.

From the present study it becomes evident that GSH is also involved in regulating the level of GR. The involvement of GSH in stress signalling has been described before (Wingate et al. 1989; Foyer et al. 1997). An induction of GR can contribute to the beneficial effect of GSH, because GR will reduce GSSG produced during detoxification of H_2O_2 (Noctor and Foyer 1998), during reduction of lipid peroxidation products (Mullineaux et al. 1998) and during the increased synthesis of cysteine in a situation of chilling stress (Kocsy et al. 1996). It was postulated that regulation of nuclear gene expression is influenced by reactive oxygen species and the redox state of antioxidant pools (Creissen et al. 1999). Indeed, our results clearly show that oxidative stress alone does not induce the formation of GR. This induction seems to be at least partially dependent on a high GSH level.

The plant hormone ABA is thought to play a crucial role in plant responses to chilling stress (Janowiak and Dörffling 1996). Consistent with results presented by Ristic et al. (1998), however, our results indicate that the protective effect of GSH during chilling stress of maize seedlings is not dependent on ABA. This is consistent with the model of Ishitani et al. (1997), in which ABA-dependent and ABA-independent pathways converge in cold-stress signalling.

Our results prompted us to analyze if a chemically increased GSH level would increase chilling tolerance of a chilling-sensitive maize genotype. Indeed, corresponding experiments using safeners to increase GSH levels (Farago and Brunold 1994) resulted in increased chilling tolerance (data not shown), corroborating the findings presented here.

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