B. Zhu · D.-W. Choi · R. Fenton · T. J. Close

Expression of the barley dehydrin multigene family and the development of freezing tolerance

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Abstract Dehydrins (DHNs; LEA D11) are one of the typical families of plant proteins that accumulate in response to dehydration, low temperature, osmotic stress or treatment with abscisic acid (ABA), or during seed maturation. We previously found that three genes encoding low-molecular-weight DHNs (Dhn1, Dhn2 and Dhn9) map within a 15-cM region of barley chromosome 5H that overlaps a QTL for winterhardiness, while other Dhn genes encoding low- and high-molecular-weight DHNs are located on chromosomes 3H, 4H and 6H. Here we examine the expression of specific Dhn genes under conditions associated with expression of the winterhardiness phenotype. Plants grown at 4 °C or in the field in Riverside, California developed similar, modest levels of freezing tolerance, coinciding with little low-MW Dhn gene activity. Dicktoo (the more tolerant cultivar) and Morex (the less tolerant) grown in Saskatoon, Canada expressed higher levels of expression of genes for low-MW DHNs than did the same cultivars in Riverside, with expression being higher in Dicktoo than Morex. Dehydration or freeze-thaw also evoked expression of genes for low MW DHNs, suggesting that the dehydration component of freeze-thaw in the field induces low expression of genes encoding low-MW DHNs. These observations are consistent with the hypothesis that the major chilling-induced DHNs help to prime plant cells for acclimation to more intense cold, which then involves adaptation to dehydration during freeze-thaw cycling. A role for chromosome 5H-encoded DHNs in acclimation to more intense cold seems possible, even though it is not the basis of the major heritable variation in winterhardiness within the Dicktoo × Morex population.

Key words Dehydrin · LEA D11 · Multigene family · Barley · Freezing tolerance

Introduction

Dehydrins (DHNs) are characterized by a consensus 15-amino acid sequence (the K-segment), EKKGIMDKIKEKLPQ, that is present near the C-terminus and is usually repeated one to many times, and a tract of serine residues (the S-segment) where phosphorylation occurs. Other domains of DHNs include a consensus sequence VDEYGNP (the Y-segment) and repeated units that are rich in polar amino acids (the φ-segments). The numbers of Y, S, K and φ-segments that compose each DHN can be used for convenient reference to sub-classes of DHNs and for comparisons between alleles of individual Dhn genes (Campbell and Close 1997; Choi et al. 1999). The abundance of dehydrins in desiccated viable embryos (Close et al. 1993), coupled with their up-regulation by abscisic acid and osmotic stresses including drought (Close et al. 1989; Guo et al. 1992) and high salt (Godoy et al. 1994), and by low temperatures (Neven et al. 1993; Arora and Wisniewski 1994; Robertson et al. 1994; van Zeel et al. 1995), tentatively points to DHNs as key components of tolerance to abiotic stresses (for reviews see Dure 1993; Close 1996, Close 1997).

Immunolocalization and sub-cellular fractionation have established that DHNs can be present in the nucleus or cytoplasm (reviewed in Close 1997). Two studies have clarified the location of DHNs in the cytoplasm. The major DHN of the maize embryo, which is a YSK₁ DHN, seems to be associated with a cytoplasmic endomembrane (Egerton-Warburton et al. 1997), while an acidic wheat DHN, which is an SK₃ type, is located in the vicinity of the plasma membrane (Danyuk et al. 1998). Most DHNs contain putative bipartite nuclear localization sequences (for example Monroy et al. 1993;
Godoy et al. 1994). Progress has been made in the purification of DHNs for in vitro biochemical studies (Plana et al. 1991; Ceccardi et al. 1994; Jepson and Close 1995; Houde et al. 1995; Lisse et al. 1996; Ismail et al. 1999a). Recently, a 26.5-kDa cowpea (*Vigna unguiculata*) DHN has been shown to take on α-helical structure in the presence of SDS (Ismail et al. 1999a), which led the authors to propose that DHNs are lipid-associating proteins.

Inheritance studies in several crop plants have revealed apparent co-segregation of *Dhn* genes with phenotypes associated with dehydrative stresses, such as drought and freezing (reviewed in Campbell and Close 1997; see also Ismail et al. 1999b). Several studies have implicated a region on group 5 chromosomes in the Triticeae, including barley 5H (Hayes et al. 1993; Pan et al. 1994) and wheat 5A and 5D (Sutka and Snape 1989; Roberts 1990; Galiba et al. 1993, 1995), as the location of major genes or gene clusters that control winter versus spring growth habit and freezing tolerance. Other traits that may be inter-related biochemically have been mapped to the same region of Triticeae group 5 chromosomes, including osmotic stress tolerance (Galiba et al. 1992) and cold- and drought-induced ABA production (Galiba et al. 1993; Quarrie et al. 1994). A cluster of genes for low-MW DHNs (*Dhn1, Dhn2, Dhn9*) on chromosome 5H also overlaps this region (Pan et al. 1994; Choi et al. 2000). However, van Zee et al. (1995) observed little or no expression of low-MW *Dhn* in a controlled environment chamber following exposure of Dicktoo and Morex barley seedlings to 20 °C for 48 h. This observation, together with a break in linkage of *Dhn1/Dhn2* and *Dhn9* from the major component of the QTL for freezing tolerance, raised doubts about a role for *Dhn1* or *Dhn2* in freezing tolerance (van Zee et al. 1995).

Freezing tolerance is determined not only by genetic but also environmental factors, among which low non-freezing temperature, dehydration, and the plant growth hormone ABA enhance tolerance (Thomashow 1990). Heritable freezing tolerance has been dissected into two genetically independent components, non-acclimated and acclimated tolerance, in tuber-bearing *Solanum* species (Stone et al. 1993). Gene x environment interactions are not yet well understood in the Triticeae. Conceivably, maximal freezing tolerance depends on maximum expression of specific genes. Both winter and spring barley cultivars can acclimate to low temperature, but spring cultivars generally develop less freezing tolerance than do winter cultivars (Brule-Babel and Fowler 1988). The critical question in regard to genetically determined freezing tolerance in the Triticeae therefore is not “How do plants acclimate to cold?”, but “What is the basis of the incremental difference between cultivars in their potential for freezing tolerance”?

Freezing tolerance of barley plants can be induced in controlled environments and in the field (Hayes et al. 1993). The effects of individual parameters such as low temperature or day length can be studied in a controlled environment, but such conditions generally do not induce the full potential for freezing tolerance, nor the full differential between cultivars observed in the field. Additional field parameters, such as mild water stress, long-term low, non-freezing, temperatures, and freeze-thaw cycles above lethal temperatures, have synergistic actions on freezing tolerance (Sutka and Snape 1989; Robertson et al. 1994). The phenotypic expression of the *Sgh2/Winl* and *Fr1* genes on group 5 chromosomes of the Triticeae is greater when the acclimation conditions include sub-zero temperatures (Sutka and Snape 1989; Hayes et al. 1993; Pan et al. 1994). The aim of this study was to characterize the expression of the barley *Dhn* multigene family, with an emphasis on growth conditions most relevant to heritable variation in freezing tolerance. We included low non-freezing and sub-zero temperatures in a controlled environment, as well as exposure to moderate to severe cold in the field.

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**Materials and methods**

**Plant material**

Barley seeds ([*Hordeum vulgare* L., cvs. Dicktoo (winter barley) and Morex (spring barley)] were provided in the summer of 1993 by Patrick Hayes (Oregon State University, Corvallis). Seed numbers were expanded in a greenhouse at the University of California at Riverside, in the autumn to mid-winter of 1993/1994, and then in the field in the late winter and spring of 1994. For this study, seeds were planted at two locations: (1) Riverside (latitude 34°N) in October 1994; and (2) Saskatoon, Saskatchewan, Canada (52.2°N) in September 1994. Plants were also grown in greenhouses at both locations as reference controls. Plants were sampled on Nov. 22, Dec. 9, Dec. 28, Jan. 10, Jan. 18, and Feb. 6 in Riverside, and on Oct. 17 and Nov. 5 in Saskatoon. Following a determination of freezing tolerance, plants grown in Saskatoon were frozen and shipped to Riverside by Ping Fu and Lawrence Gusta (University of Saskatchewan, Saskatoon, Canada) for further analysis. For seedling studies, barley seeds were surface-sterilized, then germinated on half-strength MS medium (Murashige and Skoog 1962) in the dark. Seven days after germination, the seedlings were acclimated at approximately 4 °C for 0.5, 1, 2, 4, 8, or 14 days, with a photoperiod of 16 h light/8-h dark in a cold chamber. For detached leaf studies, barley leaves were sampled from the field in Riverside on Feb. 6, 1995, and either air-dried in a partially enclosed petri dish or subjected to freeze-thaw at −2 or −4 °C.

**Determination of freezing tolerance**

The freezing tolerance of leaf and crown tissues of plants grown in Riverside, either in the field or in growth chambers or greenhouse, was determined by measuring electrolyte leakage after freeze-thaw cycles at various temperatures according to a leaf-disk method (Sukumaran and Weiser 1972). Freezing tolerance was expressed as \( LT_{50} \) (the temperature that results in 50% electrolyte leakage). A re-growth assay was used to determine the freezing tolerance (the temperature that results in 50% survival) of barley plants grown in Saskatoon as described by Robertson et al. (1994).

**RNA and protein extraction**

Total RNA was extracted from leaf and crown tissues by a hot-phenol procedure (Vervoort et al. 1989). Proteins were precipitated from the phenol/chloroform phase of RNA extractions with four volumes of methanol containing 0.1 M ammonium acetate at

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