Allelic variation of a dehydrin gene cosegregates with chilling tolerance during seedling emergence

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Dehydrins (DHNs, LEA D-11) are plant proteins present during environmental stresses associated with dehydration or low temperatures and during seed maturation. Functions of DHNs have not yet been defined. Earlier, we hypothesized that a ≈35-kDa DHN and membrane properties that reduce electrolyte leakage from seeds confer chilling tolerance during seedling emergence of cowpea (*Vigna unguiculata* L. Walp.) in an additive and independent manner. Evidence for this hypothesis was not rigorous because it was based on correlations of presence/absence of the DHN and slow electrolyte leakage with chilling tolerance in closely related cowpea lines that have some other genetic differences. Here, we provide more compelling genetic evidence for involvement of the DHN in chilling tolerance of cowpea. We developed near-isogenic lines by backcrossing. We isolated and determined the sequence of a cDNA corresponding to the ≈35-kDa DHN and used gene-specific oligonucleotides derived from it to test the genetic linkage between the DHN presence/absence trait and the DHN structural gene. We tested for association between the DHN presence/absence trait and both low-temperature seed emergence and electrolyte leakage. We show that allelic differences in the *Dhn* structural gene map to the same position as the DHN protein presence/absence trait and that the presence of the ≈35-kDa DHN is indeed associated with chilling tolerance during seedling emergence, independent of electrolyte leakage effects. Two types of allelic variation in the *Dhn* gene were identified in the protein-coding region, deletion of one Φ-segment from the DHN-negative lines and two single amino acid substitutions.

Tropical crops such as cotton, maize, and cowpea, are sensitive to chilling soil temperatures often encountered during early sowing in subtropical regions in spring. Early spring sowing can be beneficial in the subtropics because it results in a longer growing season and higher yields. However, early sown seeds that are subjected to chilling temperatures suffer reductions in rate of emergence and maximal emergence. Variation in chilling sensitivity at germination has been found among genotypes of warm-season annuals such as cowpea (1), soybean (2), cotton (3), and maize (4). Numerous studies have suggested positive associations between the extent of electrolyte leakage from seeds and pre-emergence mortality of embryos at chilling temperature for chilling sensitive crops (5, 6).

Soluble sugars (7, 8) and proteins, typically LEA (late embryogenesis-abundant) (9–12) are known to accumulate during seed development and are thought to play a role in protecting the embryo during desiccation. Studies with soybean indicated that accumulation of LEA proteins during embryogenesis might reduce the extent of desiccation-induced electrolyte leakage in immature seeds, suggesting a role in membrane protection (9). Dehydrins (DHNs, LEA D11 family) are among the most commonly observed proteins induced by environmental stress associated with dehydration or low temperature (10, 11). Distinct subclasses of DHNs have been noted (11). Several lines of evidence suggested a role of DHNs in membrane interactions and/or protein stabilization (1, 11, 13, 14).

In cowpea, two closely related lines (F_6 siblings) were found to vary in maximal emergence under chilling field conditions (1) but also were found to vary in other characters (15). Dry, mature seeds of the chilling-tolerant line, 1393–2-11, were found to contain a substantial quantity (estimated to be ≈1% of total soluble protein) of a ≈35-kDa DHN protein that was not detected in the seeds of the genetically similar line, 1393–2-1. Also the chilling-tolerant line had slower electrolyte leakage from its seeds during imbibition at low temperature. Based on studies with reciprocal hybrids, the chilling tolerance of 1393–2-11 was hypothesized to be caused by additive and independent effects of the DHN under dominant nuclear inheritance and a maternal effect associated with slower electrolyte leakage from seeds during imbibition compared with line 1393–2-1 (1). In this study, we further tested this hypothesis by using pairs of near-isogenic cowpea lines that differ in both the presence or absence of the ≈35-kDa DHN and parental cytoplasms, developed by backcrossing lines 1393–2-11 and 1393–2-1.

Materials and Methods

Development of Near-Isogenic Lines and Emergence Test. Genetic lines were bred that would enable us to distinguish between nuclear and cytoplasmically inherited maternal effects associated with differences in electrolyte leakage while providing near-isogenic lines with and without the specific DHN. Reciprocal crosses were made between cowpea line 1393–2-11 and the genetically related line 1393–2-1. Replicates of the reciprocal F_1s were backcrossed to 1393–2-1, and BC_1F_1 seeds were harvested and screened for the presence of the ≈35-kDa DHN protein by using an immunoblot assay of a chip taken from a cotyledon (1). Plants were propagated from seeds containing the DHN. The backcross and immunoblot assays were performed two more times. Plants then were allowed to self-pollinate. The BC_3S_1 families were screened for the presence of the DHN followed by three generations of selfing. Seeds of BC_3S_4 families were assayed for the presence of the DHN. Those originating from seeds in BC_3S_1 families that did not contain the DHN bred true for absence of the DHN. In addition, lines homozygous for the presence of the DHN were identified from those originating from BC_3S_1 seeds with the DHN. One pair of BC_3S_4 lines with individuals either having or not having the DHN in their seed was chosen from each of the original reciprocal crosses in the cytoplasms of 1393–2-11 and 1393–2-1.

Seeds from the two pairs together with the two parental lines were incubated over a saturated solution of lithium chloride for 4 wk to reduce seed moisture content to 6% on a fresh weight basis (1). Samples of 10 seeds/line were tested for electrolyte leakage from individual seeds at 14°C (G-2000 Seed Analyzer, Wavefront, Ann Arbor, MI). Seeds also were tested for emergence at 14°C in reach-in growth chambers in the dark as described (1).

Abbreviations: DHN, dehydrin; LEA, late embryogenesis-abundant.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF159804).

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Construction and Screening of the cDNA Expression Library. Cowpea pods were harvested from line 1393–2-11 at the color break stage. At this stage, there is a low, but detectable, level of the ≈35-kDa DHN in developing seeds of this genotype (16). Seeds were ground to a fine powder in liquid nitrogen. Total RNA was prepared by using the method of Verwoerd *et al.* (17). Poly(A)$^+$ mRNA was isolated from total RNA by using Promega polyATtract mRNA isolation system IV and then converted to a double-stranded cDNA by using a Stratagene ZAP Express cDNA synthesis kit.

Approximately 100 ng of the cDNA was ligated to *Eco*RI-predigested ZAP express vector and then packaged *in vitro* using the Gigapack III gold-packaging extract (Stratagene). The library contained a total of 2.1×10^6 plaque-forming units. Plaques were lifted by using nitrocellulose membrane and screened by using affinity-purified antidehydrin Abs (18).

Phagemid Excision and DNA Sequencing. Several positive clones (35 clones) were selected and further purified. Phagemids from four positive clones were excised *in vivo* from the Λ ZAP vector using a helper phage (Stratagene). Phagemid DNAs were isolated by an alkaline lysis method (19) from 25-ml overnight cultures. DNA inserts from the four clones were sequenced on both strands using the dideoxy chain termination method. The nucleotide sequences and deduced amino acid sequences were analyzed using the DNASIS and PROSIS programs (Hitachi Software Engineering, San Bruno, CA).

Genomic DNA Amplification. A gene-specific oligonucleotide primer set was designed by using PRIMER-MASTER (Version 100, Institute of Influenza, St. Petersburg, Russia) program; it was composed of GGATCAGGGTTGCAAGATCGAC (5′ primer) and CCCTTACGTTGGAGCCAACACC (3′ primer). Primers were synthesized by Genosys (The Woodlands, TX). Genomic DNA was isolated from etiolated young shoots by using DNAzol and following the instructions provided by the manufacturer (Life Technologies, Gaithersburg, MD). Genomic DNA was prepared from parental cowpea lines 1393–2-11 and 524B, whose seeds contain the ≈35-kDa DHN, parental lines 1393–2-1 and IT84S-2049, whose seeds do not contain the ≈35-kDa DHN, a set of 79 recombinant inbred lines, and the two pairs of near-isogenic lines developed by backcrossing. The recombinant inbred lines came from a cross involving 524B and IT84S-2049 and previously were used to develop a cowpea linkage map (20). Genomic DNA amplifications were performed by using a GeneAmp PCR System 9600 (Perkin–Elmer) in 50 μl of reaction containing 1.25 units of *Taq* DNA polymerase (Qiagen, Valencia, CA), 1× PCR buffer (10 mM Tris·HCl, pH 8.3/50 mM KCl_2/1.5 mM $MgCl_2$), 0.4 μM primer, 200 μM each of dNTP, and 200 ng of genomic DNA from each of the cowpea lines. PCRs were started at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 62.4°C for 30 sec, and 72°C for 1 min. The reaction then was terminated at 72°C for 10 min. PCR products were analyzed by using 1.5% agarose gels. The PCR products from the four parental lines were purified by using QIAquick PCR purification kit (Qiagen) and then sequenced on both strands in their entirety, using the dideoxy chain termination method.

Results

Emergence of the Near-Isogenic Lines at Low Temperature. We evaluated the chilling tolerance of two pairs of near-isogenic lines together with their parents. The first pair consists of A4–3-2–4 (DHN present) and A4–3-2–5 (DHN absent) with the cytoplasm of 1393–2-11 in the 1393–2-1 genetic background, and the second pair consists of lines B13–4-4–2 (DHN present) and B13–4-4–3 (DHN absent) with the cytoplasm and genetic background of 1393–2-1. The genetic similarity between each pair of isolines should be >99% because the original parental lines were F_6 siblings, and they then were backcrossed three times to provide the isolines. Emergence of the seeds of the two pairs of near-isogenic lines and parents were determined at 14°C. Lines containing the DHN had significantly greater (33 percentage points) maximal emergence than lines not having the DHN (Fig. 1). These results provide strong support for the hypothesis that the ≈35-kDa DHN protein confers an increment of chilling tolerance during emergence in cowpea. It should be noted that these cowpea seeds had 6% moisture content and would be more sensitive to chilling than seeds with higher moisture contents, such as 12%, which is typical of commercial seed (1). Although this chilling tolerance trait is more pronounced with drier seed, it is present at the higher moisture contents typical of commercial seed (1).

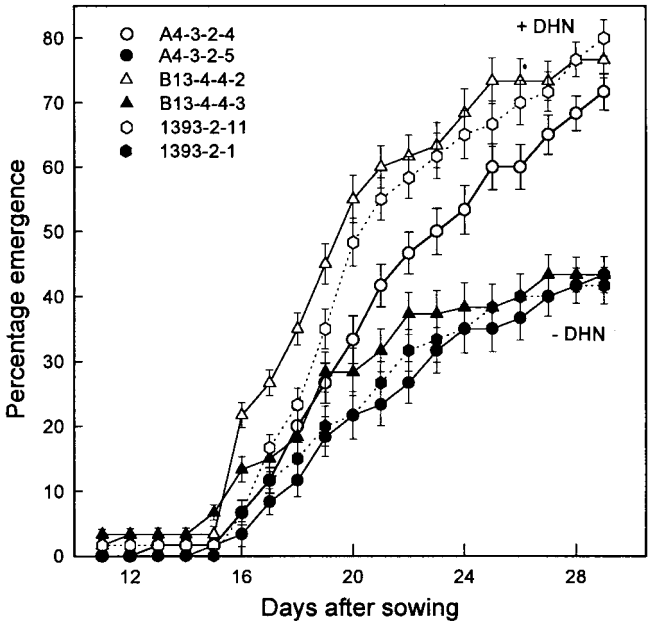

Fig. 1. Percentage emergence of cowpea lines with (open symbols) or without (closed symbols) the ≈35-kDa DHN at 14°C using seeds with 6% moisture content. Parental lines are indicated by dotted lines and the vertical bars are the standard errors.

Cloning and Sequencing of the ≈35-kDa DHN cDNA. Previously, we had purified and partially characterized the cowpea ≈35-kDa DHN (16) and had shown that its presence/absence in seeds mapped as a single gene on a cowpea linkage map (20). Our objective here was to determine whether the presence/absence of this DHN in the mapping population is caused by allelic variation in a *Dhn* structural gene or a trans-acting *Dhn* regulatory locus. For this purpose, it was necessary to identify a cDNA encoding this protein and develop a gene-specific probe. A cDNA expression library was constructed using poly(A) RNA from developing seeds. The cDNA expression library was screened by using affinity-purified antidehydrin Abs (18). Approximately 7% of all cDNA plaques were immuno-positive. A representative set of plaques then was purified and used for further analysis by DNA sequencing. The cDNA phagemids were excised from the λ ZAP vector and four cDNAs were sequenced. The four cDNAs were found to be identical to each other in sequence except for the position of the poly(A) site and the 5′ end, which is similar to observations made before with cereal *Dhn* cDNAs (21). The deduced amino acid sequence contained the polypeptide identified by partial amino acid sequencing of a cyanogen bromide fragment (16) of the ≈35-kDa DHN protein

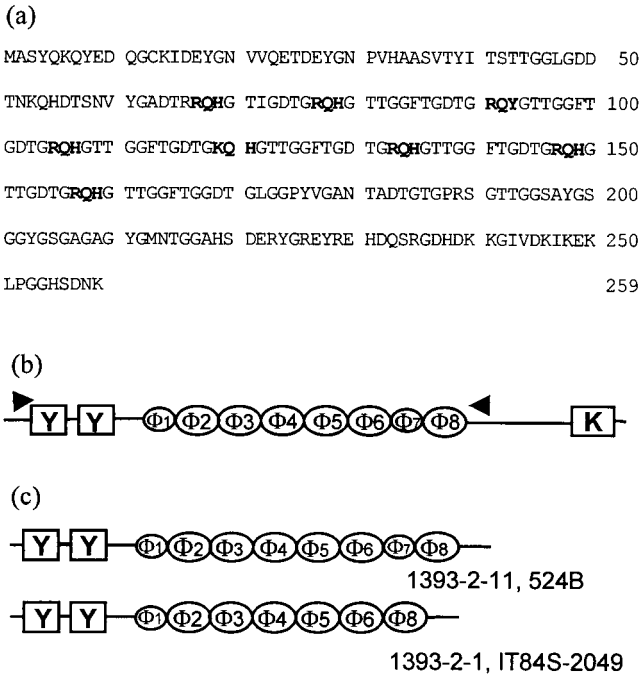

Fig. 2. (*a*) Deduced amino acid sequence of the ≈35-kDa cowpea dehydrin cDNA with the amino acid sequence of the putative-coding region presented in a single-letter code. The Φ-segment landmarks are shown in boldface. (*b*) Schematic representation showing the Y_2K structure with eight repeats (Φ-segments), six of them with 14-aa residues and two with 10 residues each. Arrows indicate positions of the PCR primers. (*c*) PCR products from each line; note that Φ_7 is deleted in the alleles represented by smaller PCR products.

(the last two T residues in ref. 16 are now considered to be spurious). One sequence was submitted to GenBank (accession no. AF159804). A high similarity, but not 100% identity, was observed between this *Dhn* cDNA and a previously published *Dhn* cDNA sequence, CPRD22, from cowpea leaves subjected to dehydration (22).

The deduced amino acid sequence of the 1393–2-11 *Dhn* cDNA is shown in Fig. 2*a*. The encoded protein consists of 259 aa (actual M_r of 26.5 kDa compared with an apparent M_r on SDS/PAGE of 35 kDa; an actual M_r 24% smaller than the apparent M_r on SDS/PAGE is typical of DHNs). Following the nomenclature scheme proposed by Close (11), this DHN is referred to as a Y_2K DHN because it has two Y-segments near the N terminus and one K-segment near the C terminus. There are eight Φ-segments between the distal Y-segment and the K-segment, two of which are smaller (10-aa residues) and six larger (14-aa residues) (Fig. 2*b*). The polypeptide is rich in Gly (25.9%), Thr (16.6%), and Asp (8.5%) and is acidic (pI = 5.98). We refer to this protein as *Vigna unguiculata* DHN1 (1393–2-11, 259) following the allele nomenclature of Choi *et al.* (23).

Mapping of the Structural Gene. To map the structural gene encoding the Y_2K DHN, gene-specific primers were designed from the nucleotide sequence and used to detect a polymorphism between cowpea lines, using PCR. Inbred lines 524B (DHN present) and IT84S-2049 (DHN absent) were the parents of a set of recombinant inbred lines used for a cowpea linkage map (20). The *Dhn*1-specific primer set amplified a single major PCR product from each of the two lines and yielded a higher M_r band from the line with the Y_2K DHN in the seed than from the one without it (Fig. 3*a*). Genomic DNA from a set of 79 recombinant inbred lines developed from IT84S-2049 and 524B were screened by using these primers; a subset of the results is shown in Fig. 3*a*. A 100% cosegregation was observed between the DHN protein and the PCR products, indicating that both the locus controlling the presence/absence of the DHN protein and the structural *Dhn* gene map to the same position or are tightly linked.

The two parental lines, 1393–2-11 and 1393–2-1, were used for developing the near-isogenic lines used in this study. The near-isogenic lines and parents (six total lines) were examined by using the same PCR primers. Similar to the recombinant inbred lines, PCR products from DHN-positive lines yield a higher M_r band compared with those without the DHN (Fig. 3*b*).

To further define the allelic variation in the *Dhn* gene between DHN-positive and DHN-negative cowpea lines, PCR products from the four parental lines were purified and sequenced. Two types of allelic variation were identified at the protein level: deletion of one Φ-segment (Φ_7) from the DHN-negative lines (Fig. 2*c*) and two single-amino acid substitutions (T replaced by S, and K replaced by R, in the DHN-negative lines).

Electrolyte Leakage from Seeds of the Near-Isogenic Lines. Electrolyte leakages under chilling conditions from seeds of the first pair of near-isogenic lines, A4–3-2–4 and A4–3-2–5, were 153 and 133 mS $m^{-1}\cdot g^{-1}$, and from the second pair, B13–4-4–2 and B13–4-4–3, were 134 and 158 mS $m^{-1}\cdot g^{-1}$, respectively. The two parents, 1393–2-11 and 1393–2-1, had electrolyte leakages of 104 and 143 mS $m^{-1}\cdot g^{-1}$, respectively, and the $LSD_{0.05}$ was 20 mS $m^{-1}\cdot g^{-1}$. These data indicate that electrolyte leakages from seeds of lines derived from both backcrosses were high and statistically similar to that of the recurrent parent, 1393–2-1, indicating nuclear inheritance and independence from the presence or absence of the DHN, which is also under nuclear control. These results confirmed our previous model (1) in regard to independence of the DHN protein effect on chilling tolerance and differences in electrolyte leakage and excluded cytoplasmic inheritance as the basis of the maternal effect on electrolyte leakage.

Discussion

In previous studies, we found that there is a genetic association between the presence of a ≈35-kDa protein in mature seed and chilling tolerance during seedling emergence of cowpea line 1393–2-11 (1). Accumulation of this protein in developing seeds is coordinated with the start of the dehydration phase of embryo development, and the characteristics of the purified protein indicated that it is a DHN (16). Among other properties, this DHN has been found (16) to adhere to other polypeptides under specific salt conditions and also to adopt an α-helical structure in the presence of SDS.

Here we provide genetic evidence of the association between the structural gene, *Dhn1*, encoding this DHN protein and the chilling tolerance trait. Two types of allelic variations in the *Dhn1* gene were identified at the protein level: deletion of one Φ-segment from the DHN-negative lines and two amino acid substitutions. These types of allelic variations are similar to differences observed between *Dhn* alleles in barley, where it was noted that the most common allelic variations involve deletions or duplications of entire Φ-segments or single amino acid substitutions (23). The genetic mechanism responsible for the presence/absence of DHN1 in mature seed is not yet known but could involve differences in gene regulation. We can be confident that the DHN1 protein encoded by the "absence" allele is not produced in seed because the Abs that detected the "presence" allele are specific to the K-segment (18), and the K-segment is identical in both allelic forms of the DHN1 protein. In addition, others have shown that the absence allele of *Dhn1* can indeed be expressed in other tissues; *Dhn1* mRNA (CPRD22) is expressed in leaves of plants subjected to drought (22). Consequently, the allelic difference that was used to study

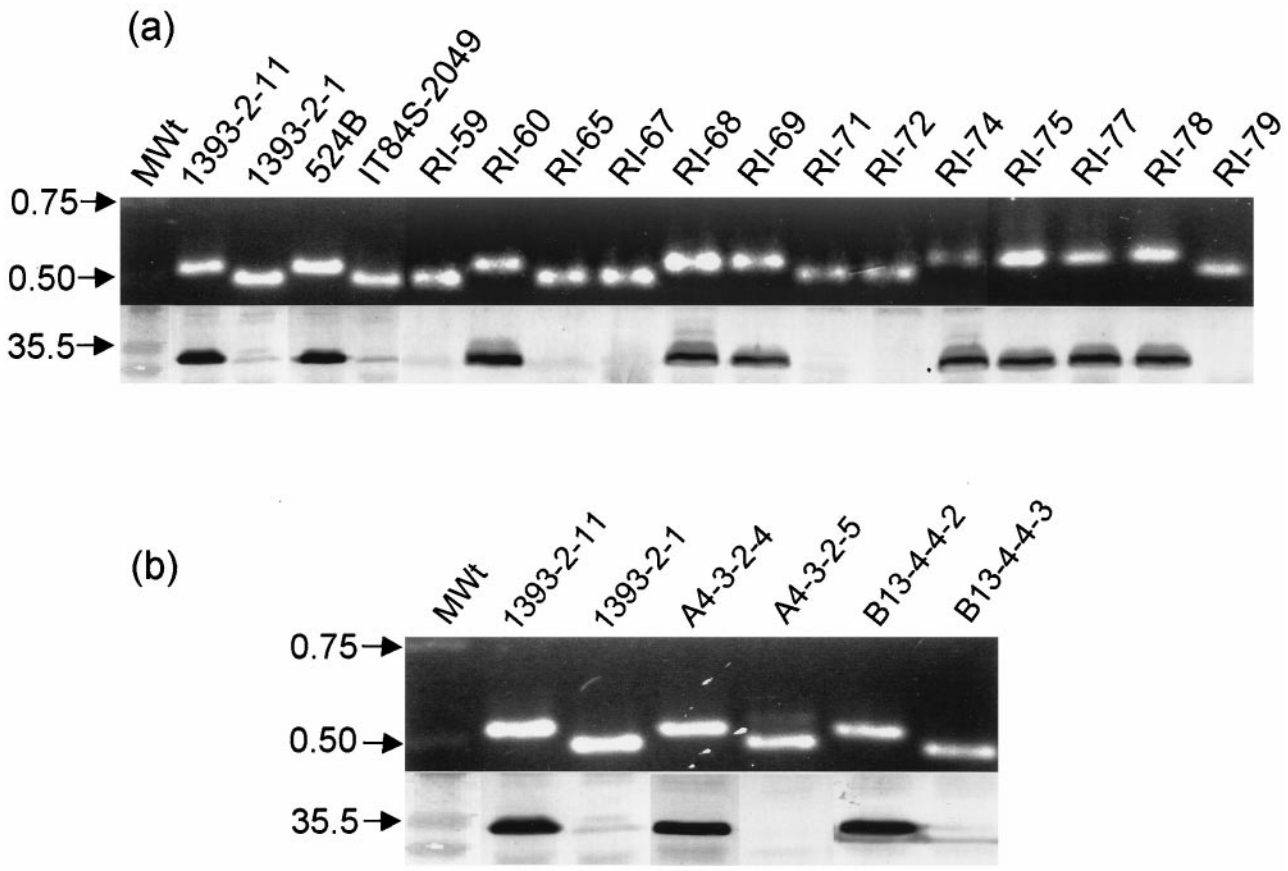

Fig. 3. PCR products (*Upper*) and Western blots (*Lower*) from parental cowpea lines and a subset of the recombinant inbred (RI) lines (*a*) and cowpea near-isogenic lines and parents (*b*). Total DNA was isolated from etiolated young shoots for PCR analysis. Total protein was extracted from dry, mature seeds for Western blot analysis.

the inheritance of the *Dhn1* gene (variable number of ϕ segments) seems unlikely to be the basis of the phenotypic effect. In addition, we cannot yet fully exclude the possibility that a gene mapping very near to the *Dhn1* gene is the true phenotypic determinant, in which case it could be only coincidental that there is allelic variation in the *Dhn1* gene and strong expression of the DHN1 protein in close parallel with the seed properties.

The mechanism whereby the ≈35-kDa DHN protein enhances chilling tolerance during emergence may not be related to the protection of plasma membrane because the effect was not associated with differences in electrolyte leakage. Previous studies provided evidence in favor of the presence of lipid-binding amphipathic α-helices in DHN proteins (16). The propensity of this DHN to adopt α-helical structure in the presence of SDS together with its apparent polypeptide adhesion suggests that it may be exerting its effect through the stabilization of proteins in an endomembrane environment. This DHN, and others, may function biochemically in a manner similar to α-synuclein, which is a protein linked to both Parkinson's and Alzheimer's diseases, in the former as the major component of Lewy bodies and in the latter as a nonamyloid component of amyloid plaques (24). α-Synuclein is a small, soluble protein of 140–143 aa that is highly enriched in presynaptic nerve terminals but not tightly associated with either the synaptic vesicle or the synaptic plasma membrane. Like the ≈35-kDa DHN (16), α-synuclein appears in solution to have a largely random conformation, unless associated with negatively charged sulfonated lipids or phospho-lipids, in which case the protein contains α-helices (24). Presumably then, the mode of action of the Y_2K cowpea DHN involves a comparable type of phospholipid bilayer surface interaction (within the interior of the cell but not the plasma membrane) that minimizes dehydration-associated lesions, which can result in diminished emergence of seedlings at low temperature.

Cosegregation of the Y_2K *Dhn* gene with chilling tolerance during emergence represents a case in which the functional significance of allelic variation in a stress-related gene has been strongly implicated by inheritance data in plants. Because several other classes of LEA and related proteins have similar conformational and compositional properties to DHNs (10, 25), it seems likely that further studies of natural allelic variation in genes encoding these proteins will explain different facets of heritable variation in stress tolerance.

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