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A view of plant dehydrins using antibodies specific to the carboxy terminal peptide

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Abstract

Dehydrins are characterized by the consensus KIKEKLPG amino acid sequence found near the carboxy terminus, and usually repeated from one to many times within the protein. A synthetic peptide containing this consensus sequence was used to produce specific antibodies that recognize dehydrins in a wide range of plants. This range covered two families of monocots, viz. Gramineae (*Hordeum vulgare* L., *Triticum aestivum* L., *Zea mays* L., *Oryza sativa* L.) and Liliaceae (*Allium sativa* L.), and five families of dicots, Malvaceae (*Gossypium hirsutum* L.), Solanaceae (*Lycopersicon esculentum* L.), Brassicaceae (*Raphanus sativus* L.), Fabaceae (*Vigna unguiculata* L.), and Cucurbitaceae (*Cucumis sativus* L.). Two families of gymnosperms, Pinaceae (*Pinus edulis* Engelm.) and Ginkgoaceae (*Ginkgo biloba* L.), were also included. For several plants in which dehydrin cDNA and genomic clones have previously been characterized, it now appears that the dehydrin family of proteins is larger, and the regulation of dehydrin expression much more complex, than earlier studies have shown.

Introduction

In the past two decades, especially the last five years, much attention has been given to the role of proteins that accumulate during plant cell dehydration. One constant that has emerged from these studies is that, regardless of whether the environmental cue is evaporation, low temperature, increased solutes, embryo desiccation, or application of the plant growth regulator abscisic acid (ABA), similar proteins accumulate [6, 9, 11, 24]. Many of the known families of such proteins are often referred to as LEA proteins, named after their initial observation as 'late embryogenesis abundant' during cotton embryo development [5, 6, 15]. Dehydrins, also known as the LEA D-11 family [1, 3, 6], are characterized by a consensus 15 amino acid sequence EKKGIMDKIKEKLPG that is present near the carboxy terminus, and in additional copies upstream of the terminus, in many cases as a slightly modified 14 amino acid consensus KKGIKEKIKEKLPG. Evidence of dehydrins has been gathered in a wide range of angiosperms, and numerous alternative names have been used [3, 6]. Many dehydrins contain a tract of serine residues, and it is at these positions where phosphorylation has been demonstrated [21, 25].

In this work we describe procedures for generating anti-dehydrin consensus region antibodies. We also present results regarding the occurrence of dehydrins in both gymnosperms and

angiosperms, and the spatial distribution of barley dehydrins.

Materials and methods

Plant material

The seeds used, and their sources, were as follows: barley (*Hordeum vulgare* L. cv. Himalaya), Department of Agronomy and Soils, Washington State University, Pullman; wheat (*Triticum aestivum* L. cv. Chinese Spring), Dr Giles Waines, Department of Botany and Plant Sciences, University of California, Riverside (UCR); maize (*Zea mays* L. inbred B73), Dr Julia Bailey-Serres (UCR); rice (*Oryza sativa* L. cv. 1R36), Dr T.T. Chang, International Rice Research Institute, Manila, Philippines; tomato (*Lycopersicon esculentum* L. cv. Ailsa Craig), Dr Elizabeth A. Bray (UCR); cotton (*Gossypium hirsutum* L. cv. Royale), Dr Jody Holt (UCR); cowpea (*Vigna unguiculata* L. CB46), Dr Anthony E. Hall (UCR); cucumber (*Cucumis sativus* L. cv. Marketmore 80), Dr Henry Munger, Cornell University, Ithaca; radish (*Raphanus sativus* L. cv. White Icicle), The Seed Bin, Ontario, California; onion (*Allium cepa* L. cv. Yellow Globe) and pinon pine (*Pinus edulis* Engelm.), Susan Sanders (UCR); *Ginkgo biloba* L., Claremont, California. For monocot seedling dehydration studies, seeds were surface-sterilized, imbibed for two days in the dark on wet filter pads, transferred to a 16 h/8 h light/dark cycle for three days, then slowly dehydrated in chambers maintained at approximately 90% relative humidity (RH), as described previously [1]. For radish seedling dehydration, surface-sterilized seeds were imbibed in the dark at 4 °C for two days, transferred to the light for three days, then dehydrated as with monocots. In some instances, seedlings were also placed in deionized water in chambers at 100% RH. The extent of seedling dehydration was monitored by periodically measuring seedling fresh weight. Plant materials were either frozen in liquid nitrogen and stored at −80 °C or used fresh.

Peptide coupling reactions

A peptide with the sequence CTGEKKGIMDKIKEKLPGQH was synthesized at the Peptide and Oligonucleotide Facility, University of California, San Diego, La Jolla with [^{14}C] glycine incorporated during synthesis.

For antigen production, BSA (Boehringer-Mannheim 100377) was treated with succinimidyl 4-(*p*-maleimidophenyl)butyrate (sulfo-SMPB) (Pierce) and then coupled to the peptide, essentially according to instructions provided by the coupling agent supplier. Of numerous reaction conditions tested, the optimum coupling was ca. 6.6 mol of peptide per mole of BSA, as determined from relative areas under [^{14}C] peaks from gel filtration. Briefly, the optimum reaction conditions were: 1) 20 mg BSA and 7 mg sulfo-SMPB in 100 mM sodium phosphate buffer pH 7 for 30 min at 25 °C; 2) reduction of 73 mg peptide in 100 mM sodium phosphate buffer pH 7.5, 2 mM EDTA, 20 mM DTT for 1.5 h at 25 °C; 3) coupling to BSA in 100 mM sodium phosphate buffer pH 7.5 for 4 h at 25 °C. Another protein-peptide conjugate, substituting ovalbumin as the carrier and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Pierce) as the coupling agent, was produced for ELISA quantification of anti-peptide antibodies using similar reaction conditions.

To produce a chromatographic support for the affinity purification of anti-peptide IgG, 58.4 mg peptide was coupled to 3 ml of SulfoLink Coupling Gel (Pierce), following the instructions of the coupling gel supplier. Immediately prior to use the peptide-SulfoLink gel was pre-conditioned by a series of washes in: 1) 10 mM Tris-HCl buffer pH 7.5, 2) 100 mM glycine buffer pH 2.5, 3) 10 mM Tris-HCl buffer pH 8.8, 4) 100 mM triethylamine buffer pH 11.5, 5) 10 mM Tris-HCl buffer pH 7.5.

Antibody production and affinity purification

Antibodies were prepared in rabbits using the peptide-BSA conjugate and Freund's adjuvant

by conventional means [12]. Anti-peptide antibody titers and specificity were determined by ELISA [12] using: 1) ovalbumin, 2) ovalbumin plus coupling agent, and 3) ovalbumin-peptide conjugate.

For affinity purification of anti-peptide IgG, crude sera were diluted 1/10 in 10 mM Tris-HCl buffer pH 7.5, then passed three times over peptide-SulfoLink gel (see 'Peptide coupling reactions'). The column was then washed with 10 mM Tris-HCl buffer pH 7.5, followed by 500 mM NaCl, 100 mM Tris-HCl buffer pH 7.5. Antibodies were eluted with 100 mM glycine buffer pH 2.5, with eluent immediately neutralized in 1.0 M Tris-HCl buffer pH 8.8. Eluted antibodies were dialyzed against 10 mM Tris-HCl buffer pH 7.5, concentrated by filtration, assayed for protein concentration by a dye-binding assay [12] with bovine gamma globulin (BioRad) as a standard, examined for purity by Coomassie Blue R-250 or silver staining [12] of SDS-PAGE gels, checked for specificity by ELISA, as described above, and stored either at −80 °C or refrigerated. Affinity purification yielded a fraction that was approximately 90% IgG with a non-specific background reaction of less than 1% of initial levels. All purification and storage solutions contained 0.02% NaN_3.

Immunoblots

Crude protein extracts were made by grinding in 30 mM TES buffer pH 7.5 with 0 to 500 mM NaCl and 1 mM PMSF, followed by centrifugation at ca. $5000 \times g$. In some cases supernatants were further clarified by centrifugation at $90000 \times g$ for 20 min. 'Heat-stable' protein fractions were prepared as previously described [1] by immersing extracts in a 100 °C bath for 10 min, transferring to ice, then centrifuging in a microcentrifuge for 10 min at 4 °C. Protein concentrations were determined by a dye-binding assay [12] using bovine gamma globulin (BioRad) as a standard. SDS-PAGE [12] gels were run using Mini-Protean II Electrophoresis Cells (BioRad), transferred electrophoretically in Mini Trans-Blot Cells (BioRad) to nitrocellulose, blocked with 3% (w/v) gelatin in TBS buffer, incubated with rabbit anti-peptide antibodies, then incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (Fisher) essentially as described previously [12]. Secondary antibody was detected using 4-nitroblue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Protein bands in duplicate gels were made visible using Coomassie Blue R-250 dye or by silver staining [12].

Results

Nature of the synthetic dehydrin antigen

The consensus carboxy terminal peptide of dehydrins that emerges from an alignment of available data [3] is: E, K, K, G/S, I/V/M/L/F, M/L/V, D/E, K, I, K, E/D, K, L/I, P, G where adjacent positions in the amino acid sequence are separated by a comma, the most frequently observed amino acid listed first, and each amino acid at a single position separated by a slash. Without exception, alternative residues are neutral in effect. For example, in addition to absolute conservation of amino acids at positions 1, 2, 3, 8, 9, 10, 12, 14 and 15, only hydrophobic amino acids have been found at positions 5, 6, 9, and 13, only acidic amino acids at 7 and 11, and only the basic amino acid, lysine, at 2, 3, 8, 10 and 12. The conservation of this sequence in higher plants is so rigorous that in cereals as diverse as rice, maize and barley there are examples of dehydrins with identical 19 residue termini TGEKKGIMDKIKEKLPGQH. The antibodies described in the present study are based on this amino acid sequence. Because all coupling chemistry was based on linkage through a sulfhydryl group, an N-terminal cysteine was included in the synthetic peptide.

Observation of plant dehydrins

Proteins were extracted from tissues expected to contain LEA proteins over a wide spectrum of

plant families. This included: (1) seeds of two families of monocots (Gramineae and Liliaceae), five families of dicots (Malvaceae, Brassicaceae, Solanaceae, Fabaceae and Cucurbitaceae), and two gymnosperms (Pinaceae and Ginkgoaceae) (Fig. 1); (2) seedlings of several cereal plants (Fig. 2); (3) seeds and seedlings of barley (Gramineae) and radish (Brassicaceae) (Fig. 3). In all cases, proteins immunologically related to dehydrins were detected.

Seed proteins were examined by immunoblotting (Fig. 1). Since mature seeds may contain not only intact, but also degraded proteins, the number of bands observed in seed extracts is likely to provide only an estimate of the number and range of mass of dehydrins in each type of seed. For example, barley embryos contain two predominant bands (25 and 60 kDa) that have consistently been observed in various tissues during stress (see also Fig. 2), and many fainter bands, which could be either intact proteins or degradation products. The approximate ap-

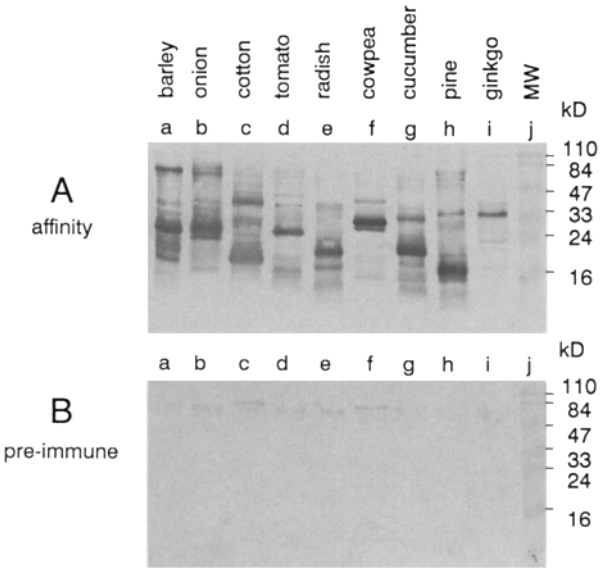

Fig. 1. Immunoblot of dehydrins from seeds. Samples were extracted from whole mature seeds of onion (20 seeds) and radish (10 seeds), whole mature seeds with seed coats removed of cotton (2 seeds), cowpea (2 seeds), cucumber (5 seeds), pinon pine (1 seed), and ginkgo (3 seeds), from embryos of mature seeds of barley (20 embryos). All extracts were prepared by grinding in 30 mM TES pH 7.5, 50 mM NaCl, 0.5 mM PMSF then clarifying at 12000 × *g* for 10 min at 4 °C, followed by ultracentrifugation of the supernatant at 90000 × *g* for 20 min. Supernatants were then heated to 100 °C for 10 min and centrifuged at 10000 × *g* for 10 min. From 9 to 35% of total protein was 'heat-stable'. Sources and quantities (μg) of 'heat-stable' [16] protein extracts loaded per lane were: a, barley (1); b, onion (1); c, cotton (4); d, tomato (2); e, radish (3); f, cowpea (1); g, cucumber (5); h, pine (1); i, ginkgo (5). Lane j contained pre-stained standards (BioRad). Panels show the results of probing with two different sources of IgG: A, affinity-purified anti-peptide IgG (5 μg/ml IgG); B, pre-immune serum diluted 1/250 ca. 50 μg/ml IgG).

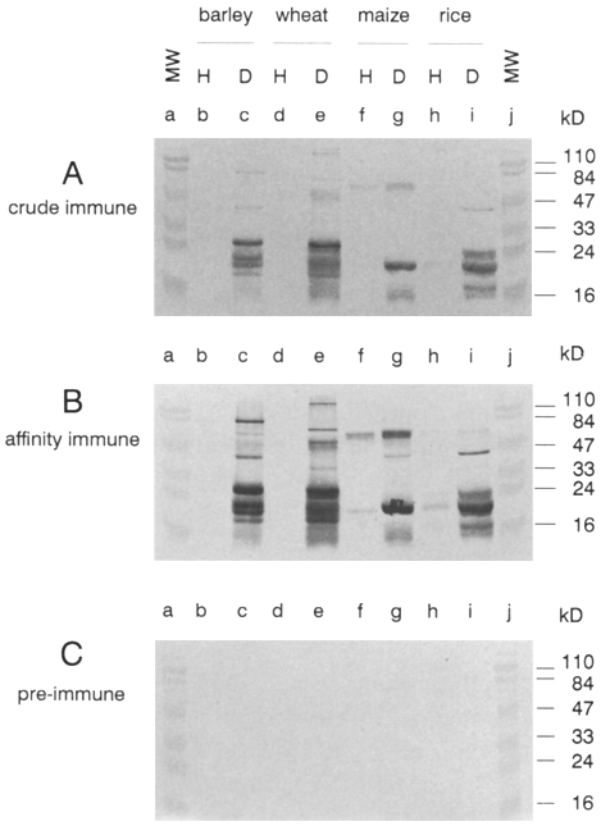

Fig. 2. Detection of barley, wheat, maize and rice dehydrins. Pairs of samples from well-hydrated or dehydration-stressed seedlings. Barley leaves (lanes b and c), wheat leaves (d and e), maize shoots (f and g), and whole rice seedlings minus endosperm (h and i) were compared to BioRad pre-stained molecular mass standards (a and j). Seedling fresh weights were reduced to the following levels of initial fresh weight by two days of dehydration: barley, 63%; wheat, 65%; maize, 69%; rice, 70%. 8 μg total protein was loaded per lane. Panels show the results of probing with three different sources of IgG from a single rabbit: A, crude immune serum diluted 1/200 (ca. 63 μg/ml IgG); B, 5 μg/ml of affinity-purified anti-peptide IgG; C, crude pre-immune serum diluted 1/200 (ca. 63 μg/ml IgG).

parent molecular mass of the most prevalent bands observed in heat-treated seed extracts were as follows: barley (25 and 60 kDa); onion (*Allium sativa* L.) (26 kDa); cotton (18 and 40 kDa); tomato (*Lycopersicon esculentum* L.) (26 kDa); radish (20 kDa); cowpea (*Vigna unguiculata* L.) (29 kDa); cucumber (*Cucumis sativus* L.) (21 kDa); pine (*Pinus edulis* Engelm.) (17 kDa); ginkgo (*Ginkgo biloba* L.) (32 kDa). None of these proteins were detected by pre-immune serum. In some cases cDNAs have been identified that correspond to the observed predominant proteins. Such is the case for barley [1], cotton [7], and radish [22].

The results of immunoblotting of proteins from hydrated and dehydrated barley, wheat, maize, and rice seedlings are shown in Fig. 2. Conditions that induce dehydrin mRNA and protein accumulation in cereal seedlings have been described previously [1, 2]. These and other studies [19, 20, 23, 25, 26] have established the presence of dehydrins in dehydration stressed seedlings of barley, wheat, maize and rice. For each plant species (Fig. 2), proteins detected by crude serum were also detected by affinity purified anti-peptide IgG, and none were detected by pre-immune serum. In Himalaya barley, four low-molecular-mass dehydrins ranging in apparent molecular mass from about 18 to 25 kDa were observed, in accordance with previous studies [1, 2]. In addition, larger dehydrins ranging up to about 90 kDa were observed. The wheat pattern is more complex than barley, presumably because of the hexaploid nature of the wheat genome. In addition to a prominent wheat dehydrin band at about 25 kDa [23], we observe dehydrins covering the full range of apparent mass between about 18 and 150 kDa. That these bands are indeed dehydrins has been further substantiated by the recent identification of three wheat dehydrin cDNAs encoding proteins of 15, 39 and 120 kDa [10, 14, 17]. In maize, in addition to a ca. 20 kDa dehydrin reported previously [1, 25], we observe bands at about 40 and 60 kDa, the largest of which are consistently present in hydrated as well as dehydrated shoots. Finally, in rice, in addition to bands between 18 and 24 kDa [19, 26] we observed larger dehydrins at about 40 and 60 kDa. In short, anti-peptide antibodies indeed detect the full range of previously described cereal dehydrins on immunoblots, and they reveal numerous immunologically related proteins whose existence has not been documented previously.

The induction patterns and solubility properties of proteins detected in barley and radish seedlings and seeds are shown in Fig. 3. For both plants (panels A and C versus panels B and D), major protein bands detected in mature seeds (lanes a) decrease during germination and seedling growth (lanes b and c), and increase during seedling dehydration (lanes d). Thus, the typical pattern of degradation during seedling growth and induction by dehydration is apparent for at least the 25 kDa barley protein and the 20 kDa radish protein. However, other bands increase and decrease with quite different patterns. All major cross-reacting proteins that were soluble in unheated extracts were also soluble in heated extracts. Thus, 'heat stability' seems to be a general property of these immunologically related proteins in barley and radish, but the pattern of gene regulation does not.

Spatial distribution of barley seedling dehydrins

Stressed and unstressed barley seedlings were dissected into five parts, and proteins were extracted and examined on immunoblots (Fig. 4). The results showed that seedling tissues contain at least some dehydrins whether stressed or not, and that the basal levels of dehydrins vary widely depending on each species of dehydrin and on the tissue. Clearly each dissected part of the seedling has a unique pattern of dehydrin accumulation, implying extensive developmental as well as environmental control. For example, the scutellum contains nearly equal quantities of a ca. 60 kDa protein whether isolated from well-hydrated or stressed seedlings, but many other dehydrins are induced by stress from undetectable levels to levels equal to the 60 kDa protein. In contrast, mainly the 25 kDa dehydrin band is detected in the aleurone layer of well hydrated seedlings, but

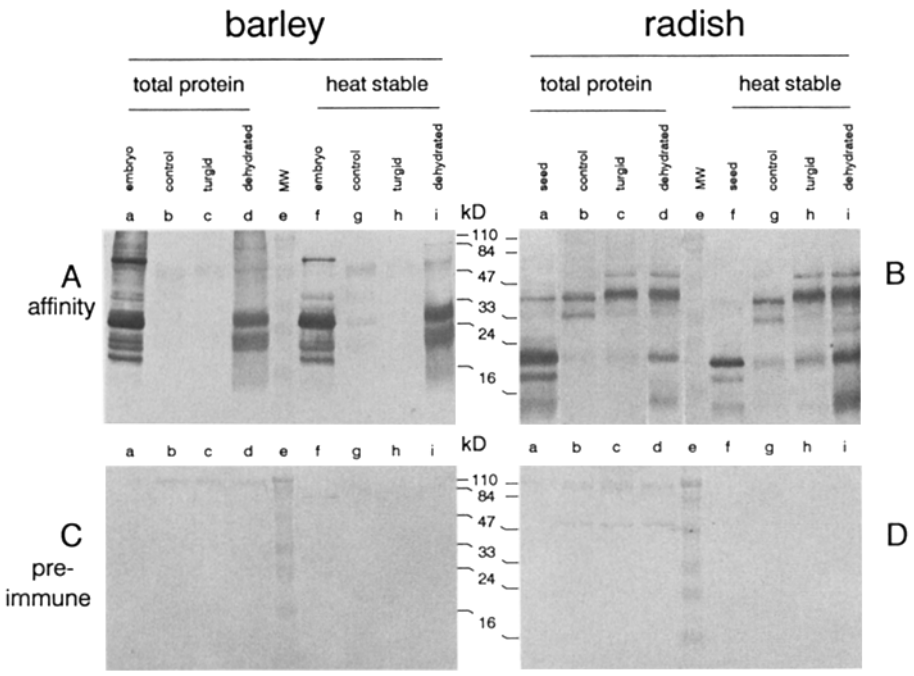

Fig. 3. Immunoblot of total and 'heat-stable' proteins from radish and Himalaya barley. All extracts were made in buffer containing no NaCl. Embryos from mature barley seeds or whole radish seeds were used for some samples (lanes a and f). Germinated seedlings were either (1) frozen as controls (after removing endosperms for barley, whole seedlings for radish) (lanes b and g); (2) placed in water inside chambers at 100% RH (lanes c and h); or (3) desiccated inside chambers at 90% RH (lanes d and i). The duration of treatments 2 and 3 above was 18 h for radish seedlings and 54 h for barley seedlings. Seedling fresh weights at harvest as a percentage of day 0 fresh weight were: turgid barley, 119%; dehydrated barley, 47%; turgid radish, 107%; dehydrated radish, 34%. Total protein (lanes a–d) or 'heat-stable' protein fractions (lanes f–i) were compared with BioRad pre-stained standards (lanes e). Barley proteins (panels A and C) were separated by 13% and radish proteins (panels B and D) by 12% acrylamide SDS-PAGE. Quantities of total protein loaded were: barley embryos (9 μg), radish seeds (4.5 μg), barley seedlings (minus endosperm) and whole radish seedlings (27 μg). The 'heat-stable' lanes contained volumes of heat-treated supernatants equal to the corresponding unheated samples. Panels A and B show the results of probing with affinity-purified antibodies (5 μg/ml IgG); panels C and D with pre-immune serum diluted 1/250 (ca. 50 μg/ml IgG).

at least eight distinct bands are observed in the aleurone layer of stressed seedlings. Once again, it is apparent that there is no universally constant pattern of gene regulation for barley dehydrins. From this it follows that it would be impossible to deduce the fundamental biochemical role of dehydrins by studying their transcriptional and translational control mechanisms.

Discussion

We have developed antibodies that recognize dehydrins in a wide range of plants, including monocots, dicots and gymnosperms. Studies with these antibodies reveal substantial complexity of the dehydrin gene family in plants.

Over all plants examined, the size of dehydrins ranges from about 15 to 150 kDa. About 10 size species are detected in Himalaya and other (not shown) barley cultivars. The spatial distribution of barley dehydrins and the response of individual genes to environmental influences constitutes another tier of complexity beyond protein size. Some dehydrins are present in some tissues whether or not seedlings are stressed, and the relative quantity of individual dehydrin species varies widely depending on the location within the seedling (Fig. 4). It must be emphasized that barley is highly inbreeding, and individual Himalaya bar-

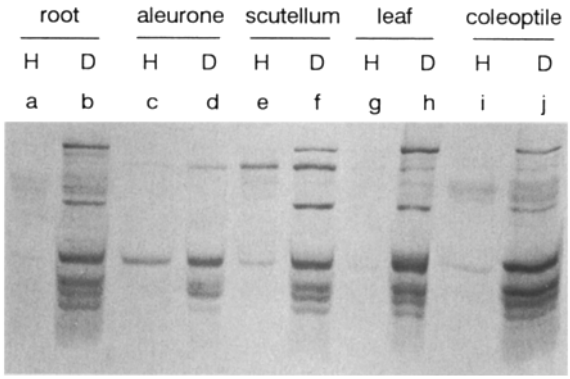

Fig. 4. Spatial distribution of barley seedling dehydrins. Pairs of samples are from hydrated (H) and dehydrated (D) barley seedlings. Extracts were prepared in buffer containing 500 mM NaCl. Total protein (8 μg each lane) was electrophoresed, transferred to nitrocellulose, reacted with 5 μg/ml affinity-purified anti-peptide IgG, and developed with goat anti-rabbit IgG. Himalaya barley extracts from roots (a and b), aleurone (c and d), scutellum (e and f), leaf (g and h), coleoptile (i and j). Protein standards are as in Fig. 2. Dehydrations were as in Fig. 2 with the fresh weight declining to 68% of the initial amount.

ley seedlings have the full range of proteins reported in this study. Therefore, this estimated number and size range applies to a single haploid genome of barley. The accuracy of this estimate is strengthened by the fact that eight distinct dehydrin nucleic and acid sequences have been characterized from Himalaya barley in a collection that is statistically unlikely to be complete. Four such sequences (Dhn1 through Dhn4) are represented by published cDNA sequences [1], and the others have not yet been published (T. Close and M. Robertson, unpublished data). In addition, it is known that three of the seven barley chromosomes (4, 6, and 7) contain dehydrin genes [2, 3]. The position of one *Dhn* locus on chromosome 6 has been accurately determined [13], as has a *Dhn* locus on chromosome 4 [18].

One dehydrin locus (*Dhn1*) was placed on the maize genetic linkage map to the long arm of chromosome six in 1988 [1], but it is not clear whether this locus contains a single gene or is multigenic. Only one maize genomic clone has been described in the literature to date [25], and it was very similar to the two cDNA clones that were described in other maize materials [1, 25]; both correspond to a 20 kDa band observed on immunoblots (Fig. 2). The fact that we observe additional protein bands at ca. 40 and 60 kDa (Fig. 2) implies that either the *Dhn* locus on maize chromosome 6 is multigenic or there are additional unlinked *Dhn* loci elsewhere in the maize genome. Similarly, in rice one locus has been described at the molecular genetic level [26], encoding four dehydrins (RAB proteins) all with an apparent molecular mass around 21 kDa, while an additional cDNA clone has indicated the existence of a 25 kDa rice RAB protein. The possibility that more than one dehydrin locus exists in maize and rice, as is certainly the case in the Triticeae, has important ramifications for any inheritance studies where one might wish to track this family of genes.

The molecular masses of dehydrins observed in seeds of several dicots (Fig. 1) suggest that the published information on dehydrins is quite incomplete in these plants also. Deduced protein sequences represent two 15 kDa dehydrins in cotton [7], one 14 kDa in tomato [4, 8], and one 19 kDa in radish [22]. Considering that dehydrins typically migrate with an apparent molecular mass up to 3–5 kDa greater than the molecular mass predicted from amino acid sequences [1], our antibodies detected all of the expected bands, and more, in protein extracts from mature seeds. The expected bands appeared at 18 kDa in cotton, 16 kDa in tomato (among the less abundant seed dehydrins), and 20 kDa in radish. Furthermore, for several species of plants we have observed additional variability by examining more than one cultivar or line. Every barley cultivar that we have examined contains a unique dehydrin pattern, as defined by migration patterns on SDS-PAGE and immunoblotting. Similarly, dehydrin patterns in maize inbreds, rice lines, and cowpea lines in most cases differ from each other (data not shown). The simplest interpretation of these observations, in conjugation with the data presented in Figs. 1–4, is that plant dehydrins are generally encoded by multigene families, whose individual members are present as multiple alleles in the total gene pool of each plant species.

Our aim is to determine the fundamental biochemical role of dehydrins. The lack of a uniform mode of regulation among the vast number of dehydrin genes that exist in plants seems to indicate that gene regulatory studies cannot lead to the fundamental biochemical role of dehydrins. In contrast, the carboxy terminal peptide seems to be a constant. Further studies that focus on the role of the consensus peptide are underway.

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