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Genetic study of glutathione accumulation during cold hardening in wheat

Gábor Kocsy[1], Gabriella Szalai[1], Attila Vágújfalvi[1], László Stéhli[1], György Orosz[2], Gábor Galiba[1]

[1]Agricultural Research Institute of the Hungarian Academy of Sciences, POB 19, H-2462 Martonvásár, Hungary
[2]Research Group of Peptide Chemistry of the Hungarian Academy of Sciences, Eötvös University, POB 32, H-1518 Budapest 112, Hungary

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Abstract. The effect of cold hardening on the accumulation of glutathione (GSH) and its precursors was studied in the shoots and roots of wheat (*Triticum aestivum* L.) cv. Cheyenne (Ch, frost-tolerant) and cv. Chinese Spring (CS, moderately frost-sensitive), in a *T. spelta* L. accession (Tsp, frost-sensitive) and in chromosome substitution lines CS (Ch 5A) and CS (Tsp 5A). The fast induction of total glutathione accumulation was detected during the first 3 d of hardening in the shoots, especially in the frost-tolerant Ch and CS (Ch 5A). This observation was corroborated by the study of de novo GSH synthesis using [^{35}S]sulfate. In Ch and CS (Ch 5A) the total cysteine, γ-glutamylcysteine (precursors of GSH), hydroxymethylglutathione and GSH contents were greater during the 51-d treatment than in the sensitive genotypes. After 35 d hardening, when the maximum frost tolerance was observed, greater ratios of reduced to oxidised hydroxymethylglutathione and glutathione were detected in Ch and CS (Ch 5A) compared to the sensitive genotypes. A correspondingly greater glutathione reductase (EC 1.6.4.2) activity was also found in Ch and CS (Ch 5A). It can be assumed that chromosome 5A of wheat has an influence on GSH accumulation and on the ratio of reduced to oxidised glutathione as part of a complex regulatory function during hardening. Consequently, GSH may contribute to the enhancement of frost tolerance in wheat.

Key words: Chromosome 5A – Frost tolerance – Glutathione – Cold hardening – *Triticum*

Introduction

Glutathione (GSH), as an important component of the ascorbate-glutathione cycle, participates in the removal of hydrogen peroxide (for reviews, see Foyer et al. 1994; Alscher et al. 1997; Noctor and Foyer 1998; Noctor et al. 1998; May et al. 1998) which may be accumulated during low-temperature-induced oxidative stress (Prasad et al. 1994; Fadzillah et al. 1996; O'Kane et al. 1996). Glutathione is synthesized in two steps (for reviews, see Rennenberg and Brunold 1994; Brunold and Rennenberg 1997): first cysteine and glutamate are bound to γ-glutamylcysteine (γEC) by γEC synthetase (EC 6.3.2.2), then a glycine is added to the dipeptide by GSH synthetase (EC 6.3.2.3). In *Gramineae* a homologue of GSH, hydroxymethylglutathione (hmGSH), is also present, in which glycine is replaced by serine (Klapheck et al. 1991).

Under natural conditions the low-temperature-induced accumulation of GSH has been observed in spruce (*Picea abies* L.) and white pine (*Pinus strobus* L.) during the winter (Esterbauer and Grill 1978; Anderson et al. 1992) and in alpine plants with increasing altitude (Wildi and Lütz 1996). In experiments carried out in growth chambers, cold treatment at 5 °C increased the total glutathione content in the roots of jack pine (*Pinus banksiana*) seedlings (Zhao and Blumwald 1998), in the leaves of soybean (Vierheller and Smith 1990) and in the fruits of squash (Wang 1995). Similar changes were observed in *Arabidopsis* callus at 4 °C (O'Kane et al. 1996) and in the leaves of *Triticum durum* at 10 °C (Badiani et al. 1993). However, GSH decreased in shoot cultures of rice at 4 °C (Fadzillah et al. 1996).

Under normal growth conditions about 90% of the glutathione is in the reduced form, but under stress conditions the ratio of reduced to oxidised glutathione (GSH/GSSH) changes (Foyer et al. 1997). The GSH/GSSG ratio decreased in the leaves of *Triticum durum* L. at 10 °C, in *Glycine max* (L.) Merr. at 5 °C and in *Lycopersicon esculentum* at 2 °C (Badiani et al. 1993; Vierheller and Smith 1990; Walker and McKersie 1993). However, this ratio increased at 5 °C in the roots of

Abbrevations: Ch = *Triticum aestivum* L. cv. Cheyenne; CS = *Triticum aestivum* L. cv. Chinese Spring; γEC = γ-glutamylcysteine; GR = glutathione reductase; GSH = reduced glutathione; GSSG = oxidised glutathione; hmGSH = hydroxymethylglutathione; Tsp = *Triticum spelta* L.

Correspondence to: G. Kocsy;
E-mail: kocsyg@fsnew.mgki.hu; Fax: 36 (22) 460 213

Pinus banksiana Lamb. (Zhao and Blumwald 1998) and in preconditioned (15 °C, 2 d) fruits of zucchini squash (Wang 1995).

The activity of glutathione reductase (GR, EC 1.6.4.2), regenerating the reduced form of glutathione (Foyer et al. 1997), increased during the winter in *Picea abies* (Esterbauer and Grill 1978). Under controlled conditions long-term hardening (4 °C, 59 d) increased the GR activity in spinach (de Kok and Oosterhuis 1983), while short-term hardening (4 °C, 7 d) resulted in greater GR activity in pre-emergent maize seedlings (Prasad 1997). The Activity of GR did not change during chilling in the leaves of tomato and maize (Walker and McKersie 1993; Kocsy et al. 1996) but it decreased in rice (Fadzillah et al. 1996). From these controversial results it can be established that the levels of GSH and GR activity depend on the developmental stage, the organs examined and the low-temperature sensitivity of the different genotypes.

To obtain further evidence on the role of GSH in the stress response, a genetic approach was used in the present study for its investigation during cold hardening in wheat. It is well established that chromosome 5A of the frost-tolerant variety Cheyenne (Ch, donor) can increase the frost tolerance of the moderately frost-sensitive recipient variety Chinese Spring (CS) (Sutka 1981; Veisz and Sutka 1989; Janda et al. 1994). Major genes influencing frost tolerance (*Fr1*) and vernalization requirement (*Vrn1*) have been mapped on the long arm of chromosome 5A (Galiba et al. 1995). In contrast to the effect of the Ch 5A chromosome, when the 5A chromosome of CS is replaced by the corresponding chromosome of the highly frost-sensitive *T. spelta* L. (Tsp) accession the tolerance of CS decreases to an even lower level (Galiba et al. 1995). Therefore, thiol accumulation, de novo GSH synthesis and GSH/GSSG ratio have been studied during hardening in this genetic system, and the results are reported here.

Materials and methods

Plant material and growth conditions. The frost-tolerant *Triticum aestivum* L. cv. Cheyenne, the moderately sensitive *T. aestivum* cv. Chinese Spring, a frost-sensitive *T. spelta* L. accession and the chromosome substitution lines Chinese Spring (Cheyenne 5A) and Chinese Spring (*T. spelta* 5A) were used in the experiments. The seeds were obtained from the Martonvásár Cereal Gene Bank (Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary). The chromosome substitution lines were developed using a mono-telocentric (for chromosome 5A) line of CS as the recurrent parent and disomic (for chromosome 5A) Ch or Tsp as the donor to commence the back-cross programme (Law et al. 1987). In each hybrid generation, selection was practised for monosomic individuals for use as pollen parents in the next back-cross cycle with the mono-telocentric recipient. After a minimum of 6 back-crosses the monosomics were self-pollinated and plants with 42 chromosomes were selected, in which the 5A chromosome pair was derived from the donor Ch or Tsp.

For the long-term hardening experiment (51 d), after germination in Petri dishes (25 °C, 3 d) the seedlings were planted in wooden boxes measuring 42 cm × 31 cm × 18 cm. They were raised in a 2:1:1 (v/v/v) mixture of garden soil, humus and sand. The plants were grown in an autumn-winter type growth chamber (Conviron PGV-36; Controlled Env., Winnipeg, Canada) at 15/10 °C day/night temperature for 2 weeks with 16 h illumination at 260 μmol photons m^{-2} s^{-1}, then at 10/5 °C for a week (prehardening) and after that at 4/2 °C (cold hardening) for 51 d. The plant material for the thiol determination was harvested at the start and end of prehardening, after 3 d hardening and subsequently every eighth day.

To compare the effects of short-term hardening at 4/2 °C (day/night) for 3 d on thiol accumulation in control and treated plants the seedlings were grown after germination (25 °C, 3 d) in hydroponic culture using half-strength Hoagland nutrient solution (Hoagland and Arnon 1950). The cultivation conditions were similar to those described above. For the investigation of in-vivo sulfate assimilation, 46 MBq [^{35}S]sulfate was added to 350 ml nutrient solution at the beginning of hardening. For the biochemical investigations, samples were taken from plants grown under control conditions (15/10 °C day/night) or at 4/2 °C (day/night) for 3 d.

Test of frost tolerance. The frost tolerance of the seedlings was tested after 0, 19, 35 and 51 d hardening. In this investigation the plants were frozen at −10 °C for 2 d, then the leaves of the plants were cut off and after 18 d cultivation at 17/16 °C the re-growth was scored on a scale between 0 (no re-growth) and 5 (very good re-growth).

Determination of cysteine, γEC and GSH. The plant material was ground with liquid nitrogen in a mortar, then 1 ml of 0.1 M HCl containing 1 mM Na_2EDTA was added to each 100-mg plant sample. After mixing, the samples were centrifuged for 20 min at 15 000 *g* at 4 °C.

For the determination of total thiols, 120-μl aliquots of supernatant were added to 180 μl of 0.2 M 2-[cyclohexylamino]-ethanesulfonic acid (pH 9.3) and reduced with 30 μl of a freshly prepared 400 mM $NaBH_4$ solution. The mixture was kept on ice for 20 min. For derivatisation, 15 μl of 15 mM monobromobimane was added and the samples were kept in the dark at room temperature for 15 min. The reaction was stopped with 250 μl 5% (v/v) acetic acid.

When determining reduced and oxidised thiols, for measurements on the total disulfides, the reduction was carried out with 3 mM dithiothreitol instead of $NaBH_4$. For the detection of oxidised thiols, 600 μl of 0.2 mM 2-[cyclohexylamino]ethane sulfonic acid (pH 9.3) was added to 400 μl extract, and the free thiols were blocked with 30 μl 50 mM N-ethylmaleimide. The excess of N-ethylmaleimide was removed by extracting five times with equal volumes of toluene, after which 300 μl of extract was reduced with 30 μl of 3 mM dithiothreitol. Derivatisation was done as described for total thiols and the reaction was stopped with 250 μl of 0.25% methane sulfonic acid.

The derivatised samples were analysed as described by Schupp and Rennenberg (1988) and modified by Rüegsegger and Brunold (1992) after separation by reverse-phase HPLC (Waters) using fluorescence (W 474 scanning fluorescence detector; Waters) and radioactive (Flow Scintillation Analyzer; Canberra-Packard, Meriden, Conn. USA) detection, respectively. A mixture of the four thiols at different concentrations (0, 1, 2, 3, 4, 5 mM) was used for their qualitative and quantitative identification, as well as for the recovery experiments. To determine the recovery, the amount of the individual thiols was measured using the plant sample (T_p), the standards (T_s) and the mixture of plant sample and internal standards (T_{p+s}). The recovery was calculated for each thiol with the following formula: recovery(%) = $(T_{p+s} - T_p) \times 100/T_s$. The recovery of the individual thiols varied between 90 and 95% in shoots and roots, respectively.

Protein determination. For the measurement of total protein (Brunold and Suter 1984), 50 mg of plant material was extracted in 1 ml 0.6 M NaOH. After 12 h at 4 °C, 400 μl of 11.64 M $HClO_4$ was added and the extracts were kept at 4 °C for 4 h. Following

centrifugation at 2200 *g* for 10 min and the removal of the supernatants the pellets were dissolved in 1 ml 1 M Tris-HCl (pH 9.0) and the protein content was estimated as described by Bradford (1976) using bovine serum albumin as the standard. The reaction mixture consisted of 995 µl of protein assay reagent and 5 µl of extract. These extracts were used for the detection of the ^{35}S incorporated in the proteins.

Assay for GR. The plant material was homogenised 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) polyvinyl-polypyrrolidone in an ice-cooled glass homogeniser and centrifuged at 30 000 *g* for 10 min at 4 °C. The supernatant was used for measuring GR activity according to Smith et al. (1988). The assay mixture contained 100 mM Na-K-phosphate (pH 7.5), 0.2 mM diethylenetriamine pentaacetic acid, 0.75 mM 5,5′dithiobis(2-nitro-benzoic acid), 0.1 mM NADPH, 0.5 mM GSSG and 50 µl of plant extract in a total volume of 1 ml. To achieve maximum GR activity, 10 mM dithiothreitol was added.

Statistics. Data of six measurements from three independent experiments were compared using two-component (treatments, genotypes) analysis of variance. The significant differences were calculated with the student's t-test, the mean differences were compared pairwise with the Tukey test (Systat for windows).

Chemicals. [^{35}S]Sulfate was obtained from NEN Life Sciences (Boston, Mass., USA), monobromobimane from Calbiochem, γEC from Nacalai Tesque (Kyoto, Japan), 2-[cyclohexylamino]ethane sulfonic acid from Fluka, N-ethylmaleimide and methane sulfonic acid from Sigma and methanol from Baker (Deventer, The Netherlands). Hydroxymethylglutathione was synthesised by the Research Group of Peptide Chemistry at the Eötvös University (Budapest, Hungary). All other chemicals were purchased from Reanal (Budapest, Hungary).

Results

After 3 d of hardening, when GSH synthesis as well as in vivo sulfate assimilation were compared in cold-hardened and non-hardened seedlings, no significant difference was found in the fresh weights (data not shown).

The maximum frost tolerance was observed after 19 d hardening in Ch and after 35 d hardening in the other genotypes (Table 1). The greatest increase in frost tolerance was detected in Ch and CS (Ch 5A) during hardening. After achieving the maximum level, the frost tolerance decreased again except for Ch, where it remained at a high level during the whole experiment. The substitution of chromosome 5A of CS with the same chromosome of Ch or Tsp resulted in increased and reduced frost tolerance, respectively, compared to CS.

Thiol accumulation was induced by hardening, as shown by the increased total cysteine and glutathione contents of the shoots (Fig. 1). During prehardening (10/5 °C, day/night, 7 d) a slight increase in these

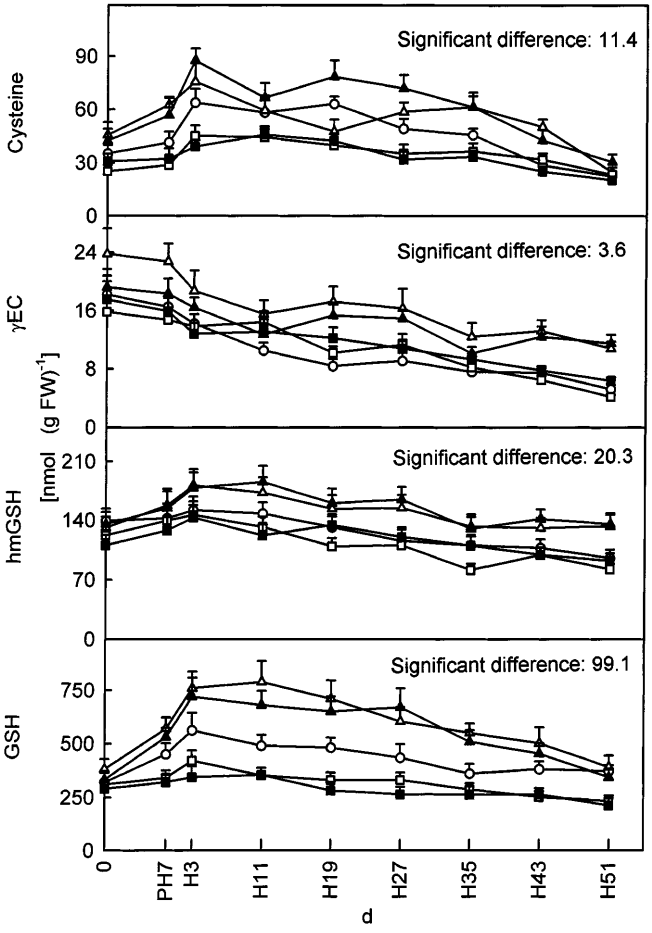

Fig. 1. Total cysteine, γEC, hmGSH and GSH contents in shoots of five wheat genotypes during prehardening (*PH*, 10/5 °C, 7 d) and a subsequent hardening (*H*, 4/2 °C, 51 d). Mean values and significant differences of six measurements from three independent experiments are presented. △, Ch; ○, CS; □, Tsp; ▲, CS (Ch 5A); ■, CS (Tsp 5A)

Table 1. Survival of wheat seedlings prehardened at 10/5 °C for 7 d, subsequently hardened at 4/2 °C for different durations and subjected to a freezing test at −10 °C for 2 d. Survival was evaluated after 18 d re-growth at 16/17 °C. 100%: all plants survived, very good re-growth; 0%: all plants died, no re-growth. Three parallel experiments with five plants were tested after different durations of hardening. Mean values ± SD of six measurements from three independent experiments are presented. Values carrying different letters are significantly different at the $P < 5\%$ level

Days at 4/2 °C	Survival (%)			
	0	19	35	51
Ch	0.0 ± 0.0^a	99.7 ± 9.6^a	100.0 ± 9.8^a	99.0 ± 8.6^a
CS	0.0 ± 0.0^a	16.0 ± 3.7^b	55.7 ± 6.4^b	4.3 ± 1.3^b
Tsp	0.0 ± 0.0^a	5.2 ± 1.8^c	13.9 ± 3.2^c	0.0 ± 0.0^c
CS (Ch 5A)	0.0 ± 0.0^a	67.0 ± 8.5^d	87.0 ± 8.9^a	6.1 ± 1.7^b
CS (Tsp 5A)	0.0 ± 0.0^a	7.1 ± 1.4^c	24.6 ± 5.7^d	1.0 ± 0.3^d

compounds was observed, but during the first 3 d of subsequent hardening (4/2 °C, day/night) a high rate of accumulation was detected in Ch and CS (Ch 5A). Subsequently, the total glutathione content remained high until the fourth week of hardening in these two genotypes, after which it decreased rapidly. In CS a moderate induction of GSH accumulation was found, while in Tsp and CS (Tsp 5A) only a slight increase in its level was measured. In all the genotypes a moderate increase in total hmGSH was observed. The total γEC content decreased gradually during the whole hardening period. The lack of an initial increase in γEC content can be explained by the relatively small size of its pool and the increased γEC demand for GSH synthesis. The size of the pool of all the thiols examined was greater in Ch and CS (Ch 5A) compared to the other genotypes during the whole experiment. The substitution of the 5A chromosome of CS with the same chromosome of Tsp reduced the total glutathione content of CS to a level similar to that observed in Tsp.

To determine changes in the redox state of the thiol pools after 35 d hardening, when the greatest frost tolerance was detected, the levels of reduced and oxidised thiols were also investigated (Fig. 2). Like the total thiol contents in the time-course experiment, the greatest reduced cysteine, hmGSH and GSH contents were detected in the frost-tolerant genotypes Ch and CS (Ch 5A). Under the control conditions the ratio of reduced to oxidised thiols (about 10) was not significantly different in the genotypes examined (data not shown). Hardening resulted in a greater ratio of reduced and oxidised hmGSH and GSH, as well as in a correspondingly greater GR activity in Ch and CS (Ch 5A) compared to the other genotypes (Table 2).

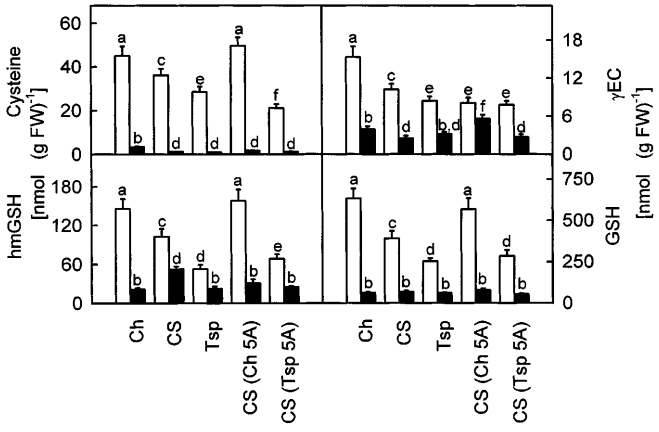

Fig. 2. Reduced (*open columns*) and oxidised (*checkered columns*) cysteine, γEC, hmGSH and GSH contents in shoots of five wheat genotypes after 7 d prehardening at 10/5 °C and a subsequent 35 d hardening at 4/2 °C. Mean values ±SD of six measurements from three independent experiments are presented. Values carrying different letters are significantly different at the $P < 5\%$ level

Since the greatest changes in glutathione accumulation were detected during the first 3 d of hardening, more-detailed examinations were carried out in this period. The total thiol levels were compared in the shoots and roots of control and hardened seedlings (Fig. 3). Under control conditions the total cysteine and γEC in the shoots and the hmGSH content in the roots of Ch were greater compared to the other genotypes. Although the total glutathione level was similar without hardening in all the genotypes, cold treatment induced a greater increase in this level in the shoots and roots of Ch and CS (Ch 5A). This large induction of GSH accumulation could be the result of a correspondingly large increase in cysteine synthesis, because the level of this thiol was also greater in these two genotypes. The total hmGSH content was 2–3 times less than the total GSH content and its level was greatest in the shoots and roots of Ch and CS (Ch 5A) after hardening. The total γEC content did not change or was even lower after 3 d hardening compared to the control seedlings in all the genotypes.

To check whether the cysteine used for GSH synthesis was derived from the breakdown of proteins or from de novo synthesis, [^{35}S]sulfate was added to the nutrient solutions and the incorporation of ^{35}S into cysteine, γEC, hmGSH and GSH was investigated. The uptake of radioactive sulfate by the roots and its transport to the shoots decreased during hardening in all the genotypes (Fig. 4). As in the non-radioactive experiment (Fig. 3) the amounts of ^{35}S-labelled cysteine and GSH were greater after 3 d hardening in the shoots and roots of Ch and CS (Ch 5A) than in the other genotypes (Fig. 5). Hardening also increased the level of ^{35}S in hmGSH and this increase was significantly greater in the roots of Ch and CS (Ch 5A) compared to the other genotypes. The incorporation of radioactivity into γEC was less intense

Table 2. Activity of GR and ratios of the contents of reduced and oxidised cysteine, γEC, hmGSH and GSH in shoots of five wheat genotypes after 7 d prehardening at 10/5 °C and a subsequent 35 d hardening at 4/2 °C. Mean values ± SD of six measurements from three independent experiments are presented. Values carrying different letters are significantly different at the $P < 5\%$ level

	GR [μkat . (g prot.)$^{-1}$]	Reduced/Oxidised			
		Cysteine	γEC	hmGSH	GSH
Ch	5.8 ± 0.6^{a}	14.7 ± 2.1^{a}	3.9 ± 0.4^{a}	6.7 ± 0.7^{a}	10.4 ± 1.6^{a}
CS	3.2 ± 0.4^{b}	31.5 ± 4.5^{b}	4.1 ± 0.3^{a}	1.9 ± 0.2^{b}	5.8 ± 0.6^{b}
Tsp	2.5 ± 0.2^{c}	30.2 ± 3.9^{b}	2.6 ± 0.2^{b}	2.5 ± 0.3^{c}	4.2 ± 0.5^{c}
CS (Ch 5A)	4.8 ± 0.5^{a}	28.9 ± 3.1^{b}	1.4 ± 0.1^{c}	5.4 ± 0.6^{a}	7.3 ± 0.8^{d}
CS (Tsp 5A)	2.2 ± 0.3^{c}	18.3 ± 2.7^{a}	2.8 ± 0.3^{b}	2.7 ± 0.2^{c}	5.1 ± 0.4^{a,b}

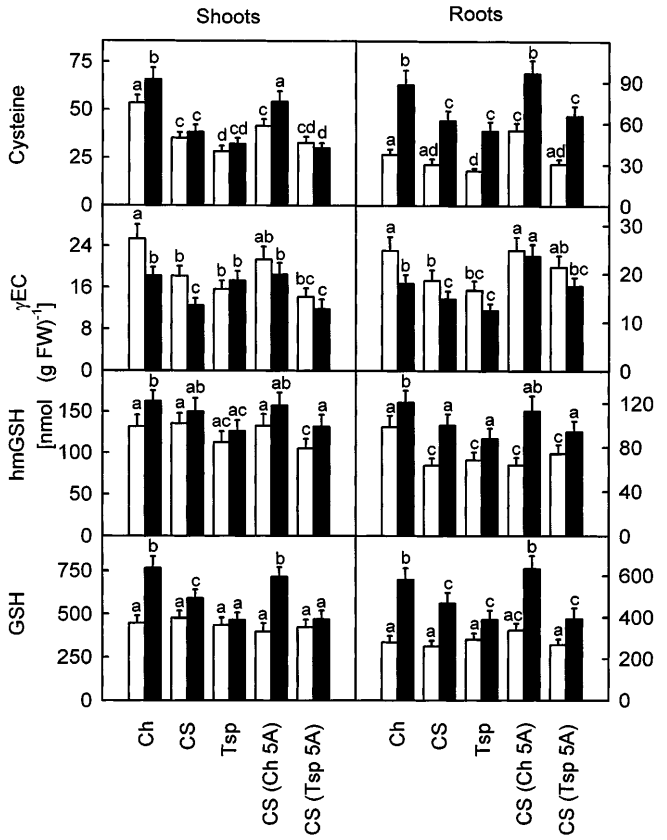

Fig. 3. Total cysteine, γEC, hmGSH and GSH contents in shoots and roots of five wheat genotypes after 10 d at 15/10 °C (*open columns*) or 7 d prehardening at 10/5 °C and a subsequent 3 d hardening at 4/2 °C (*checkered columns*). Mean values ±SD of six measurements from three independent experiments are presented. Values carrying different letters are significantly different at the $P < 5\%$ level

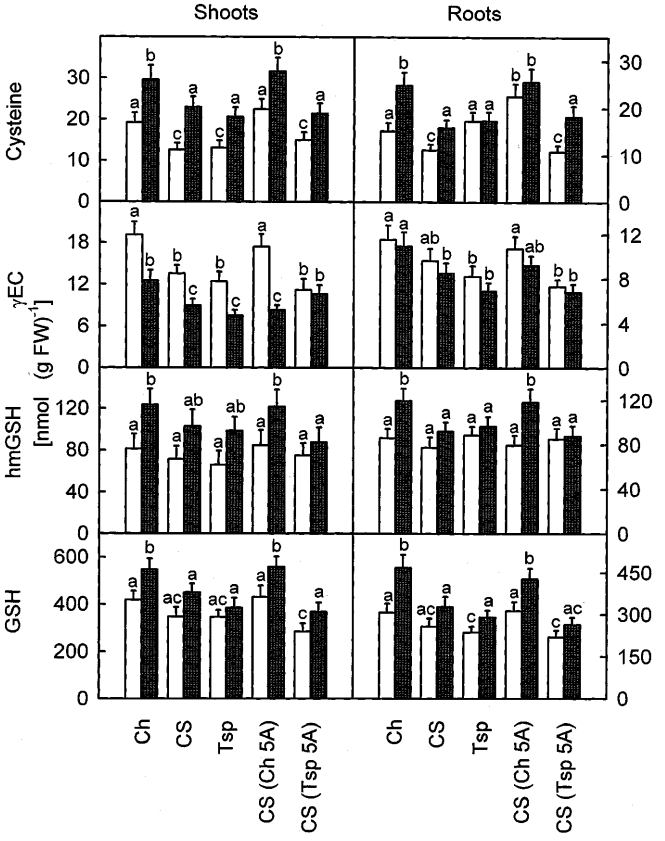

Fig. 5. ^{35}S-Radiolabelled compounds in shoots and roots of five wheat genotypes after 10 d at 15/10 °C (*open columns*) or 7 d prehardening at 10/5 °C and a subsequent 3 d hardening at 4/2 °C (*checkered columns*) with [^{35}S]sulfate. The amounts of radioactive compounds were calculated using the specific radioactivity of the [^{35}S]sulfate applied. Mean values ±SD of three independent experiments are presented. Values carrying different letters are significantly different at the $P < 5\%$ level

in the shoots of hardened seedlings than in the control ones, but no difference was observed in the roots.

To record the changes induced by hardening in the amount of cysteine used for GSH and protein synthesis, the incorporation of ^{35}S into proteins was also studied (Fig. 6). Hardening resulted in a great reduction in the radioactivity of the proteins in the shoots of all the genotypes. The ^{35}S-incorporation in the proteins of the roots also decreased except in Ch and CS. The total protein content did not change during the first 3 d of hardening (data not shown).

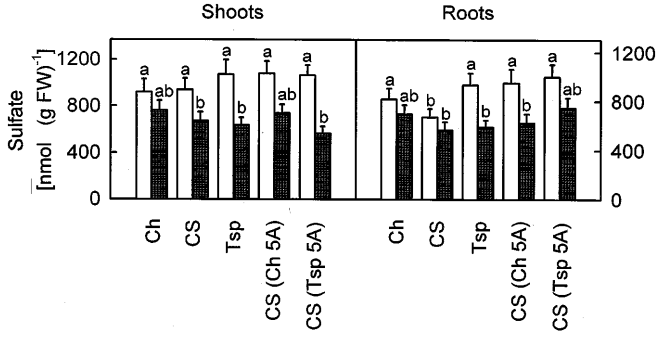

Fig. 4. [^{35}S]Sulfate in shoots and roots of five wheat genotypes after 10 d at 15/10 °C (*open columns*) or 7 d prehardening at 10/5 °C and a subsequent 3 d hardening at 4/2 °C (*checkered columns*). Mean values ±SD of three independent experiments are presented. Values carrying different letters are significantly different at the $P < 5\%$ level

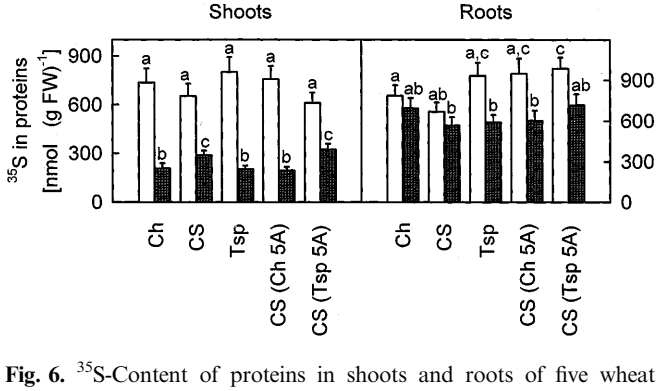

Fig. 6. ^{35}S-Content of proteins in shoots and roots of five wheat genotypes after 10 d at 15/10 °C (*open columns*) or 7 d prehardening at 10/5 °C and a subsequent 3 d hardening at 4/2 °C (*checkered columns*) with [^{35}S]sulfate. Mean values ±SD of three independent experiments are presented. Values carrying different letters are significantly different at the $P < 5\%$ level

Discussion

The results obtained in these experiments are consistent with the hypothesis that GSH enhances tolerance to low-temperature stress, since cold hardening induced both frost tolerance and GSH accumulation. Moreover, the data presented here prove this hypothesis from the genetical point of view, because the level of accumulation in chromosome substitution lines depended on the tolerance of the wheat genotype from which the 5A chromosome was derived. Like the results obtained in wheat, a greater GSH accumulation was found in tolerant genotypes of maize and tomato compared to the sensitive ones (Walker and McKersie 1993; Kocsy et al. 1996).

The accumulation of GSH due to its increased synthesis is only possible if sufficient precursors are available. Indeed, a higher activity of adenosine 5′-phosphosulfate sulfotransferase, the regulatory enzyme of sulfate reduction, was found in maize during low-temperature stress, resulting in an increased cysteine level (Kocsy et al. 1996). Low-temperature stress also induced an increase in glutamate and glycine (Szalai et al. 1997). Similar to the results obtained in maize, low temperature increased the rate of cysteine biosynthesis in wheat as shown in the sulfate-labelling experiments presented here. These higher cysteine levels, allowing a higher rate of GSH synthesis, might be responsible for the accumulation of GSH; however, a reduced GSH turnover cannot be excluded. The increased serine synthesis shown for maize at low temperature (Szalai et al. 1997) was shown here for wheat by the moderate increase in hmGSH. Since this increase was greater in the frost-tolerant Ch and CS (Ch 5A) genotypes than in the more sensitive genotypes, the accumulation of hmGSH, like the accumulation of GSH, contributes to the reduction of the low-temperature-induced injuries.

Cysteine can be used for both protein and GSH biosynthesis, so low temperature may result in an imbalance between the two biochemical pathways, as assumed for the increase in GSH content during cold acclimation in spinach (Stuiver et al. 1992). This hypothesis does not explain the present results, because during hardening the incorporation of ^{35}S into shoot proteins greatly decreased [300–500 nmol $(g\ FW)^{-1}$] in all the genotypes, while the radioactivity in GSH only showed a relatively large increase [120 nmol $(g\ FW^{-1})$] in Ch and CS (Ch 5A), with a moderate [60 nmol $(g\ FW^{-1})$] increase being detected in the other genotypes.

Similar to the present results, in other plant species the GSH level first increased then decreased during low temperature stress, but there were differences in the duration necessary for the attainment of maximum GSH content. In time-course experiments the maximum total glutathione content was detected after 3 d at 4/2 °C (day/night) in the leaves of wheat (present study) and after 2 weeks at 5 °C in the roots of jack pine (Zhao and Blumwald 1998). In callus tissue of *Arabidopsis* the greatest GSH content was measured after 2 d at 4 °C (O' Kane et al. 1998) and in soybean leaves after 1 week at 5 °C (Vierheller and Smith 1990). Although these results indicate the participation of GSH in the defence mechanisms induced during the first 1–2 weeks of low-temperature stress, Guy et al. (1984) found that increased GSH synthesis was not accompanied by an elevated level of frost tolerance.

While the initial large changes in total glutathione content indicate its role as an inducer of the stress response, as suggested by Foyer et al. (1997), the maintenance of the high GSH/GSSG ratio after 5 weeks of hardening in the tolerant genotypes also shows that the maintenance of this ratio may be important in ensuring a better adaptation to low-temperature stress. Consistent with this hypothesis, the GSH/GSSG ratio exhibited a dramatic decrease in the leaves of chilling-sensitive soybean during cultivation at 5 °C for 3 weeks (Vierheller and Smith 1990). Short-term chilling (2 °C, 3 d) of tomato decreased the GSH/GSSG ratio only in the sensitive genotype and the GR activity was also correspondingly less in this genotype (Walker and McKersie 1993). Thus, greater GR activity is important in the maintenance of an appropriate GSH/GSSG ratio, as observed in tolerant genotypes of wheat.

Maximum thiol levels were measured after 3 d hardening, while maximum frost tolerance was observed only after 19 d in Ch and after 35 d in the other genotypes, so the activation of the antioxidative system may be one of the first adaptive reactions during low-temperature stress, initiating the hardening process. Maximum frost tolerance will be achieved later, because of the induction of other adaptive mechanisms, such as the accumulation of carbohydrates (Galiba et al. 1997), free amino acids and polyamines (Galiba et al. 1989). Since chromosome 5A was also involved in the accumulation of these compounds (Galiba et al. 1992, 1993, 1997) it can be postulated that this chromosome carries regulatory gene(s) which induce a whole series of adaptive responses during low-temperature stress.

In conclusion, the results show that GSH accumulation and the maintenance of a high GSH/GSSG ratio, together with other biochemical changes, contribute to enhanced frost tolerance in wheat, and that chromosome 5A participates in the regulation of these stress-induced processes.

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