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Mapping of genes involved in glutathione, carbohydrate and COR14b cold induced protein accumulation during cold hardening in wheat

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Abstract

Using some of the chromosome substitution lines developed from the crosses of the donor Cheyenne to Chinese Spring we showed that the accumulation of water soluble carbohydrates during different stages of hardening was time dependent. Moreover there was a significant correlation between the rate of carbohydrate accumulation and the frost tolerance. The expression and regulation of a wheat gene homologous to the barley cold regulated *cor14b* gene was compared in frost sensitive and frost tolerant wheat genotypes at different temperatures. Studies made with chromosome substitution lines showed that the threshold induction temperature polymorphism of the *cor14b* wheat homologous gene was controlled by loci located on chromosome 5A of wheat, while *cor14b* gene was mapped, in *Triticum monococcum*, onto the long arm of chromosome $2A^m$. Our study on the effect of cold hardening on glutathione (GSH) metabolism showed that chromosome 5A of wheat has an influence on the GSH accumulation and on the ratio of reduced and oxidised glutathione as part of a complex regulatory function during cold hardening. In addition, the level of increase in GSH content during hardening may indicate the degree of the frost tolerance of wheat.

Introduction

Cultivation of bread wheat (*Triticum aestivum* L., 2n = 6x = 42), particularly the winter wheat has a decisive role in European, North-American and Asian food production. For the successful cultivation tolerance to frost has been considered as the primary limiting factor for survival in most regions (McKersie & Leshem, 1994). The frost tolerance of winter wheat is controlled by a polygenic system (see details in the paper of Sutka et al., in this volume). Of the 21 chromosome pairs of wheat at least 10 are involved in the control of frost tolerance. Veisz and Sutka (1989) found that chromosomes 5A, 5B, 5D, 4B and 7A of the frost-tolerant variety Cheyenne increased the frost tolerance of the recipient frost-sensitive variety Chinese Spring to various levels. Major genes influencing frost tolerance (*Fr1*) and vernalisation requirement (*Vrn-A1*) were localised on the long arm of chromosome 5A (Galiba et al., 1995).

To achieve the full genetic potential of frost tolerance the plant must be hardened. Under natural conditions the cold hardening (acclimatisation) takes place in autumn when the temperature gradually decreases to 0 °C over several weeks. Temperatures of 2–5 °C and photoperiods of about 12 h are considered to be optimal for cold hardening under controlled environmental conditions. During cold acclimation a complex of responses in plants at the cellular, physiological and developmental levels took place resulting the enhancement of the frost tolerance. Among these we investigated the genetic regulation of carbohydrate accumulation, the expression and regulation of the wheat

homologue of the barley cold-regulated gene *cor14b* and the regulation of glutathione (GSH) biosynthesis.

It is well established that the accumulation of various osmotically active solutes, such as soluble carbohydrates, quaternary ammonium compounds, proline etc., takes place during cold hardening (Galiba, 1994; McKersie & Leshem, 1994). These may function as cryo-protectants, helping to avoid cellular dehydration during extracellular ice formation, and many of these compounds may also stabilise membranes. Carbohydrate changes during hardening are of particular importance because of their direct relationship with such physiological processes as photosynthesis, translocation and respiration. Sucrose can act in water replacement to maintain membrane phospholipids in the liquid-crystalline phase and to prevent structural changes in soluble proteins. Next to their role as plant carbohydrate reserves, fructans, as sucrose derived oligosaccharides, also play role in the stress-induced metabolic process (Housley & Pollock, 1993). Fructans accumulate in large amounts in *Gramineae* at low temperature, which might be the consequence of the low demand for photosynthates at low temperature. Numerous authors found a positive correlation between carbohydrate content and the degree of tolerance to abiotic stresses in cereals, suggesting that a higher sugar content increase hardiness (Hurry et al., 1995). We studied the Chinese Spring (Cheyenne) CS(Ch) chromosome substitution lines to elucidate the possible chromosomal location of gene(s) responsible for the elevated carbohydrate levels during cold stress conditions (Galiba et al., 1997; Vágujfalvi et al., 1999).

Glutathione (GSH), as an important component of the ascorbate-glutathione cycle, participates in the removal of hydrogen peroxide which may be accumulated during low temperature-induced oxidative stress (Prasad et al., 1994). GSH is synthesized in two steps (for reviews see Rennenberg and Brunold, 1994): first cysteine and glutamate are bound to γ-glutamylcysteine (γEC) by γEC synthetase, then a glycine is added to the dipeptide by GSH synthetase. 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). Glutathione reductase (GR) regenerates the reduced form of glutathione. 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 (Kocsy et. al., 2000).

Messenger RNAs corresponding to the cold-regulated gene *cor14b* (formerly *pt59*) are accumulated in barley leaves when plants are exposed to low temperature. The expression of *cor14b* is strictly regulated by cold (Cattivelli & Bartels, 1990), although it is enhanced by light-dependent factor(s) (Crosatti et al., 1999). Further studies have demonstrated that the *cor14b* gene encodes for the COR14b protein, which is cold regulated and imported into the chloroplast. (Crosatti et al., 1996). Several experimental evidences suggest a relationship between the accumulation of the COR14 proteins and frost resistance. In particular, it has been demonstrated in barley, that the threshold induction temperature for COR14 proteins is lower in frost sensitive than in frost tolerant cultivars (Crosatti et al., 1996) and, when evaluated under field conditions, winter barley accumulated more COR14b than spring ones (Giorni et al., 1999). A gene homologous to *cor14b* is expressed in response to low temperature in other monocots including wheat (Cattivelli & Bartels, 1990). Here we report on the regulation of the wheat gene homologous to the barley *cor14b*, the localization of the *cor14b* gene, and the approximate map position of two genes that regulate the temperature dependent expression of wheat *cor14b* homologous gene (Vágújfalvi et al., 2000).

Materials and methods

Plant material and growth conditions

Frost resistant and sensitive bread wheat (*Triticum aestivum*) genotypes and a sensitive *Triticum spelta* (*Tsp*.) accession were tested. Chinese Spring/Cheyenne (CS/Ch) 5A, 5D, and 7A, and CS/*Tsp.* 5A chromosome substitution lines as well as single chromosome recombinant lines, developed from the cross between CS/*Tsp.* 5A and CS/Ch 5A (Galiba et al., 1995) were used. The *cor14b* gene was mapped using an F_2 population from a cross between a cultivated *T. monococcum* (DV92) and a wild *T. monococcum* ssp. *aegilopoides* (G3116) (Dubcovsky et al., 1996).

For studying the time dependent changes of carbohydrates and GSH biosynthesis the seedlings were raised in garden soil in wooden boxes. The plants were grown in growth chamber (Conviron, Canada) at 15/10 °C day/night temperature for 2 weeks with 16 h illumination at 260 μmol 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 carbohydrate and thiol determination was harvested at the start and end of prehardening, after 3 d hardening and subsequently every 8^{th} day. The frost tolerance of the seedlings was tested in the same time as described by Vágújfalvi et al. (1999).

To study cold induced *cor14b* gene expression seedlings were raised hydroponically on modified Hoagland solution (Nagy & Galiba, 1995) for two weeks at 18 °C 16 h light (260 μmol m^{-2} s^{-1})/13 °C 8 h dark and subsequently cold hardened at 2 °C. Alternatively, plants were also grown at 25 °C for 6 d.

Biochemical and molecular analysis

Total water-soluble carbohydrate, glucose, fructose, sucrose, fructan and glucan contents were determined on fresh leaves of the plants according to Kerepesi et al. (1996). Determination of thiols GR, protein and ^{35}S-radiolabelled compounds written in details by Kocsy et al. (2000). The details of the western analysis of COR14b protein and the northern analysis of *cor14b* mRNA are described by Crosatti et al. (1999).

Results and discussions

Genetic regulation of carbohydrate content in the course of cold hardening

The frost resistance and endogenous carbohydrate contents of the plants were measured every 8 days in the course of cold treatment (Vágújfalvi et al., 1999). There was a continuous rise in the total water-soluble carbohydrate (WSC) content and in the fructan content as the hardening period proceeded. The increase in concentration was greater in the frost-resistant variety Ch and in the substitution lines CS(Ch 5A), CS(Ch 5D) and CS(Ch 7A) than in the sensitive genotypes CS and CS(*T. sp* 5A). A significant positive correlation was found between frost resistance, WSC and fructan content after 19 days of cold treatment. A significant correlation was exhibited between the fructose and sucrose contents and frost resistance after 43 days of cold treatment. In the chromosome substitution lines the accumulation of carbohydrates began after 11 days of cold hardening and reached a maximum after 35–43 days.

The sucrose and fructan contents in recombinant lines arising from a cross between the substitution lines CS(Ch 5A) and CS(*T. sp* 5A) were determined after cold hardening (Galiba et al., 1997). The *Fr1* (frost sensitivity) and *Vrn-A1* (spring habit) alleles originating from *T. spelta* did not increase the sucrose and fructan contents, while their concentrations increased significantly in recombinant lines carrying the alleles *vrn-a1* (winter habit) and *fr1* (frost resistance). In the 7-3 line, carrying the *vrn-a1* and *Fr1* genes (where recombination took place between the *Fr1* and *Vrn-A1* genes), a large accumulation of sugar was observed, indicating that the allele influencing carbohydrate accumulation was closely linked to the *Vrn-A1* gene.

Genetic regulation of glutathione content during cold hardening

The effect of cold hardening on the accumulation of glutathione (GSH) and its precursors was studied in the shoots and roots of different wheat genotypes. The fast induction of total GSH accumulation was detected during the first 3 days 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]sulphate. In Ch and CS (Ch 5A) the total cysteine, γ-glutamylcysteine (precursors of GSH), hydroxymethylglutathione and GSH contents were greater during the whole 51-day treatment than in the sensitive genotypes. After 35 d hardening, when the maximum frost tolerance was observed, a greater ratio of reduced and oxidised hydroxymethylglutathione and glutathione was detected in Ch and CS (Ch 5A) compared to the sensitive genotypes. Correspondingly, a greater GSH reductase activity was also found in Ch and CS (Ch 5A). It can be assumed that chromosome 5A of wheat has an influence on the GSH accumulation and on the ratio of reduced and oxidised glutathione as part of a complex regulatory function during hardening. Consequently, GSH may contribute to the enhancement of frost tolerance in wheat.

Mapping of the regulator and structural genes of the COR14b *protein in wheat*

Among the wheat plants raised under control (18/13 °C day/night) conditions the accumulation of *cor14b* mRNA was observed in the leaves of frost-resistant genotypes, but not in those of frost-sensitive varieties and lines (Vágújfalvi et al., 2000). At higher temperatures (25/18°C) there was no detectable quantity of *cor14b* mRNA in any of the genotypes. At low (2 °C) temperature all the varieties accumulated mRNA. As experienced previously in barley, gene expression was temperature- and tolerance-dependent, though the

temperature threshold at which the gene became expressed was higher in wheat than in barley. This result was confirmed by the Western blot analysis of the COR 14b protein. This was followed by the mapping of the gene regulating the expression of the *cor14b* gene. The results of Western and Northern analyses showed that the 5A chromosome carries genes responsible for the sensing of the threshold temperature. In the CS genetic background at 2°C the Ch 5A chromosome increased the quantity of *cor14b* mRNA. At 18/13 °C the COR 14b protein was only present in demonstrable quantities in the Ch variety and in the CS(Ch 5A) line, but not in the CS parent or in the CS(*T. sp* 5A) line.

The analysis of single chromosome recombinant lines derived from the cross between Chinese Spring/*Triticum spelta* 5A and Chinese Spring/Cheyenne 5A identified two loci with additive effect involved in the genetic control of *cor14b* homologous mRNAs accumulation. The first locus was positioned tightly linked with marker *psr911*, while the second one was located between marker *Xpsr2021* and *Frost resistance* gene *Fr1*. It was known that the structural gene was located on chromosome 2 in barley, but its position on the chromosome was unknown (Crosatti et al., 1996). The RFLP mapping of the structural gene was carried out using a *cor14b* gene cDNA probe on *Triticum monococcum* mapping population. The locus of the *cor14b* allele examined was mapped on the long arm of the $2A^m$ chromosome.

We can only speculate on the role of the COR14 protein. It probably helps to prepare the plants for cold hardening. Its accumulation in both wheat and barley was associated with a well-defined threshold temperature, so it is considered to be a useful biochemical marker for the selection of frost-resistant and frost-sensitive genotypes.

In conclusion, the adaptation processes related to frost resistance are regulated by polygenic manner. Dubcovsky et al. (1995) mapped dehydrin and *Esi* genes involved in osmoregulation and the loci of heat shock proteins to the region of the *Vrn* gene locus on the long arm of the fifth chromosome of *T. monococcum* ($5A^mL$). These results indicate that the 5A chromosome carries an 'adaptation gene complex' in the region of the *Vrn-A1* and *Fr1* genes. The development of this gene family regulating adaptation may have represented an advantage for the evolution. Recombination frequency is low for genes located close to each other, so the genes are inherited together and form a relatively large functional unit. As the result of continual selection pressure due to the winter climate, the grouping of the genes ensured a selective advantage for progeny generations.

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