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ARTICLE

Redox regulation of cold acclimation and vernalization in wheat

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ABSTRACT Winter cereals require cold acclimation to reach their maximum freezing tolerance and vernalization for the initiation of the transition from the vegetative to the reproductive phase. The aim of this study was to investigate whether these processes are under redox regulation and whether they effect the expression of the genes involved developmental and defence processes. A relationship was found between frost tolerance and redox changes. The developmental phase of shoot apices in examined plants was independent from the redox state of crown tissue. In the double ridge phase the frost tolerance was significantly increased. The expression of the genes involved in cold acclimation was induced by cold but their expression levels decreased from spike initiation. Moreover treatment of plants with reactive oxygen species and antioxidants improved the freezing tolerance and affected the expression of several genes, which confirms the assumed redox regulation of freezing tolerance.
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KEY WORDS

antioxidants
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reactive oxygen species
vegetative to generative transition

Cold acclimation is necessary for winter cereals to achieve their genetically determined maximum freezing tolerance and it also fulfils their vernalization requirement which is necesseray for the vegetative to generative transition and the correct timing of this process. Under natural conditions these processes occurs during autumn when the temperature gradually decreases and they can be induced by cold treatment under control conditions.

In the regulation of cold acclimation redox changes may have an important role (Foyer and Noctor 2005, 2009). Comparing wheat genotypes with different frost tolerance, a relationship was found between the level of their tolerance and alteration in the amount of glutathione (GSH) and in its ratio to glutathione disulphide (GSSG) during cold acclimation (Kocsy et al. 2000). Based on this observation it was supposed that other antioxidants and reactive oxygen species (ROS) may have important role in the acclimation process. In addition, their possible involvement in the vernalization was proved, too. The important role of ROS was verified in strawberry by Zhung et al. (2008) their results suggested that cold-hardening triggers an increase of ROS which induced the antioxidant defence system.

Materials and Methods

Cultivation and treatment of plants

The plant material consisted of the moderately freezing-sensitive spring wheat (*Triticum ae. ssp. aestivum*) variety Chinese Spring [CS] and CS chromosome 5A substitution lines in which the 5A chromosome derived either from the freezing-sensitive *T. aestivum ssp. spelta* (Tsp) [CS(Tsp5A)] or from the freezing-tolerant *T. ae. ssp. aestivum* cv. Cheyenne (Ch) [CS(Ch5A)]. The freezing tolerance of the substitution lines resembled to that of the parental genotype (Vágújfalvi et al. 1999). The seeds were obtained from the Martonvásár Cereal Gene Bank. After germination in Petri dishes at room temperature (25°C) for 3 days the seedlings were grown for 21 days in half-strength modified Hoagland nutrient solution (Kocsy et al. 2000) in a growth chamber (Conviron PGV-36; Controlled Env., Ltd., Winnipeg, Canada) at 18/15°C day/night temperature and 75/75% relative humidity, with 16 h illumination at 270 μmol m^{-2} s^{-1} and after that at 2°C (cold acclimation) for 22 days. To study the effect of cold treatment on various biochemical parameters the sampling was done after 1, 3, 7, 11 and 22 d in the middle of the light period in order to avoid the effects of circadian changes. For the investigation of the influence of developmental stage on the gene expression and antioxidants the plants were cultivated for 3 weeks at optimal temperature and later at 3°C, and the samples were collected at the vegetative, double ridge, and spike initiation stage. To see the effect of possible redox regulation of cold acclimation and vernalization the following chemicals were added to the nutrient solution for 3 d before the start of the 3-week cultivation at 5°C: no chemical, 1 mM glutathione (GSH), 1 mM glutathione disulphide (GSSG), 1mM buthionine sulphoximine (BSO), 1 mM H_2O_2, 10 μM

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paraquate, 1mM ascorbate, 100 mM NaCl, 15% polyethyleneglycol (PEG).

Morphology of shoot apices

The apices were isolated from the crowns before the start and at the end of the various treatments to see their effect on the development or weekly if the sampling had to be done at a certain developmental stage. The isolation was done under a Zeiss Stemi 2000-C stereomicroscope (Carl Zeiss Mikroskopie, Jena, Germany). Photos were taken using a Canon digital camera (Canon PowerShot G6).

Study of electrolyte leakage

This measurement was done according to Murray et al (1983). One cm long leaves pieces were cut from every treated and untreated plant. Each piece was placed in a 12 ml plastic vial to which 10 ml distilled water was added. These vials were mixed over night. Next day 4 ml was pipetted out into measuring cell and the actual conductivity (C_0 if the sample came from treated plant, C_{Intact} if the sample originated from untreated plant) was determined. Then the solution was put back in a vial and all samples were boiled in pressure cooker for 40 minutes to destroy all cell membranes. After this treatment total conductivity (C_{T0}, $C_{TIntact}$) value was obtained. The water had own conductivity (C_W) so correction was made by C_W. Relative conductivity was calculated according to the next formula: $\{((C_0-C_W)/(C_{T0}-C_W))*100\}-\{((C_{Intact}-C_W)/(C_{TIntact}-C_W))*100\}$, the result was given as percentage.

Determination of thiols and H_2O_2

The plant material was homogenized to powder with liquid nitrogen in a mortar, then 1 ml of 0.1 M HCl containing 1 mM Na_2EDTA was added to each 200 mg sample. After mixing by vortex the samples were centrifuged for 10 min at 15000 g at 4°C. For determination of total thiol concentrations, 120 µl aliquots of supernatant were added to 200 µl of 0.2 M 2-(cyclohexylamino) ethanesulfonic acid (CHES) pH 9.3 and the thiols were reduced with 10 µl 9 mM dithiothreitol. The dithiothreitol was freshly prepared. This mixture was kept on room temperature for one hour. Then thiols were derivatised by bromobimane. 20 µl dye was added to the samples and the labelling was carried out for 15 minutes in the dark. The reaction was stopped with 250 µl 0.25% methane sulfonic acid.

When determining thiol disulphides, free thiols were first blocked with N-ethylmaleimide. 200 µl extract were added to 300 µl of 0.2 M CHES and 15 µl 50 mM N-ethylmaleimide. The reaction mixture was kept at room temperature for at least 15 minutes. The excess of N-ethylmaleimide was removed by extracting three times with equal volumes of toluene, after which 300 µl of extract was reduced with 10 µl 9 mM dithiothreitol and 20 µl water was added, too. Then the reaction mixture was left at room temperature for one hour.

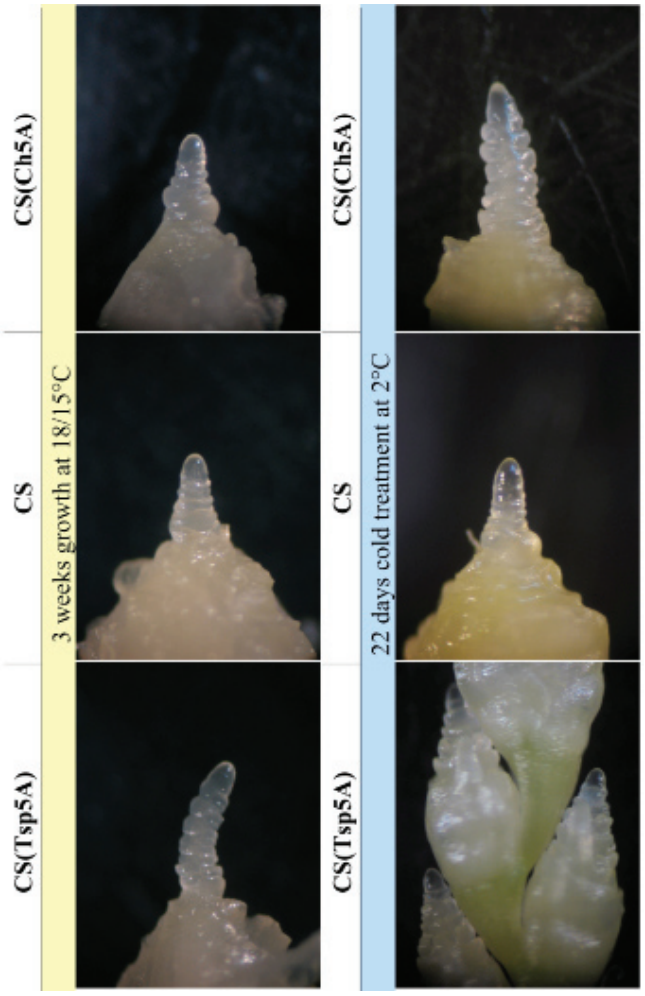

Figure 1. Effect of three weeks long cold treatment on vegetative/reproductive transition. Photos were taken from apices in fivefold magnification.

Derivatization was done as described for total thiols.

The prepared samples were analysed after separation by reverse-phase HPLC using fluorescent detection (W474 scanning fluorescence detector, Waters). The half-cell reduction potential of the GSH/GSSG redox couples was calculated according to Schafer & Buettner (2001).

H_2O_2 was determined as described previously (Kellős et al. 2008).

Gene expression studies

Total RNA was isolated by TRIzol reagent (Invitrogene) according to manufacturer instructions. The sample total RNA content was measured by NanoDrop2000 (Thermo Scientific). One µg RNA was used for cDNA synthesis. Reverse transcription was made using M-MLV Reverse Transcriptase and Oligo(dT) 15 primer (Promega) according to manufacturer

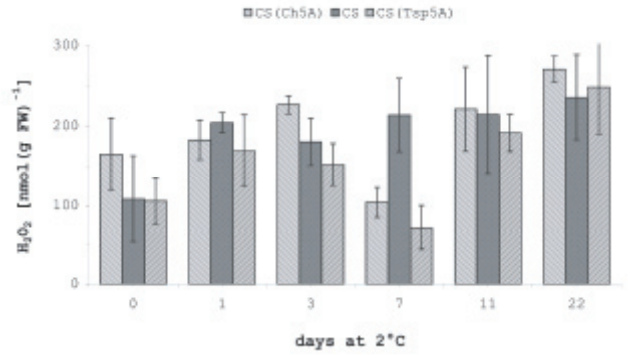

Figure 2. Effect of cold treatment on hydrogen peroxide content in the crowns. The H_2O_2 content was supplied in nmol/fresh weight explicated in grams. On the abscissa time (explicated in days) was represented.

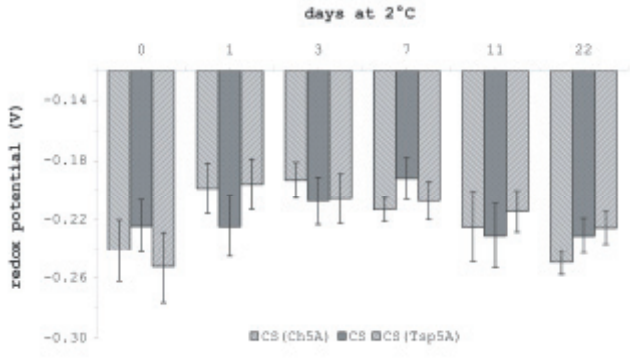

Figure 3. Regulation of reduction potential in the crowns by cold. Crowns of the freezing-tolerant CS(Ch5A) and the moderately freezing-sensitive CS and freezing sensitive CS(Tsp5A) wheat genotypes were compared. The redox potential was supplied in V. On the abscissa time was represented.

instructions. At the end of the synthesis the total amount was diluted 1.6-fold. For the expression studies usually 2µl cDNA was used and semiquantitative RT-PCR was done as described previously (Kellős et al. 2008).

Result and Discussion

The effect of three weeks long cold hardening (2°C) on development and biochemical parameters was determined. The three selected genotypes reacted differently to cold. Before the treatment the plants were grown at 18/15°C day and night temperature and CS and CS(Ch5A) genotypes were in the vegetative phase (VP) (Fig. 1). The frost sensitive spring genotype (CS(Tsp5A)) developed faster than the others and it reached the double ridge (DR) stage before cold treatment and the spike initiation (SI) phase after cold treatment. Spring sown cereals have shorter time for development, so they have to develop faster and they do not need growth at low temperature for the vegetative to generative transition. The frost-tolerant substitution line CS(Ch5A) was switched its developmental stage only after cold treatment. The moderately frost-sensitive CS developed very slowly irrespectively of the cold hardening.

Hydrogen peroxide content of the crowns was also measured in this experiment. Accumulation of H_2O_2 indicates increased oxidative stress in plants. Every abiotic stress agent cause oxidative stress in cells, so measuring of H_2O_2 is convenient for determining the strength of the actual effect of abiotic stress agents. In CS crowns amount of H_2O_2 was slightly increased in the course of cold treatment (in this experiment it was done at 2°C) (Fig. 2). In CS(Ch5A) after an early increase, on the 7th day sharp decrease was detected. Similar changes were noticed in the other substitution line. Maximum amount of H_2O_2 (approximately 250nmol/FW (g)) was detected at the end of the cold hardening in all of genotypes.

The half-cell reduction potential is dependent on the concentration of GSH and GSSG in the cells. After measuring the quantity of these thiols the half-cell reduction potential was calculated according to Schafer and Buettner (2001). It increased in all three genotypes during the first week of cold hardening. In CS its maximum, -192 mV was detected on the seventh day of treatment. (Fig. 3) Three interesting differences were determined among substitution and parental genotypes. In the beginning of the treatment CS had higher reduction potential compared to the substitution lines, this relation changed until the 3rd day and turned back on the 7th day. After the initial increase, the half-cell reduction potential decreased, and after 3 weeks cold treatment its level was lower in the freezing-tolerant genotype compared to the sensitive ones.

In another experiment freezing tolerance was determined during growth at 3°C at different developmental stages. Apices were checked to decide the suitable sampling time. CS(Tsp5A) reached the double ridge stage (vegetative/generative transition) after 50 day, while CS(Ch5A) after 80 days. This variance between the developmental speeds was due to their different growth habit. The vegetative/generative transition of spring habit CS(Tsp5A) line happened earlier. Electrolyte leakage measurement was used to determine frost tolerance. A relationship was found between the frost tolerance and developmental phase in CS(ChA5) (data not shown). The level of frost tolerance reached its maximum when the double ridges appeared on the apices. Similar changes were not observed in the other line. There were expressive differences among relative conductance which were detected at different freezing temperatures.

Expression of some genes, which belonged to four different groups, developmental, acclimation, antioxidative and apoptotic processes, were determined by semiquantitative RT-PCR. Samples were collected from leaves and crowns

separately. The transcript level of genes involved in the cold acclimation was induced by cold and decreased after the vegetative/reproductive transition. The *Vrn1* gene (Loukoianov et al. 2005) was expressed without cold treatment only in the CS(Tsp5A). Expressive differences were not found in the transcript level of genes which are coding important antioxidant enzymes (for example: γ-glutamylcysteine synthase, glutathione reductase, dehydroascorbate reductase etc.), but at low temperature these genes had higher expression levels in the leaves of CS(Tsp5A). Chromosome 5A-specific expression differences could be detected in the case of some development-, acclimation- and apoptosis-related genes (data not shown).

Beside these experiences, chemical agents were added to the nutrient solution of the plants (*Triticum spelta* and *T. ae.* cv. *Cheyenne*) before of cold treatment in order to check their possible effects on freezing tolerance, developmental stage and gene expression. The used chemical agents were GSH, GSSG, BSO, H_2O_2, paraquate, ascorbate, NaCl and PEG. According to first result most of the chemicals can increase freezing tolerance in both lines.

Triticum spelta (Tsp) had faster development both under cold and warm conditions, and after 6 weeks this genotype reached spike initiation phase independently of the temperature. Ascorbic acid and NaCl increased the speed of growth at cold and at warm conditions, respectively.

Some interesting changes were induced by the applied chemical treatments in the gene expression levels. Hydrogen peroxide induced the *Vrn1* gene in warm condition in Ch. This chemical agent also induced *Cbf12* gene in both lines. *Cbf12* was induced by PEG, too. All of the chemicals increased the expression level of galactonolactone dehydrogenase (*Gldh*) in both lines in warm conditions and in Tsp in cold except GSH which did not affected the expression of this gene.

In summary, the freezing tolerance of the examined genotypes showed a correlation with the level of hydrogen peroxide and antioxidants. This observation was confirmed by exogenous application of reactive oxygen species and antioxidants which affected freezing tolerance, too. The activation of defence processes was also shown at the level of gene expression. The vegetative to generative transition of shoot apex was modified by the addition to these chemical. The present experiments indicate the redox control of cold acclimation and vernalization.

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