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Changes in the Callus Soluble Proteins of Winter and Spring Wheat Cultivars following Cold Treatment

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Abstract

Quantitative changes in total soluble proteins were determined in the embryogenic callus derived from a spring (cv. Niknejad) and in a winter (cv. Sabalan) cultivars of bread wheat (*Triticum aestivum* L.) exposed to 4°C for two weeks. Mature embryos were excised and inoculated on MS supplemented with 2.0 mg/l 2,4-D, 0.2 mg/l BAP, 3% sucrose at pH 5.8 and solidified with 8 g/l purified agar. They were incubated in darkness at 25 ± 1°C for five weeks. Calli were subcultured on fresh medium and grown in the above mentioned conditions for 14 days. Half of the Petri dishes were transferred to 4 ± 1°C on day 14 for two weeks (cold treatment), and they were returned to 25°C. The other half was maintained at 25°C throughout the experiment (control treatment). Over the experimental period (14 - 40 days), calli were sampled at random every 48 h (3 X 0.5 g) for total fresh weight measurements. Total callus soluble proteins were extracted and their concentration was determined by a colorimetric method. Significant cold-induced increases in protein quantity occurred during the low temperature treatment, irrespective of the cultivar compared with the controls. Both wheat cultivars showed differences in their callus soluble proteins during the experimental period in response to temperature alterations.

Introduction

Temperature is an important environmental factor that limits the productivity and survival of plants in large areas of the world (Boyer 1982). Exposure to low, non-freezing temperatures induces a variety of changes in plants that can result in the development of cold hardiness (Ougham and Howarth 1988, Howarth and Ougham 1993, Hughes and Dunn 1996). For example, low

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temperature can result in the synthesis of cold shock proteins (Hughes and Dunn 1996). In many plant species e.g. *Lolium temulentum*, progressive alterations in gene expression occur following their exposure to low temperatures around 5°C; these alterations occurred in parallel with an increase in chilling or freezing tolerance (Ougham and Howarth 1988). Such tolerance is referred to as hardening; altered gene expression is an essential component of the hardening process (Howarth and Ougham 1993). One feature of cold hardening in some species is an increase in soluble proteins, content e.g. alfalfa (*Medicago sativa*) (Mohapatra et al. 1987), wheat (*Triticum aestivum*) and rye (*Secale cereale*) (Cloutier 1983, Hughes and Dunn 1996; Sarhan et al. 1997, Kolesnichenko et al. 2000) and canola (*Brassica napus*) (Lee et al. 2002, Karimzadeh et al. 2003). The accumulation of high molecular weight (200 kDa; Howarth and Ougham 1993, Sarhan et al. 1997 and 310 kDa; Kolesnichenko et al. 1997) polypeptides is a major change that occurs during hardening of wheat.

Increases in levels of certain RNA species as a result of cold exposure (5°C) in alfalfa and spinach were detected by Mohapatra et al. (1987) and Guy and Haskell (1987). Changes in gene expression and the synthesis of cold shock or cold-acclimation proteins were reported to be correlated with enhanced cold tolerance (Hughes and Dunn 1996). Synthesis of specific proteins is an important mechanism involved in increasing freezing tolerance during cold acclimation (Crosatti et al. 1994, Sarhan et al. 1997, Guy 1999). The low temperature-induced accumulation of proteins (12, 26, 40, 50, 66, 180 and 200 kDa) encoded by the WCS 120 gene family was reported for more that 20 wheat genotypes (Sarhan et al. 1997).

In this work, we examined whether spring and winter cultivars of wheat responded differently to cold treatments through differential protein accumulation. The aim of the work reported here was to test this hypothesis by comparing quantitative accumulation the total soluble proteins in the callus of spring and winter cultivars of bread wheat in treatments of temperature shifts from 25 to 4°C and back to 25°C.

Materials and Methods

Seeds of a spring (cv. Niknejad) and a winter (cv. Sabalan) wheat (*Triticum aestivum*, 2n = 6x = 42) cultivars supplied by The Seed and Plant Improvement Institute (SPII), Karaj, Iran, were surface sterilized with a 0.1% (w/v) $HgCl_2$ solution, containing a few drops of Tween 20 for 8 min and then rinsed 3 - 5 times in sterile distilled water. The seeds were then soaked in sterile distilled water for 20 h at room temperature (Chlyah et al. 1990) before embryos were excised.

Under aseptic conditions, the seed coat was stripped off and the mature embryo was separated from the endosperm with a scalpel. In order to establish more calli, embryos were cut longitudinally into two parts by a sharp scalpel (Chlyah et al. 1990) and were then placed face down on to the nutrient agar medium. Excised embryos were inoculated on MS supplemented with 2.0 mg/l 2,4-D, 0.2 mg/l BAP, 3% sucrose at pH 5.8 and solidified with 8 g/l purified agar. They were incubated in the darkness (He et al. 1986) at 25 ± 1°C for five weeks. Five embryos were cultured in a Petri dish (100 mm diam ∇ 15 mm depth), containing 30 ml of sterile culture medium. Ten Petri dishes per cultivar per temperature treatment per sample time were established.

In early stages of formation (seven days), the embryogenic callus was glistening pale yellow with a smooth surface but eventually it became compact in appearance. The established calli were incubated for five weeks. Calli were subcultured three times at a five-week interval on fresh culture medium. Following subcultures, small roots formed and necrotic tissues were carefully and aseptically removed from the callus under aseptic conditions. For experimental treatments, calli were subcultured on a fresh culture medium and were grown for 14 days. Thereafter a half of callus pieces were transferred to a constant temperature at 4 ± 1_C for two weeks (cold treatment) and they were returned to the original growth chamber at 25°C. The other half was maintained at a constant 25°C throughout the experimental period (control treatment).

Sampling times : Over the experimental period (14 - 40 days), 3 ∇ 0.5 g of total callus fresh weight samples (Crosatti et al. 1996, Dunn et al. 1998) were sampled at random every 48 h from ten Petri dishes of five callus pieces. In other words, the sampling time for low temperature treatment combination of each cultivar was 14 days (before transfer to 4°C), 16, 18, 20, 22, 24, 26, 28 (during exposure to 4°C); 30, 32, 34, 36, 38 and 40 (after the return of cold-treated calli to 25°C). Samples were also taken from the controls following the same time schedule as above.

Protein extraction : Total soluble proteins were extracted from the calli. The method described by Guy et al. (1992) was modified to obtain better results. This method consisted of homogenizing the tissue in a chilled mortar and pestle, using a buffer containing ice-cold 50 mM Tris-HCl, pH 7.5; 2 mM EDTA and a 0.04% (v/v) 2-mercaptoethanol. The homogenate was centrifuged at 4000 rpm for 30 min at room temperature (Masoodi-Nejad and Yazdi-Samadi 1992). The supernatant was re-centrifuged for 20 min and stored at –20°C for later analysis (Hames and Rickwood 1990).

Quantification of proteins using the Bradford assay : Protein extracts were thawed and their concentration was determined by a colorimetric method based on the method described by Bradford (1976), using a commercially available reagent (Bio-Rad protein assay dye reagent). The protein concentration of the extracts was determined from the standard curve, using an Unicam 8620 UV/VIS (USA) Spectrophotometer at 595 nm.

Statistical analysis : The quantitative amounts of total proteins were statistically analysed using three-factorial balanced analysis of variance (ANOVA) on the basis of randomized complete design (RCD) with three replications. Cultivars, temperature treatments and sampling times were considered as factors with 2, 2 and 14 levels, respectively. Analysis of variance was conducted using Multi-Factorial Balanced Model in MINIAB Statistical Software (Minitab Inc., State College, PA, USA; Fry 1993, Ryan and Joiner 2001); identification of differences in response of the cultivars to the temperature treatments at the sampling time are based on the outcome of these tests. An additional analysis was carried out in order to test between temperature treatment differences within each cultivar at each sampling time (Yazdi-Samadi et al. 1998).

Results and Discussion

Quantitative proteins analysis : Considering all sampling times, in spring wheat, ANOVA analysis showed that there were significant differences between-temperature treatments, between-sampling times and their interactions ($P < 0.001$; Table 1). An additional statistical analysis was also carried out to test between-temperature treatments difference within each cultivar at each sampling time. According to this analysis, a low temperature treatment resulted in an increase in total protein amounts in the cold-treated callus of spring wheat on day 18 (four days following 4°C) compared with that in the control (Fig. 1a). When such calli were transferred from 4 to 25°C, the amount of proteins decreased considerably with time (30 to 40 days; $P < 0.01$; Fig. 1a) in comparison with the control. In spring wheat on day 40, the protein amount in the cold-induced calli was 53.5% less than that in the control calli. In agreement to our data, Chauvin et al. (1993) reported that protein accumulation in wheat cv. Glenlea, which was less tolerant to the first days of cold exposure (4_C), was faster at the start of cold treatment which then decreased with time. When such materials were transferred from 4 to 24_C the protein accumulation decreased rapidly afterwards. Such response was also detected in our data in the spring wheat cultivar, indicating that this response

probably could be one of the reasons for the non- or less tolerance of the spring wheat seedlings to cold stress.

In winter wheat, there were no significant differences between temperature treatments ($P > 0.05$) but the sampling times and interactions were significant ($P < 0.01$; Table 1), showing that the temperature effect was different on the amount of callus soluble proteins in various sample times during or after the cold exposure period. The changes of soluble proteins in the callus

Table 1. Mean squares of analysis of variance of temperature treatments effect on total soluble proteins amount in the callus of a spring (cv. Niknejad) and a winter (cv. Sabalan) wheat cultivars.

Winter wheat	Spring wheat	df	S.O.V.
0.001^{ns}	0.088**	1	Temperature treatments (TT)
0.011*	0.039**	13	Sampling times (ST)
0.010*	0.016**	13	TT ∇ ST
0.001	0.001	56	Error

ns, * and ** = Non significant at 0.05, significants at 0.01 and 0.001, respectively.

of winter wheat in response to temperature alteration is shown in Fig. 1b. Additional analysis showed that the amount of proteins in cold-treated callus of winter wheat increased significantly ($P < 0.01$) on experimental days 22 and 24 (8 and 10 days following cold exposure, respectively; Fig. 1b) compared with the control. From day 22 the proteins content decreased with time in cold-induced calli until day 28 (end of the 14 days cold exposure period) after which these calli produced proteins to the same level as in the controls ($P < 0.05$). In other words, the return of cold-treated materials to 25°C resulted in the production of proteins in the callus to levels similar to those in the controls in the winter wheat cultivar (Fig. 1b). In agreement to our data on winter wheat, Houde et al. (1992) reported a gradual accumulation of proteins in winter wheat (cvs. Fredrick and Norstar) cultivars during the first days of cold exposure period (21 days at 4°C). Following the return to 20°C, cold-induced seedlings produced proteins to the same level as in the control. In the present work, this response of cold-treated winter wheat in sustaining proteins level in the callus to that in the control level may reflect the potential of winter callus to withstand low temperature stress better than the spring wheat cultivar.

Karimzadeh et al. (2000) studied low temperature-induced accumulation of proteins per DNA C-value in roots and in callus of a spring and a winter cultivar of bread wheat under the same temperature treatments as described in the present report. They also reported the accumulation of proteins

in root meristems and calli. They reported a 2.5-fold increase in total proteins in the cold-induced embryogenic callus of winter wheat at seven days at 4°C in comparison with the controls (20°C), and a 2.2-fold increase of proteins

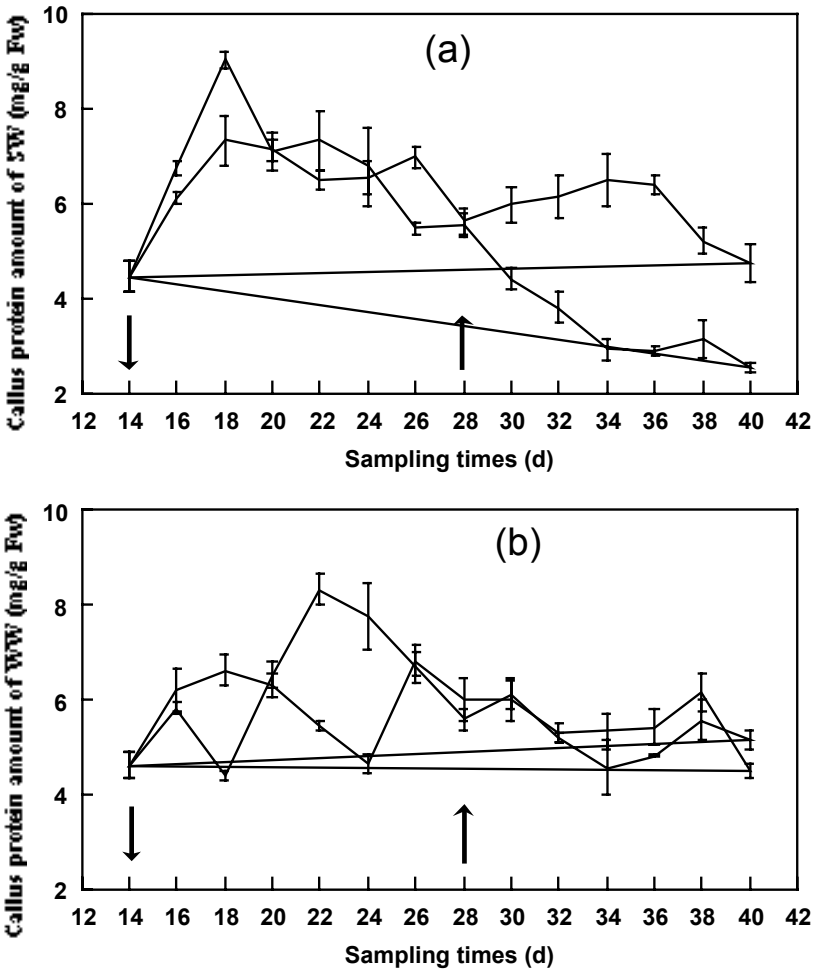

Fig. **1**. The curve showing the changes of mean quantitative amount (mg/g Fw) of total callus soluble proteins of (**a**) spring wheat cv. Niknejad and (**b**) winter wheat cv. Sabalan grown either at a constant 25°C (solid line with filled symbols) or at 25_C followed by transfer to 4°C on day 14 for 14 days and then transferred back to 25_C on day 28 (dotted line with open symbols). The arrows show the timing of the downshift and upshift of temperatures. Bars represent S.E. but where absent, the variation about the mean was less than the diameter of the symbol (n = 3). SW = Spring wheat, WW = Winter wheat, d = day.

compared with those recorded in cold-treated calli of spring wheat during the same period of time. They attributed this accumulation to low temperature with inherent cellular response, to the proliferating potential of callus cells. In another work on barley, low temperature induced increases in leaf soluble protein quantity over the second week after the exposure to 4°C, irrespective of

spring and winter cultivars: these changes were detected in the winter cultivar two days before comparable changes in the spring cultivar (Unpublished data). Such cold-induced response was also detectable in non-cereal plants, e.g. canola. spring and winter cultivars of canola seedlings were exposed to 4°C for three weeks. Karimzadeh et al. (2003) reported clear cold-induced increases in soluble proteins at low temperature treatments in the leaves of winter canola, but not in those of spring canola. In winter canola, these increases appeared eight days following the cold exposure and were sustained until two days following their return to 15/10°C (day/night).

Sarhan and Perras (1987) studied the changes of epicotyl protein patterns of three bread wheat (cv. Clenlea, spring wheat; cvs. Fredrik and Norstar, winter wheat) cultivars, following 10, 20 and 30 days at 2°C in the darkness and compared them with the seedlings maintained at 22°C for three, four and five days, correspondingly. They reported that low temperature resulted in the production of a new low temperature-induced protein with a molecular weight of 200 kDa. This protein was observed at a higher density in both winter wheat cultivars and in a lower density in the spring wheat. They also reported increases in the amount of three polypeptides (42, 47 and 48 kDa) and decreases in other five polypeptides (63, 67, 80, 89 and 93 kDa) in cold-acclimated epicotyles compared with non-acclimated epicotyls (Sarhan and Perras 1987).

Ougham and Howarth (1988) and Howarth and Ougham (1993) reported that prolonged exposure of *Lolium temulentum* to 5°C resulted in re-programming of gene expression. In this species, 5°C-grown seedlings synthesized polypeptides which were qualitatively different from those at 20°C and, together with low temperature-induced mRNAs, were linked to cold hardening (Howarth and Ougham 1993). Mohapatra et al. (1987) also detected increases in levels of certain RNA species as a result of cold exposure (5°C) in alfalfa. Moreover, Tabaei-Aghdaei et al. (2000) reported that dehydrin and non-specific lipid transfer protein (nsLTP) mRNA sequences and polypeptides increased more during acclimation to cold (6/2°C day/night for 14 days) and drought (watering withheld for six days) in *Agropyron desertorum* than in *Lophopyrum elongatum* crowns.

In wheat, cold acclimation rapidly induces the expression of a specific set of *Wcs* genes. Subsequently, their effects disappear upon de-acclimation. *Wcs* are regulated by low temperatures at the transcriptional level and winter wheat cultivars exhibit higher levels of expression than spring wheat cultivars (Limin et al. 1995). In this species and other cereals, the expression of several genes during cold acclimation was found to be positively correlated with the capacity of each genotype to develop freezing tolerance. Among

these, the *wcs 120* gene family in wheat encodes a group of highly abundant proteins ranging in size from 12 to 200 kDa (Sarhan et al. 1997).

Cloutier (1983) reported that two protein patterns of 30 and 40 kDa play an important role in freezing tolerance in six wheat cultivars following four weeks exposure to 2°C compared with seedlings kept at 24°C for two days. A feature of hardening of wheat is attributed to the accumulation of a HMW (200 kDa) polypeptide purported to protect important enzymes (Cloutier 1983, Perras and Sarhan 1989, Howarth and Ougham 1993). These reports indicate that the spring and winter wheat cultivars reported here may differ in producing polypeptides responding to low temperature treatments.

Limin et al. (1997) using the ditelocentric series of the hexaploid Chinese Spring wheat, mapped the *wcs 120*, *wcs 200* and *wcs 66* genes on 6DL, 6AL and 6BL, respectively. In general, wheat genotypes differ in their responses to low temperatures. Genetic studies on frost resistance in wheat are difficult because the effects are quantitative in nature. The diallel analyses indicated that the inheritance of frost resistance is polygenic and mostly additive (Sutka 2001). Using monosomic, ditelosomic and substitution lines, Sutka (2001) identified the chromosomes 5A and 5D to carry major genes (i.e. *Vrn-A1* (vernalization) and *Fr1* (frost resistance) loci located on 5AL and *Fr2* on 5DL). It was also pointed out that *Fr2* and *Vrn-D1* genes are homoeologous to *Fr1* and *Vrn-A1*.

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