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Differential expression of a gene encoding an acidic dehydrin in chilling sensitive and freezing tolerant gramineae species

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Abstract

We have characterized a new wheat cold-regulated cDNA clone, *Wcor410*, that accumulates to equivalent levels in root, crown and leaf tissues during cold acclimation. The *Wcor410* cDNA contains an ORF encoding a dehydrin-like glutamate-rich protein of 28 kDa with a pI of 5.1. However, the acidic nature, the absence of the glycine-rich repeat and of the conserved N-terminal region, DEYGNP, suggest that *Wcor410* belongs to a different subgroup of the D11 protein family. Northern analysis showed that this gene is expressed only in freezing tolerant gramineae, whereas Southern analysis showed that the *Wcor410* gene is present in all monocot species tested. The presence of freezing tolerance-associated genes in sensitive species such as rice and corn is interesting. Characterization of the regulatory factors controlling these genes may help to establish an appropriate strategy to improve freezing tolerance.

Key words: Freezing tolerance; Acidic dehydrin; Gramineae; cDNA

1. Introduction

It is now well established that cold acclimation induces several genes which may be involved in the development of greater tolerance to subzero temperatures [1,2]. Some of these genes are specifically induced by low temperature while others are also responsive to dehydration and the plant hormone, ABA. Sequence analyses have revealed that these proteins share certain features with fish antifreeze proteins [3], with transcription factors [4] and with proteins of dehydrins and Rab families [5,6]. However, no definite functions for these genes or their products have been demonstrated. Furthermore, the isolated genes represent only a small fraction of the genetic components involved in the acclimation process. In order to learn more about this complex genetic system, all the genes involved and their functions during cold acclimation must be identified. Towards this goal, we have isolated several new cDNAs that did not cross hybridize with those already isolated.

In this report, we describe a new gramineae-specific low temperature-responsive gene. The level of *Wcor410* mRNA increased during cold acclimation and water stress. Northern and Southern analyses of different species showed that the gene is present in all monocot species tested but expressed only in the freezing tolerant ones.

2. Materials and methods

2.1. Plant material and growing conditions

In this study we used: two spring wheat genotypes (*Triticum aestivum* L. cv Glenlea, LT_{50} (lethal temperature that kills 50% of the seedlings) −8°C; and cv Concorde, LT_{50} −8°C); 4 winter wheat genotypes (*T. aestivum* L. cv Monopole, LT_{50} −15°C; cv Absolvent, LT_{50} −16°C; cv Fredrick, LT_{50} −16°C; and cv Norstar, LT_{50} −19°C); winter rye (*Secale cereale* L. cv Musketeer, LT_{50} −21°C); oat (*Avena sativa* L. cv Laurent, LT_{50}−6°C); barley (*Hordeum vulgare* L. cv Winchester, LT_{50} −7°C); rice (*Oryza sativa*, LT_{50} 4°C); corn (*Zea mays*, LT_{50} 4°C); rapeseed (*Brassica napus* cv Jet neuf, LT_{50} −16°C); and two genotypes of alfalfa (*Medicago sativa* cv Trek, LT_{50} −9°C, and cv Anik, LT_{50} −14°C).

Plants were germinated in moist sterilized vermiculite for 5 days in the dark and 2 days under artifical light at 25°C/20°C (day/night). Control plants were maintained under the same conditions while cold acclimation was performed by subjecting the germinated seedlings to 6°C/2°C (day/night) as previously described [7]. In the case of rice and corn, seedlings were transferred to 10°C/6°C (day/night). Control (7 and 12 days), and cold-acclimated (1 and 36 days) plants were at comparable physiological ages based on dry weight [8]. Deacclimation was performed by transferring 36 days cold-acclimated plants to 25°C/20°C (day/night). Heat shock was performed by incubating seedlings at 40°C for 1 and 3 h. Wounding stress was induced by cutting the seedlings into 1 cm segments and placing them in water at 20°C for 3 and 14 h. Salt-stressed plants were obtained by incubating seedlings for 18 h with a nutritive solution containing 300 or 500 mM NaCl. Water stress was induced by removing seedlings from vermiculite and leaving them at 20°C without water for different periods. The relative water content was evaluated for each time point. ABA-treated plants were obtained by transferring seedlings for 18 h to a nutritive solution containing 10^{-4} M ABA and concomitantly applying a foliar spray containing 10^{-4} M ABA in 0.02% (v/v) Tween-20.

2.2. Cloning and molecular analysis

The p*Wcor410* clone was isolated from a Lambda Zap II library constructed from poly(A)$^+$ RNA isolated from cold-acclimated winter wheat (*T. aestivum* L. cv Norstar) [9]. Differential screening of this library was done with cDNA probes synthesized from poly(A)$^+$ RNA isolated from control and cold-acclimated winter wheat. The *Wcor410* clone which hybridized preferentially with the cold-acclimated probe

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was purified and subcloned in the pBluescript vector as described (Stratagene). DNA sequence was determined on both strands. All other molecular biology techniques were performed using standard procedures [10] and modifications already published [9].

3. Results and discussion

The cDNA clone *Wcor410*, for wheat cold-regulated gene, hybridizes preferentially to a mRNA of 1.3 kb that is strongly up-regulated upon exposure to low temperature. The kinetic studies using Northern blot analysis show that the *Wcor410* transcript accumulates to a high level within 24 h and then decreases gradually during the acclimation period in the three genotypes (Fig. 1). However, the more freezing tolerant genotypes, Fredrick and Norstar, maintain a higher level of *Wcor410* mRNA at the end of the cold acclimation period (36 days) when compared to the less freezing tolerant genotype, Glenlea. When the plants are deacclimated at 24°C, the transcript declines rapidly to the non-acclimated control level. Furthermore, expression during the cold acclimation period is not tissue-specific since it was detected equally well in the leaf, crown and root tissues.

To determine if the *Wcor410* mRNA accumulation is specifically regulated by low temperature, plants were subjected to different treatments. Northern blot analysis (Fig. 2A) indicates that extensive water stress (RWC 57–44%) results in its accumulation to a level comparable to 1 day of low temperature exposure while exogenously applied ABA resulted in only partial accumulation (an ABA concentration of 10 μM gave comparable results). Heat shock, salinity and wounding also induce partial accumulation in shoots. Similar results were also obtained with the genotypes, Glenlea and Norstar (not shown). Fig. 2B shows that the accumulation of *Wcor410* mRNA after low temperature exposure is highest in winter wheat and rye which are the most freezing tolerant species. We could not detect any expression of *Wcor410* mRNA in the freezing sensitive species, rice and corn, even after a long exposure time. It is worth mentioning that the low temperature-sensitive species, rice and corn, accumulate detectable levels of a transcript homologous to *Wcor410* during water stress or ABA treatment while no significant accumulation was observed when these plants were exposed to different low temperature regimes (results not shown). In the two tolerant dicot plants examined, alfalfa and rapeseed, we did not detect any induction of *Wcor410*. This may reflect the absence of an homologous mRNA in dicots. Southern analysis (Fig. 3) shows that genes homologous to *Wcor410* are present in all monocot species examined including rice and corn but was not detected, even at low stringency (5× SSC, 55°C), in rapeseed and alfalfa. However, since dicots have a lower GC content, similar genes, such as the related D11 family of dehydrins, may not be detectable with our probe. The observation that three large bands hybridize to *Wcor410* might indicate the existence of a small gene family. Overall, these results suggest that, as in the case of *Wcs120* [9,11], the *Wcor410* gene is gramineae-specific and is expressed only in freezing tolerant species.

The inability of the *Wcor410* and *Wcs120* (this gene is also detected on Southern blots of rice and corn; result not shown) genes to respond to low temperature in sensitive species may be explained by the absence of a low temperature-responsive element or of the appropriate transcription factor interacting with this element. This may not be a generalized phenomenon since the induction of *rab21* was observed upon low temperature exposure of rice, indicating that some cold-regulated pathways may exist in rice [12]. It is also important to note that while *Wcor410* is induced by water stress, its accumulation at low temperature in wheat is not caused by changes in water balance. The water content of plants

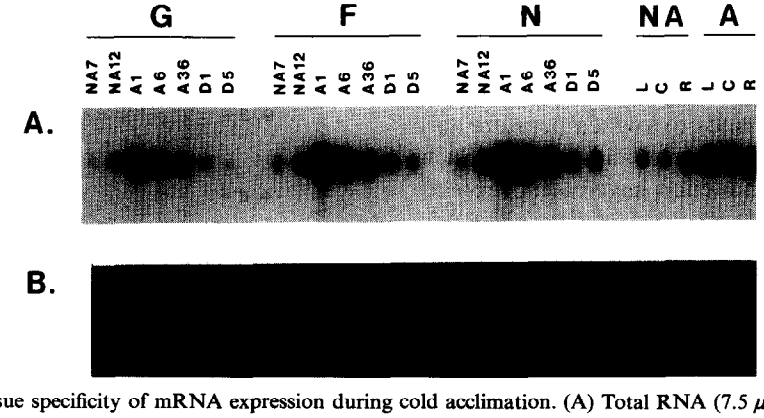

Fig. 1. Kinetic analysis and tissue specificity of mRNA expression during cold acclimation. (A) Total RNA (7.5 μg) extracted from the shoots of wheat genotypes Glenlea (G), Fredrick (F) and Norstar (N) was separated on a formaldehyde agarose gel, transferred to a nitrocellulose membrane and hybridized with a ^{32}P-labeled cDNA insert from p*Wcor410*. NA7 and NA12, control plants (non-acclimated) grown for 7 and 12 days; A1, A6 and A36, plants cold-acclimated for 1, 6 and 36 days; D1 and D5, cold-acclimated plants (36 days) were deacclimated for 1 and 5 days. Tissue specificity was determined in the genotype Fredrick for leaf (L), crown (C) and roots (R); NA, non-acclimated control plants grown for 12 days; A, cold-acclimated for 36 days. (B) Ethidium bromide-stained 28 S ribosomal band (included to show RNA loads).

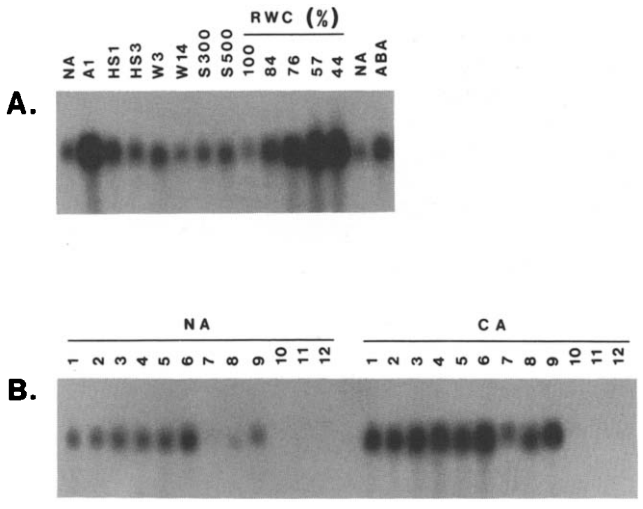

Fig. 2. (A) Northern blot analysis of *Wcor410* expression in wheat shoots (cv Fredrick) exposed to different treatments. NA, control plants (non-acclimated); A1, plants cold-acclimated for 1 day; HS1 and HS3, plants heat-shocked for 1 and 3 h; W3 and W14, 3 and 14 h after wounding plants; S300 and S500, plants salt-stressed for 18 h with 300 or 500 mM NaCl; RWC (%), relative water content after water stress for 0, 2, 4, 6, and 18 h; ABA, plants treated with 10^{-4} M ABA. (B) Northern blot analysis of *Wcor410* mRNA expression in several plant species. Total RNA was isolated from non-acclimated plants (NA) or plants cold-acclimated for 1 week (CA). Lanes: 1–6, wheat genotypes Glenlea, Concorde, Monopole, Absolvent, Fredrick and Norstar, respectively; 7, oat; 8, barley; 9, rye; 10, rice; 11, alfalfa cv Trek; 12, rapeseed.

after 1 week at 4°C decreased by 2.5%. This indicates that there is no significant water stress associated with transfer to low temperature in wheat [13]. Furthermore, after 36 days of cold acclimation, the water content is down by 8.5% while according to Northern hybridization, the amount of *Wcor410* mRNA at 36 days is actually decreasing compared to 1 day of cold acclimation (Fig. 1). We have also found that cold acclimation of wheat is not associated with increased levels of ABA compared to water stress where ABA increased by tenfold [14]. This suggests that in tolerant cereals the regulatory region of *Wcor410* would have at least two responsive elements; one that responds to low temperature and another that responds to water stress and/or ABA. Such independant pathways of induction have been proposed for the cold-regulated *lti140* gene of *Arabidopsis thaliana* [15]. The isolation of promoters from tolerant and sensitive species and the identification of *cis* and *trans* acting factors will help us to understand this differential response. The presence of freezing tolerance-associated genes in both sensitive and tolerant gramineae species with specific expression in tolerant ones is important since it may offer the possibility of activating the expression of these genes to improve cold tolerance in these sensitive species.

The nucleotide sequence of *Wcor410* and its deduced protein are shown in Fig. 4. The longest ORF is 786 nucleotides long and encodes 262 amino acids. The protein has a molecular mass of 28 kDa and a pI of 5.1. Compositional analysis indicates that the polypeptide is rich in charged amino acids (43%) with glutamate and lysine residues representing 16.4 and 14.1% of the polypeptide, respectively, while cysteine and tryptophan residues are absent. A search in the Genbank database revealed that *Wcor410* has 95.6% identity (at the protein level) with the gene, *Esi35*, which is induced early to a high level during salt-stress in the roots of *Lophopyrum elongatum* [16] compared to salt sensitive *T. aestivum* [17]. In our experiments, the *Wcor410* mRNA was strongly expressed in roots, crown, and leaves during cold acclimation. These results may suggest that freezing stress mimics salt stress in all parts of the plant by increasing the intracellular ionic concentration due to the freezing of water in the intercellular space. Clearly, this gene is modulated differently by low temperature and salt stress. Further experiments will be needed to better define the role and the regulation of each of the genes induced during salt-, drought- and cold stress.

At the protein level, WCOR410 contains certain homologous features with proteins related to the D11 family [6]. In particular, we can find the presence of a succession of serine residues and three lysine-rich repeats. However, the conserved N-terminal sequence, DEYGNP, and the glycine-rich repeats are absent in WCOR410. Certain proteins that share common features with WCOR410, such as an acidic pI and a high content of charged amino acids, have recently been identified such as pLC30–15 from tomato [18], a dehydrin cognate from pea [19] and COR47 from *Arabidopsis* [20]. Secondary structure prediction shows that these proteins would be composed mostly of α-helices [21]. Furthermore, analysis of the N-terminal region of these proteins

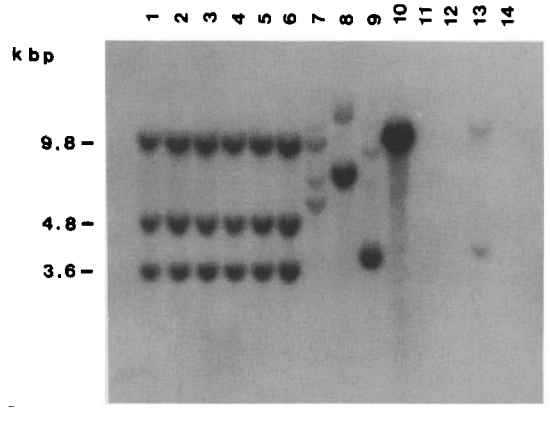

Fig. 3. Southern blot analysis of DNA from several plant species. Plant DNA (7 μg) was digested with *Bam*HI, separated by agarose gel electrophoresis, transferred to nitrocellulose and then probed with *Wcor410*. Lanes: 1–6, wheat genotypes Glenlea, Concorde, Monopole, Absolvent, Fredrick and Norstar, respectively; 7, oat; 8, barley; 9, rye; 10, rice; 11, alfalfa cv Trek; 12, alfalfa cv Anik; 13, corn; 14, rapeseed.

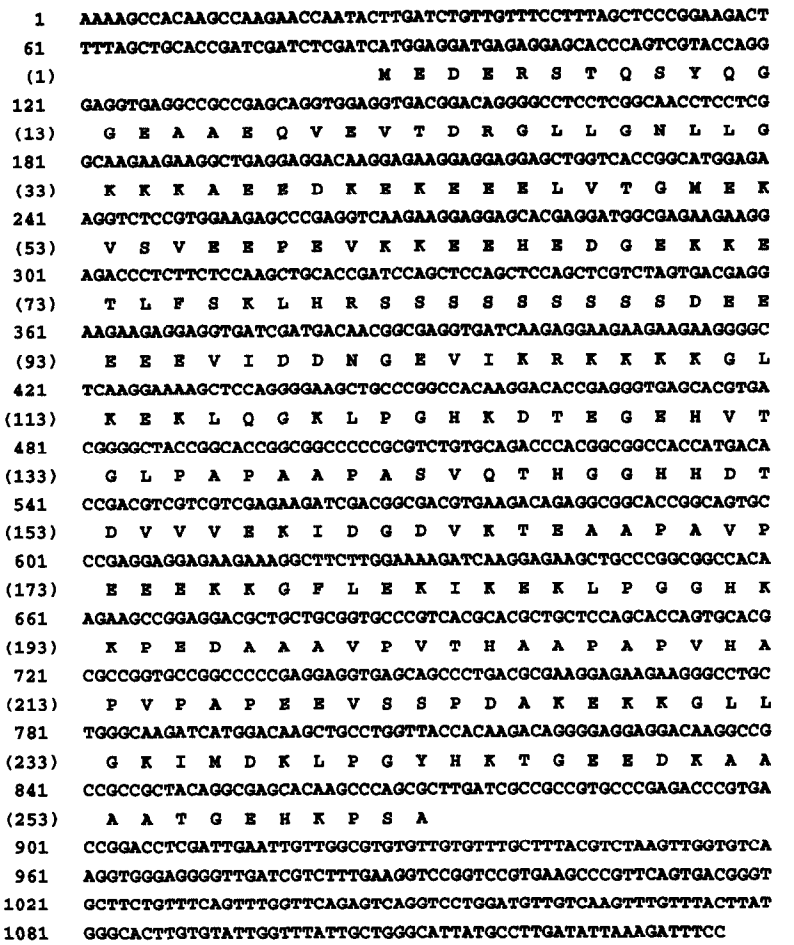

Fig. 4. DNA sequence and deduced amino acid sequence of *Wcor410*. The coding strand has been determined with T7 RNA transcription of *Wcor410* and subsequent hybridization to RNA from acclimated plants. The longest ORF is shown here (786 nucleotides). The predicted polypeptide is 262 amino acids in length, has a calculated molecular mass of 28 kDa and a pI of 5.1. Genbank accession no.: L29152.

revealed that they contain a consensus sequence [(V/T)EVTDRGLFDFLGKK(K/E)EEE] that is not present in other members of the D11 protein family. The presence of this conserved sequence suggests that it may be important for the function of this subgroup of proteins in freeze-induced dehydration or water stress. Based on these differences, we suggest that these proteins be classified into a subgroup of the D11 protein family. These proteins may still have a similar function but in different cells or subcellular compartments. On the other hand, the similarity with a salt stress-induced protein and the presence of distinctive internal sequences suggest that the role of this protein may be slightly different to that of dehydrins. We are currently developing a specific antibody against the WCOR410 protein. This antibody will be used for immunocytochemical localization in order to resolve this question.

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