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Genetic studies of Triticeae dehydrins: assignment of seed proteins and a regulatory factor to map positions

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Abstract A collection of 200 wheat (*Triticum aestivum* L. cv 'Chinese Spring') cytogenetic stocks (nullisomic, tetrasomic, nulli-tetrasomic, ditelosomic and deletion lines, addition and substitution stocks from intra- and inter-specific crosses) was utilized to determine the proteins encoded by some of the wheat and barley dehydrin genes, using a western blot procedure. Proteins extracted from seeds were reacted with antibodies that recognize dehydrins in a wide range of plants, including wheat and barley. Proteins encoded by dehydrin loci in chromosome arms 4DS, 5BL and 6AL of 'Chinese Spring' wheat were assigned by this method. There was also evidence of a regulatory factor on 5B in the vicinity of the *dhn* genes, and on 5H in wheat-barley addition lines, that is required for a normal level of expression of seed dehydrins in hexaploid wheat. Further understanding of this putative regulatory factor would be helpful for the interpretation of linkage studies that may relate dehydrin gene expression to phenotypes such as dehydration, salinity or low-temperature tolerance.

Key words Dehydrin genes · Wheat · Barley · Triticeae · Regulatory factor

Introduction

Environmental stresses in the form of drought, salinity or low temperature, as well as the normal process of seed development, cause the synthesis of specific proteins that may play a role in maintaining the living status of plant cells (Ingram and Bartels 1996). Dehydrins are one example of such proteins. Each dehydrin is abundant in hydrophilic amino acids, soluble at high temperature, glycine-rich or proline- and alanine-rich, free or nearly free of cysteine and tryptophan amino acids, and contains consensus amino acid sequence domains (Close 1997). Genetic analysis, supplemented with physiological and biochemical studies, provides information helpful in the elucidation of the function of dehydrins.

Barley, wheat and rye are members of the tribe Triticeae, which carry a basic set of seven chromosomes. At the present time the most complete information on the genetics of plant dehydrins is in barley. Barley dehydrin (*dhn*) loci have been mapped to chromosomes 4H, 5H and 6H (Close 1996; Close and Chandler 1990; Kleinhofs et al. 1993; Pan et al. 1994). In barley, *dhn* genes appear in clusters in chromosomes 5H and 6H, and there are at least three loci on each of these chromosomes (Pan et al. 1994). In wheat and wheat progenitors, *dhn* genes have been located in chromosome 6D in *Triticum tauschii* (Lagudah et al. 1991), chromosomes 4A, 5A and 6A in *T. monococcum* (Dubcovsky et al. 1995) and chromosome 5A in single chromosome substitution lines involving *T. spelta* 5A and wheat 'Cheyenne' 5A (Galiba et al. 1995). Low-temperature-induced *cor* genes of wheat, which belong to the dehydrin gene family, were recently mapped using western and Southern blot analysis to the long arms of group 6 chromosomes in the A, B and D genomes of hexaploid wheat and related species carrying the A, D or AB genomes (Limin et al. 1997). Genetic maps that include the location of all of the dehydrin genes in all members of the Triticeae would facilitate the development of comparative maps and help to define the linkage between dehydrin genes and a number of important traits in wheat and its relatives for breeding purposes (Limin and Fowler 1991; Snape et al. 1996). The objective of the study presented here was to advance the genetic analysis of Triticeae

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dehydrins by beginning to define which dehydrin protein is encoded by which genetic locus. This has not been clear from mapping by nucleic acid hybridization due to the cross-hybridization of *dhn* genes in this dispersed multigene family.

Materials and methods

Plant material

A total of 161 cytogenetic stocks of 'Chinese Spring' (CS) wheat originally developed by Dr. E. R. Sears and obtained from Dr. A. Lukaszewski, Department of Botany and Plant Sciences, University of California, Riverside, were analyzed, including: nullisomics (11 total), tetrasomics for group 5 chromosomes (three total), nulli-tetrasomics (including compensating mono-tetrasomics M4BT4A and M4BT4D; 42 total), ditelosomics (including compensating dimonotelosomics Dt2ALMt2AS, Dt2BSMt2BL, Dt4BLMt4BS, Dt5BSMt5BL, Dt5DSMt5DL; 42 total), and single-chromosome substitution lines of cultivars 'Hope' (21 total), 'Thatcher' (21 total) and 'Timstein' (21 total) into 'Chinese Spring'. In addition, 33 CS wheat deletion lines provided by Dr. T. R. Endo, Kyoto University, Kyoto, Japan, and six CS wheat – 'Betzes' barley addition lines, obtained from Dr. A. K. M. R. Islam, University of Adelaide, Australia, were examined.

Immunoblots

Crude protein extracts were made by grinding the embryo half of single dry seeds in 500 µl of 30 m*M* TES buffer, pH 7.5, 50 m*M* NaCl, 1 m*M* PMSF, followed by centrifugation in a microcentrifuge for 10 min at 4°C to remove debris. The concentration of proteins in the supernatant was determined utilizing a Bradford dye-binding assay. Gel samples were prepared by heating extracts in a boiling water bath in Laemmli sample buffer for 5 min. Aliquots containing 3 µg of protein were electrophoresed on 13% or 4–20% gradient (for wheat-barley addition lines) SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane, which was then blocked with 3% (w/v) gelatin in TBS buffer. Membranes were then incubated with rabbit anti-dehydrin antiserum, which recognizes dehydrins in a wide range of plants, including barley and wheat, followed by incubation with goat anti-rabbit IgG alkaline phosphatase conjugate as described by Close et al. (1993). In order to examine the specificity of the antibodies, we pre-blocked the antiserum with dehydrin consensus peptide (TGEKKGIMDKIKEKLPG) at a concentration of 5 mg/ml for 1 h at 37°C (Fig. 1). The detection system included 4-nitroblue-tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate. Gels for immunoblots were duplicated for silver staining (Harlow and Lane 1988) to verify equal loading of all lanes. Any seed that had a dehydrin pattern other than the normal 'Chinese Spring' pattern was replicated with at least one other seed from the same stock. In some cases several additional individual seeds were examined from a single stock.

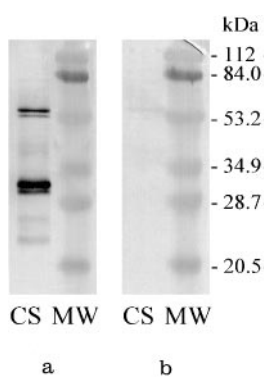

Fig. 1a, b Immunoblots of wheat seed dehydrins: **a** detected with antiserum, **b** antiserum preblocked with dehydrin consensus peptide (TGEKKGIMDKIKEKLPG). *CS* Wheat cv 'Chinese Spring', *MW* molecular-weight standards

Results

Mapping using nullisomic, tetrasomic, nulli-tetrasomic, ditelosomic and deletion lines

Western blot analysis of seed proteins detected dehydrins ranging in apparent molecular mass from about 22 to 100 kDa, with the most prevalent observed at about 30 and 53 kDa. None of these proteins were detected on immunoblots developed with antibodies pre-blocked with dehydrin consensus peptide (Fig. 1). In the mapping procedure a protein was first assigned to a chromosome arm, then to a specific chromosome region using the deletion lines for this chromosome (Mukai et al. 1990; Werner et al. 1992). Analysis of wheat cytogenetic stocks provided estimates of the sizes of dehydrins encoded by *dhn* genes located in chromosome arms 4DS, 5BL and 6AL (Fig. 2). Presumably, the homoeologous group 4, 5 and 6 chromosomes contain *dhn* genes that encode dehydrins of sizes similar to those encoded by 4D, 5B and 6L. The assignment of dehydrins to map positions by the method that we employed is limited to unique size species, which can be easily scored in the cytogenetic stocks as presence or absence, or to very pronounced differences in the quantity of a specific dehydrin. Many wheat dehydrins

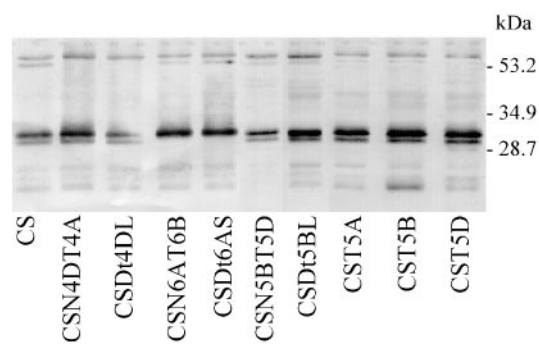

Fig. 2 Chromosome arm mapping in wheat. *CS* Wheat cv 'Chinese Spring', *CSN4DT4A* no copies of chromosome 4D (nullisomic for 4D) but four copies of chromosome 4A (tetrasomic for 4A), *CSDt4DL* long arm of chromosome 4D is present (ditelosomic for 4DL) but short arm is absent, *CSN6AT6B* nullisomic for 6A and tetrasomic for 6B, *CSDt6AS* ditelosomic for 6AS, *CSN5BT5D* nullisomic for 5B and tetrasomic for 5D, *CSDt5BL* ditelosomic for 5BL, *CST5A* tetrasomic for 5A chromosome, *CST5B* tetrasomic for 5B chromosome; *CST5D* tetrasomic for 5D chromosome. Each locus was assigned to a chromosome arm by scoring the presence or absence of a specific protein in a specific stock. Cytogenetic stocks with dehydrin profiles indistinguishable from CS (all others) are not shown

therefore could not be assigned to map positions by this method.

The first step of this analysis involved assigning protein-coding genes to chromosome arms using nullisomics and ditelosomics (Fig. 2). The complete collection (see Materials and methods) was examined, but only those revealing an obvious difference from CS are shown. For example, CSN4DT4A, which has no 4D chromosomes (N4D), but four 4A chromosomes (T4A), is missing the lower dehydrin around 53 kDa. Therefore, the gene encoding the 53-kDa dehydrin is on 4D. Further mapping limited the gene position to a chromosome arm. For example, CSDt4DL, which is a ditelosomic line carrying only the long arm of chromosome 4D (Dt4DL), is missing the 53-kDa dehydrin, indicating that the gene maps to the missing short arm of the 4D chromosome. Similarly, lines nullisomic for chromosome 6A or ditelosomic for the short arm of 6A are missing the lower protein around 30 kDa, which places the gene encoding the 30-kDa protein on 6AL. Analysis of the 5B chromosome indicated that CSN5BT5D, which is nullisomic for 5B, had an overall weaker expression of dehydrins (especially the lowest molecular weight proteins around 30 and 22 kDa) while CST5B, which is tetrasomic for 5B, had apparently normal levels of all dehydrins, except for an elevated level of the of an approximately 22-kDa protein. In addition, the ditelosomic line carrying only the long arm of chromosome 5B (CSDt5BL) has a normal CS pattern. Ditelosomic lines for 5BS are not available. From these data we concluded that 5BL encodes a 22-kDa dehydrin protein. Weaker overall expression of dehydrins in lines where the 5B chromosome is missing may reflect the effect of a regulatory factor located on this chromosome. Since overall dehydrin protein levels are not noticeably elevated in CST5B, the 5B chromosome may carry a weak homoeoallele of a negative regulator (rather than a positive regulator), which is compensated by expression of more active homoeoalleles on 5A or 5D when 5B is missing. Lines tetrasomic for 5A or 5D chromosomes (CST5A, CST5D) have normal dehydrin profiles, which would be not be inconsistent with a compensation interaction between an expressed regulator on 5B and counterpart proteins encoded by the other group 5 chromosomes.

For more precise mapping of each of the three presumed structural genes encoding the proteins identified in Fig. 2 and the putative regulator on 5B, deletion lines were used (Fig. 3). Each dehydrin locus was assigned to the chromosome region between the breakpoints of the largest deletion where the protein is present and the next larger deletion where the protein is absent. The gene encoding the 4D dehydrin around 53 kDa mapped to the tip of chromosome 4DS between breakpoint 0.82 and the end of chromosome 4D. The locus representing the 6A dehydrin around 30 kDa mapped distal to the 0.83 breakpoint in chromosome 6AL. The chromosome breakage in the 6AL-5 deletion

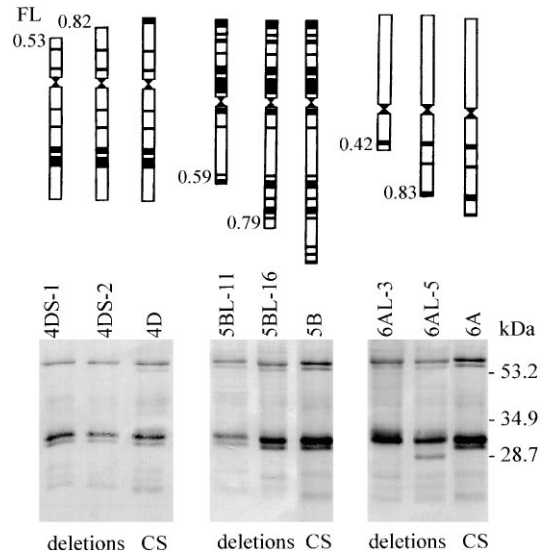

Fig. 3 Deletion mapping. Each dehydrin identified in Fig. 2 was assigned to the chromosome region between the breakpoints of the largest deletion where the protein is present and the next larger deletion where the protein is absent (Werner et al. 1992). The breakpoint for each deleted chromosome was calculated as a fraction length (*FL*) of the distance from the centromere (Mukai et al. 1990). *CS* Wheat cv 'Chinese Spring'

may have occurred within this dehydrin gene, since the protein assigned to chromosome 6AL is replaced by one of lower molecular weight in the line containing this deletion as compared to the CS pattern, which may represent a truncated dehydrin protein. For chromosome 5B, the gene encoding the 22-kDa dehydrin (the fastest migrating immunopositive protein in lane 5B) mapped distal to the 0.79 breakpoint of the 5BL-16 deletion. Interestingly, deletion of the region between the breakpoints of deletions 5BL-11 and 5BL-16 reduces expression of the overall dehydrin profile, which is consistent with a similar effect noted in CSN5BT5D (Fig. 2). This further substantiates the hypothesis that the 5B chromosome carries a regulatory factor that has a net effect of enhancing dehydrin protein levels. It should be noted that the positions of the 22-kDa dehydrin on 5B and this putative dehydrin regulatory factor are in the same general region of the 5B chromosome, but not at the same position. Also, a result of this mapping exercise is the placement of the dehydrin (*dhn*) genes on the cytogenetic map in regions consistent with previous molecular mapping data (reviewed in Campbell and Close 1997), including *T. monococcum* (Dubcovksy et al. 1995) and barley (Pan et al. 1994). In each case two recombinationally separable *dhn* loci were identified on group 5 chromosomes (Fig. 4).

Analysis of addition and substitution lines

Mapping of dehydrins in selected species of the tribe Triticeae included western blot analysis of seed

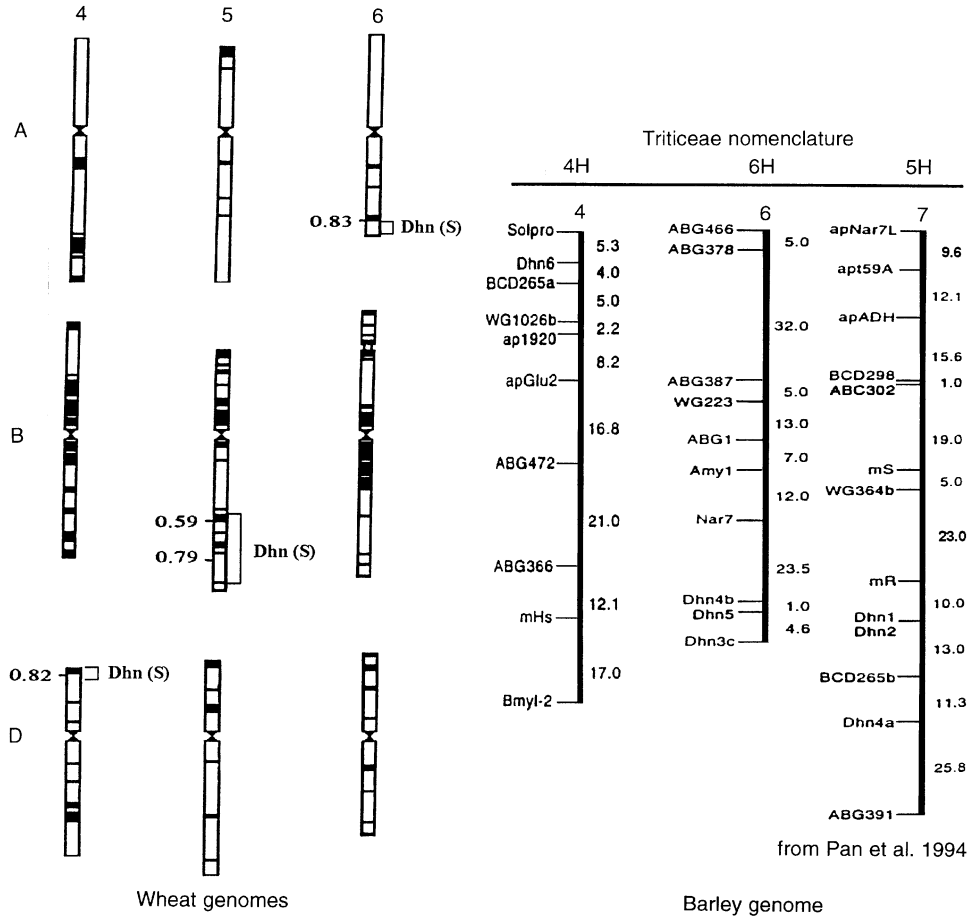

Fig. 4 Location of dehydrin genes in wheat and barley genomes. *Numbers* to the *left* of the wheat chromosome panels indicate the chromosome breakpoints calculated as a fraction length of the distance from the centromere. *Brackets* indicate boundaries of region containing loci identified by deletions shown in Fig. 3. Only chromosomes where the dehydrin protein structural genes on the cytogenetic map of wheat were located are shown. Barley chromosomes 4H, 5H and 6H correspond to barley 4, 7 and 6, respectively, in the cited reference

proteins in lines generated from intra- and inter-specific crosses. The first group of cytogenetic stocks is represented by the full collection of CS substitution lines for all chromosomes with three wheat cultivars of diverse origin: 'Hope', 'Timstein' and 'Thatcher' (three sets of 21 substitution lines for each cultivar). The second group is a collection of six wheat-barley addition lines (2H-7H). Addition line 1H from 'Betzes' barley is not available.

The wheat cultivars 'Hope', 'Timstein' and 'Thatcher' have different patterns of high-molecular-weight dehydrin proteins than CS. However, the majority of substitution lines derived from these cultivars in the CS background have entirely the CS dehydrin profile (data not shown). Exceptions are Thatcher 3D and Timstein 1A substitution lines (Fig. 5). The 'Thatcher' 3D substitution line has proteins that resemble proteins in 'Thatcher' rather than CS above and below the CS 53-kDa dehydrin, indicating possible dehydrin loci on Thatcher 3D which have been previously unidentified. The Timstein 1A substitution line has two dehydrins around 45 kDa that are similar in size to dehydrins

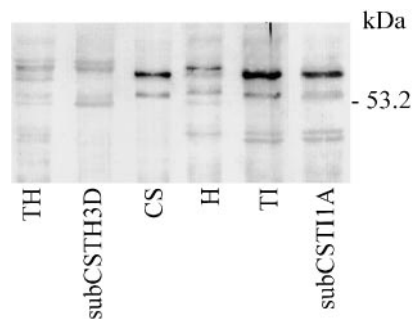

Fig. 5 Mapping in the Triticeae group using wheat substitution lines. *CS* Wheat cv 'Chinese Spring', *H* Wheat cv 'Hope', *TH* wheat cv 'Thatcher', *TI* Wheat cv 'Timstein', *subCSTH3D* 'Thatcher' substitution lines for chromosome 3D, *subCST1A* 'Timstein' substitution line for chromosome 1A

present in 'Timstein' but not in CS. This may also represent previously unidentified dehydrin loci in chromosome 1A of 'Timstein'. A caveat that applies to all of these substitution lines, however, is that these sets have

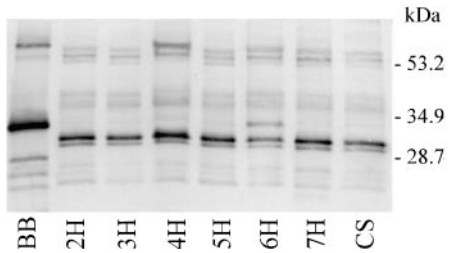

Fig. 6 Mapping in the Triticeae group using wheat-barley addition lines. *BB* Betzes barley, *CS* wheat cv 'Chinese Spring', *2H*, *3H*, *4H*, *5H*, *6H*, *7H* Triticeae nomenclature, corresponding to barley 2, 3, 4, 7, 6, 1 respectively

had only five backcrosses to CS and that there is still apparent background variation (Adam Lukaszewki, personal communication), therefore one cannot firmly conclude from these data that Thatcher 3D or Timstein 1A carry *dhn* genes.

The seed dehydrin profile of 'Betzes' barley is distinctly different from that of CS wheat. There are four readily detected seed dehydrins in barley with clearly different MW than those present in wheat, including two proteins detected as intense bands around 60 kDa and 34 kDa and two as weaker bands around 20–30 kDa (Fig. 6). In principle, it might be expected that the assignment of these barley proteins to chromosomes would be straightforward since these differences are so distinct. However, this was not always the case. Four out of the six available wheat-barley ('Betzes') addition lines have only the wheat pattern, while two (4H and 6H) have an additional seed dehydrin consistent with a barley origin. These results were expected, since 4H and 6H have been shown previously to carry dehydrin genes (for example Pan et al. 1994), but the relative abundance of the barley proteins in the wheat background was not uniform. The barley 4H dehydrin seems to be relatively immune to compensation in the wheat background, whereas the barley 6H dehydrin quantity appears to more closely parallel the total 25% contribution of 4H to group 4 chromosomes in this addition line. 'Betzes' barley dehydrins with an apparent MW around 20–26 kDa correspond with the predicted apparent MW of Himalaya 5H *dhn* genes. However, these proteins were not detected in the addition lines, so either their expression is strongly suppressed in the wheat background or they map to the 1H chromosome, for which an addition line is not available from 'Betzes' barley. Since no *dhn* genes have previously been mapped to barley chromosome 1H, and low-molecular-weight *dhn* genes have been mapped to 5H by nucleic acid hybridization, the simpler explanation is the former. This explanation is once again consistent with the presence of regulatory interactions involving group 5 *dhn* genes.

Discussion

Western blot analysis of wheat cytogenetic stock seed proteins using antibodies detecting the dehydrin consensus peptide resulted in: (1) assignment of dehydrin proteins to specific regions of wheat chromosomes 4D, 5B and 6A that are consistent with nuclei acid hybridization mapping techniques, (2) evidence of a regulatory factor in the vicinity of group 5 *dhn* genes that influences seed dehydrin levels in hexaploid wheat and in wheat-barley addition lines, (3) detection of homoeoallelic variation in dehydrin proteins existing among members of the Triticeae tribe and (4) identification of possible new dehydrin loci in wheat cultivar 'Thatcher' in chromosome 3D and in 'Timstein' in 1A.

While the western blotting technique that we employed is not as suited to comprehensive determination of the map positions of *dhn* genes in Triticeae genomes as DNA-based mapping approaches are, the western blot technique provides information related to gene expression that cannot be readily ascertained from nucleic acid techniques. The evidence, reported herein, of a genetic regulatory factor on group 5 chromosomes which influences the level of the total seed dehydrin profile is one such example. In addition, because of extensive cross-hybridization between *dhn* gene probes for restriction fragment length polymorphism (RFLP) analysis, western blot data provide a less ambiguous idea of the size of the dehydrin that is encoded by each locus. Ultimately, this limitation of DNA-based analyses by cross-hybridization can be overcome by the use of gene-specific polymerase chain reaction (PCR) primers but that will first require the isolation and sequence determination of all of the *dhn* genes from at least one Triticeae genome. The placement of *dhn* genes on cytogenetic maps could also, in principal, be accomplished with RFLP and gene-specific DNA-based probes. Nevertheless, the western blot technique that we have utilized has been a convenient way to ascertain some of this information and is directly applicable to all plants since the antibodies detect all plant dehydrins. In addition, this technique could be pushed further by two-dimensional protein gel western blot analysis.

The genetic composition of a wheat cytogenetic stock can affect the expression of seed dehydrins. In the case of chromosome 5B, there appears to be two different types of dosage effect since lack of this chromosome in the CSN5BT5D nulli-tetrasomic line weakens the overall expression of all dehydrins while two extra copies of 5B in the CST5B tetrasomic line seems to enhance the expression of only a specific approximately 22-kDa dehydrin, presumably encoded by a *dhn* gene on 5B. The 5B chromosome of wheat seems to carry both the *dhn* gene encoding the approximately 22-kDa dehydrin, which is located at or distal to the breakpoint 0.79 (Fig. 3, lane 5BL-16), and

a regulatory factor located between breakpoints 0.79 and 0.59 (Fig. 3, lanes 5BL-16 and 5BL-11), which is required for a normal level of seed dehydrin expression. Since an elevated copy number of 5B does not lead to higher than normal seed dehydrin expression (Fig. 2, lane CST5B), the absence of this factor on 5B may reflect the presence of a negative regulator on 5 A or 5D (Fig. 2, lane CSN5BT5D; Fig. 3, lane 5BL-11) that is normally held in check by the regulatory factor on 5B.

Intra-specific crosses among wheat cultivars ('Hope', 'Thatcher' and 'Timstein' crossed with CS) and inter-specific crosses between hexaploid wheat and its diploid relative, barley, also result in altered seed expression of dehydrin proteins. Evidence of suppression was observed with these materials since most of the 'Hope', 'Thatcher' and 'Timstein' wheat dehydrins were not detected in the CS background (except as noted in Fig. 5). Similarly, expression of *dhn* genes of the barley genome were expressed at non-uniform relative levels when barley chromosomes were present in the wheat background. In particular, low-molecular-weight dehydrins of barley, which presumably map to 5H, seemed to be fully suppressed (Fig. 6, lane 5H) in the CS background.

There are several reports indicating the presence of regulatory factors that influence the expression of genes in inter-specific hybrids. Stem and leaf rust resistance genes are associated with a suppressor mechanism in a number of wheat cultivars and accessions (Dyck 1987; Kerber 1983). Also, Limin and Fowler (1991) reported on modified alien gene expression affecting genetic introgression in breeding for cold hardiness in wheat. Recently, they described an altered expression of cold-induced wheat genes (*Wcs120* family, which are *dhn* genes as noted in Close 1997) in wheat-rye amphiploids and other interspecific combinations (Limin et al. 1995). Chromosome mapping of *Wcs120* genes in hexaploid wheat revealed possible gene silencing of duplicate loci (inability of the A genome *Wcs120* homoeologue to be expressed in polyploid wheat) and regulatory effects of the 5A chromosome in CS/Cheyenne substitution lines (Limin et al. 1997). Quarrie et al. (1994) also suggested the presence of a regulatory gene on wheat chromosome 5A that affects abscisic acid (ABA) accumulation under drought conditions. Our results are consistent with these reports and provide additional evidence of the presence of a regulatory gene, in this case an apparent positive influence of a regulator on 5B controlling the expression of many *dhn* structural genes in mature seeds. Since the position of one *dhn* gene in the cluster of 5H *dhn* genes identified previously (Pan et al. 1994; Dubcovsky et al. 1995) may coincide with the position of this regulatory locus, it is possible that one of the 5H *dhn* gene is itself a regulatory factor. Perhaps a *dhn* gene with a strong regulatory activity is the *Sh2* gene, as suggested previously (Campbell and Close 1997). In any case, further genetic mapping of regulatory factors affecting the profile of dehydrins would be helpful for the interpretion of linkage studies of phenotypes that may be related to dehydrin gene expression, such as dehydration, salinity or low-temperature tolerance.

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