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IDENTIFICATION OF A CHLOROPLAST DEHYDRIN IN LEAVES OF MATURE PLANTS

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Several types of proteins are known to accumulate as a result of dehydration stress in plants, and many of these are thought to serve a protective function. This includes the dehydrin family of proteins, which accumulate in cells in response to drought, low temperatures, or salinity and in embryo tissues during the maturation phase of seed development, when the seed is losing water in preparation for dormancy. Many studies to date have concentrated on the expression, localization, and function of dehydrins in seed tissues. Our study provides some of the first evidence for a chloroplast-localized dehydrin by using cell fractionation combined with immunofluorescence and immunogold electron microscopy to determine dehydrin location in mature leaf tissues of *Pisum sativum* and *Zea mays*. This article also documents constitutive expression of the chloroplast dehydrin as well as expression during different dehydrative stresses. The chloroplast-dehydrin expression pattern differs from most other dehydrins studied to date and suggests a role in basic cell metabolism for this particular dehydrin.

Keywords: chloroplast, dehydration, dehydrins, lea proteins, photosynthesis, plastids, stress proteins.

Introduction

A variety of proteins are known to accumulate in plants in response to dehydration, including the group of proteins termed dehydrins. Dehydrins accumulate in vegetative tissues during conditions that impose dehydration stress, such as drought, cold, freezing, and salinity (Bray 1993; Thomashow 1999), and in the maturation phase of embryogenesis (Close 1996). Many dehydrins also accumulate following the application of abscisic acid (ABA) (Close 1996), which is not surprising given the roles of ABA in dehydration stress and seed development in plants. Interestingly, expression of dehydrins and dehydrin-like proteins has been seen to change with photoperiod in stressed and unstressed plants (Alamillo and Bartels 1996; Cellier et al. 2000). To date, dehydrins have been primarily characterized in seed tissues and herbaceous plant whole-cell extracts.

Through protein purification and molecular analysis, distinct features of dehydrins have become evident. The defining feature of dehydrins is the presence of a predicted amphipathic α-helix-forming domain, the K-segment (Close 1996). The K-segment occurs in one to 11 copies within a single polypeptide, and one K-segment is always near the carboxyl terminus. The size of dehydrin proteins ranges from 82 to 575 amino acids, and most dehydrins are hydrophilic, rich in lysine and glycine, and free of cysteine and tryptophan (Close and Campbell 1997). Dehydrins have been identified in over 30 plant taxa, and the K-segment has been shown to be conserved in both higher and lower plants (Close and Campbell 1997). Multiple dehydrin genes have been identified within each species examined to date. For example, there are 11 identified genes in wheat and three identified genes in *Pisum sativum* (Close et al. 1993; Close 1996).

Most studies that localize specific dehydrins to their cellular compartments have been limited to seed dehydrins. Multiple seed dehydrins have been localized to the cytosol and nucleus (Asghar et al. 1994; Godoy et al. 1994; Close 1996; Egerton-Warburton et al. 1997; Colmenero-Flores et al. 1999). Some studies have investigated the localization of dehydrins in mature plant tissues. For example, the dehydrin WCOR410 of wheat localizes specifically to the plasma membrane (Danyluk et al. 1998). Dehydrins have been found to accumulate in protein bodies and starch-rich amyloplasts of birch vascular tissues (Rinne et al. 1999). Two cold-responsive dehydrins of wheat, rye, and maize seedlings localize to the mitochondria (Borovskii et al. 2000). Schneider et al. (1993) identified ABA-responsive proteins that accumulate in the chloroplast, but these were characterized as "dehydrin-like" rather than dehydrins because they did not contain K-segments. Dehydrin accumulation has also been shown to be cell-type specific. The dehydrin PCA60 of peach is found in bark cells and xylem parenchyma cells (Wisniewski et al. 1999). Increased accumulation of dehydrins has been found in the epidermis and vascular tissues of bean seedlings (Colmenero-Flores et al. 1999) or guard cells of pea (Hey et al. 1997) and Arabidopsis (Nylander et al. 2001).

Evidence of a biochemical role of dehydrin proteins remains mostly speculative, with only a few studies directly addressing the physiological role of dehydrins. It is thought that dehydrins might act at the interface between membrane phospholipids and the cytosol to stabilize membranes, act at the surface of exposed hydrophobic patches on polypeptides to prevent protein-protein aggregation when protoplasmic water activity

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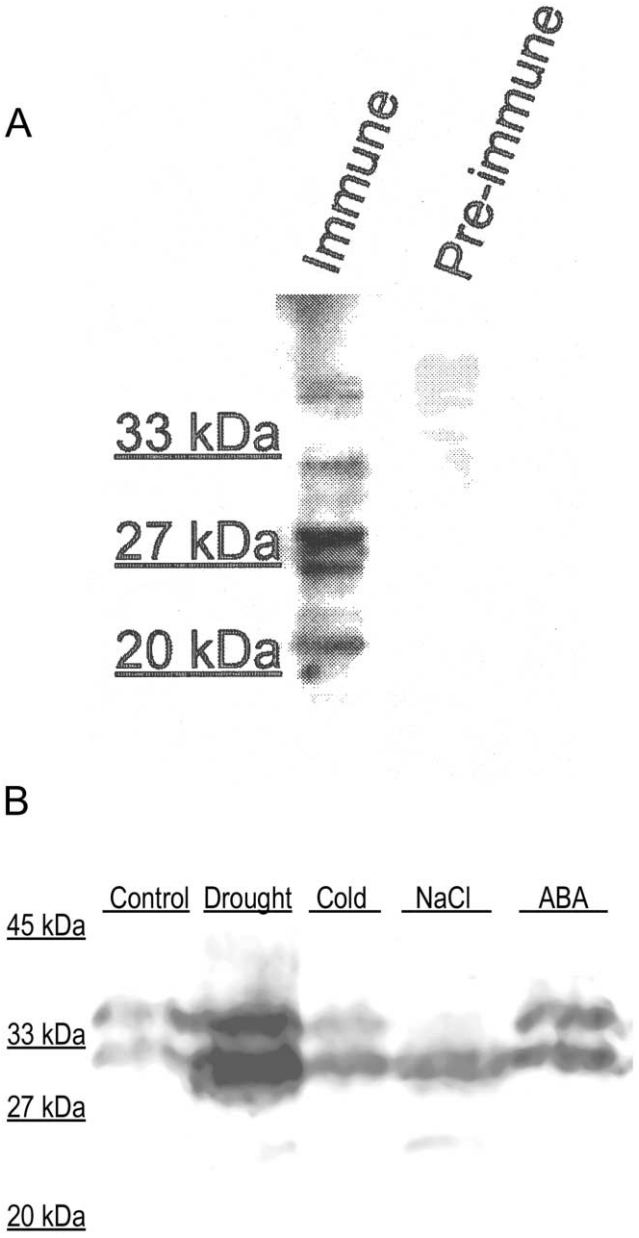

Fig. 1 *A*, Immunoblot of *Pisum sativum* seed protein extracts probed with antidehydrin immune serum and preimmune serum. Each lane was loaded with 20 μg soluble protein. *B*, Immunoblot of *P. sativum* whole-leaf protein extracts probed with antidehydrin antiserum. Each lane was loaded with 25 μg soluble protein. Experimental plants were exposed to drought, low temperature (6°C), 150 mM NaCl (soil application), and exogenous foliar application of 100 μM ABA.

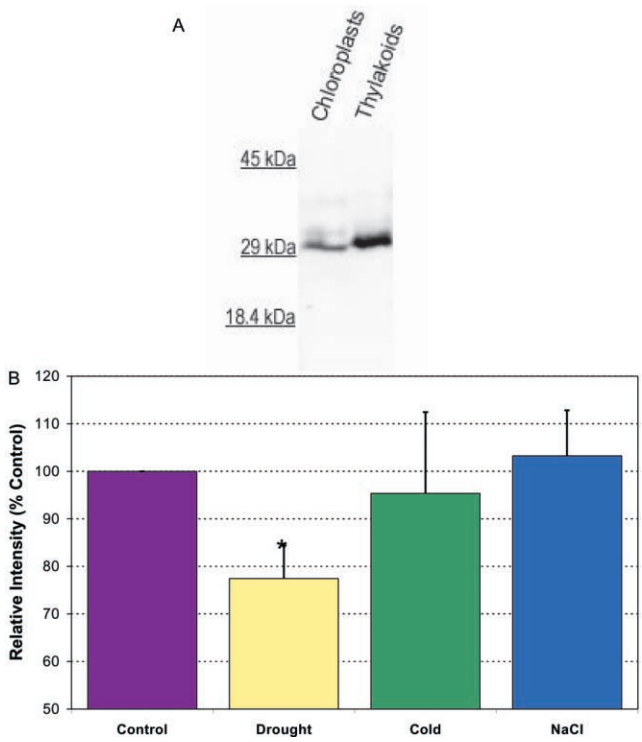

Fig. 2 *A*, Immunoblot of *Pisum sativum* chloroplast protein extracts probed with antidehydrin antiserum. Each lane was loaded with 25 μg soluble protein. Chloroplasts were isolated through differential and density gradient centrifugation. Thylakoid membranes were fractionated from whole chloroplasts with a 100 mM sorbitol solution and separated from the stromal fraction through centrifugation. *B*, Amounts of the dehydrin protein in stressed plants relative to control. Experimental plants were exposed to drought, low temperature (6°C), and 150 mM NaCl (soil application). Error bars = 1 SE. Asterisk indicates a significant difference at $P < 0.05$ (ANOVA and Kruskal-Wallis, followed by Tukey's).

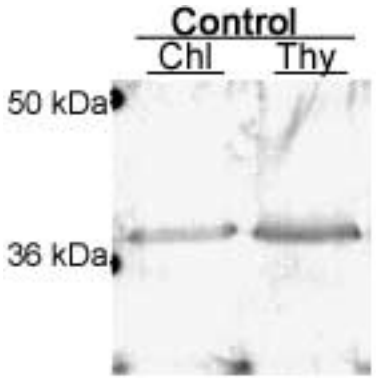

Fig. 3 Immunoblot of *Zea mays* whole-chloroplast and thylakoid protein extracts probed with antidehydrin antiserum. Each lane was loaded with 25 μg soluble protein. Chloroplasts (*Chl*) were isolated through differential and density gradient centrifugation. Thylakoid membranes (*Thy*) were fractionated from whole chloroplasts with a 100 mM sorbitol solution and separated from the stromal fraction through centrifugation.

declines, or sequester ions during water loss (Close 1996). However, there is only evidence that dehydrins have cryoprotective activity (Wisniewski et al. 1999) or slow electrolyte leakage in emerging seedlings (Close et al. 1997).

Most studies to date have concentrated on the expression, localization, and function of dehydrins in seed tissues. There is little information about dehydrin localization in mature leaf tissue or about the expression of a dehydrin in chloroplasts

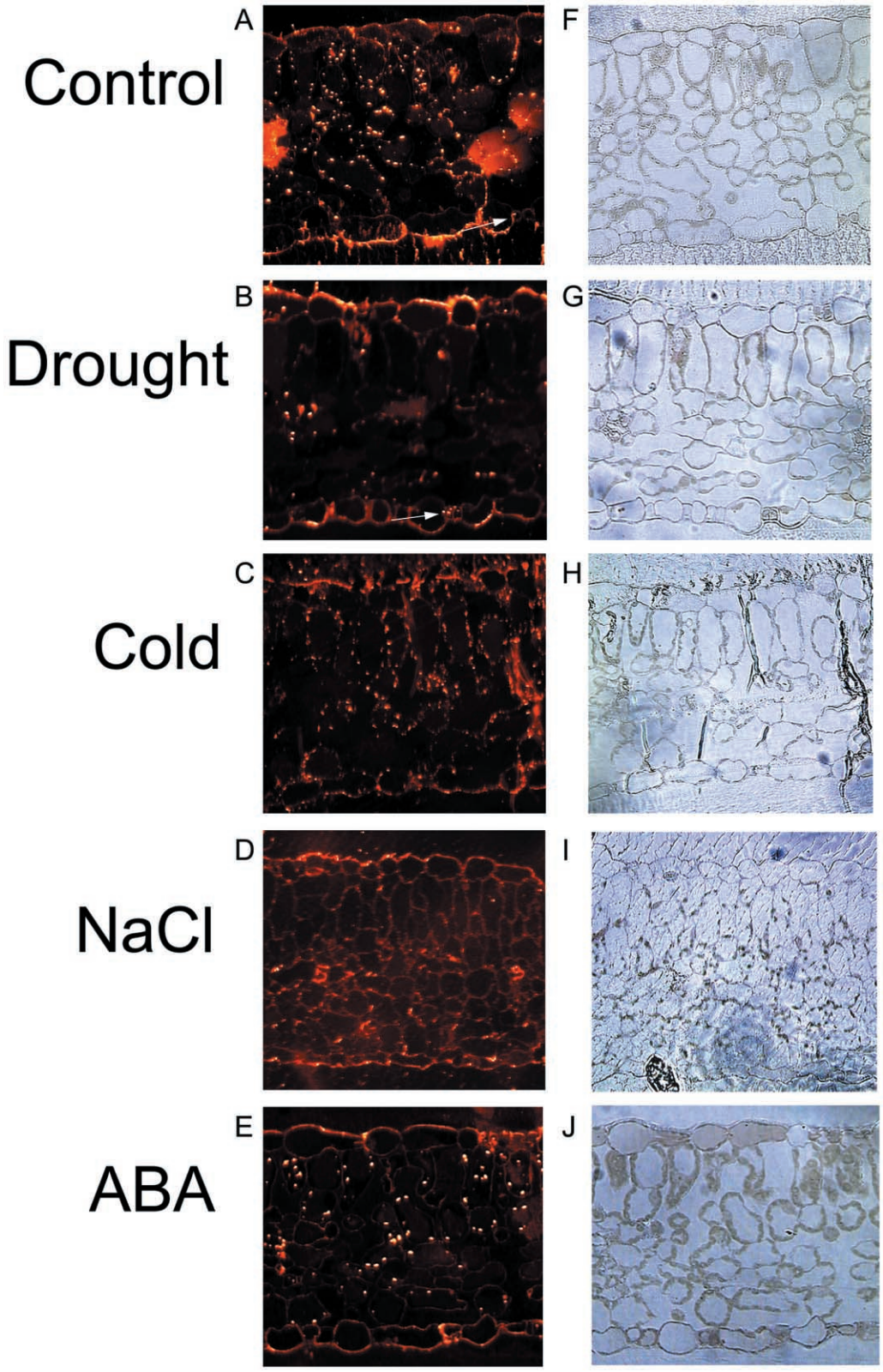

Fig. 4 *A–E*, Cross sections of *Pisum sativum* leaves viewed under a fluorescence microscope after incubation in antidehydrin antiserum and fluorescence tagging. Experimental plants were exposed to drought, low temperature (6°C), 150 mM NaCl (soil application), and exogenous application of 100 μM ABA. Sections were exposed for 1 s (control), 1 s (drought), 0.25 s (cold), 0.25 s (NaCl), and 0.25 s (ABA). Arrows in *A* and *B* indicate fluorescing guard cells. *F–J*, Same leaf cross sections viewed under white light without a filter. Cross sections were magnified to $\times 400$.

(Colmenero-Flores et al. 1999). Dehydrins have been found in the nucleus (Close 1996), mitochondria (Borovskii et al. 2000), protein bodies, and amyloplasts (Rinne et al. 1999) of higher plants. Wisniewski et al. (1999) found general distribution of dehydrins within peach cell cytosol, nucleus, and chloroplasts. We hypothesized that dehydrins are present in the chloroplasts of mature herbaceous leaves and play a protective role during stress. In this study, we describe the identification of dehydrins in chloroplasts of mature leaf tissue through subcellular fractionation and immunofluorescence and electron microscopy.

Material and Methods

Antibody Production and Affinity Purification

A 15-amino-acid sequence (EKKGIMDKIKEKLPG), known as the K-segment (Close et al. 1993), was designed, and the resulting oligopeptide was synthesized for us by BioSynthesis (Lewisville, Tex.). The peptide was conjugated to keyhole limpet hemocyanin protein and then used to induce antidehydrin antibodies in rabbits, as in Close et al. (1993). Antiserum affinity for the synthesized peptide antigen was confirmed by BioSynthesis through ELISA. To further test the accuracy of our primary antibody, pea seed proteins were separated by SDS-PAGE, blotted onto nitrocellulose, and incubated in preimmune or immune serum. The comparison blots showed that antibodies of the antidehydrin immune serum identified previously characterized pea seed dehydrins of ca. 29 and 27 kD (Robertson and Chandler 1992), and preimmune antibodies showed no affinity for the same proteins (fig. 1*A*). Additionally, seed and leaf protein blots were analyzed to compare a previous dehydrin antibody (kindly provided by T. J. Close) prepared with the same sequence, with the primary antibody synthesized for us by BioSynthesis. Insignificant differences were observed between the two antibodies (not shown), which confirmed that the antibody we used throughout the rest of the study was functionally equivalent to the antibody provided by T. J. Close.

A column of Ultralink Biosupport Medium (Pierce) was used to affinity purify the antidehydrin antiserum. The 15-amino-acid peptide used to produce the antiserum was covalently bound to media beads according to the manufacturer's instructions. Antiserum was then run through the column and washed free of the miscellaneous IgGs. The peptide-specific IgGs were eluted by washing with acid glycine.

Plants

Pisum sativum cv. Little Marvel (pea) seeds obtained from Agway (Hall, N.Y.) and *Zea mays* L. cv. 3172 (corn) grains obtained from Pioneer Hi-Bred (Johnston, Iowa) were germinated and grown in 8-cm pots in growth chambers. Pea plants were grown at 20°C : 18°C day : night temperatures with 200 μmol^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by fluorescent and incandescent bulbs. Corn plants were grown at 28°C : 22°C day : night temperatures with 400 μmol^{-2} s^{-1} PPFD. Plants were watered daily and fertilized weekly with Hoagland's solution. For both species, drought stress was imposed by withholding water until the leaf relative water content (RWC) was less than 70% (RWC = [fresh biomass − dry biomass]/[saturated biomass − dry biomass] × 100). To impose cold stress, temperatures were decreased from 18°C to 6°C over 7 h and held at 6°C for 20 h before harvest for pea plants. Corn plant temperatures were similarly reduced to 10°C and held at 10°C for 20 h before harvest. To impose a salt stress, pots were submerged to 2-cm depth in 150 mM NaCl for 7 d before harvest for both species. To determine the effects of ABA application, some plants were treated with an aqueous ABA solution (100 μM ABA, 0.01% Tween) by spraying the leaves 18 h before harvest for both species. The ABA concentration used was based on past studies (Robertson and Chandler 1992; Danyluk et al. 1998). All pea plants were harvested at the 4-wk stage of development, and corn plants were harvested at the three-leaf stage of development. Pea seeds used for protein extraction were placed on saturated filter paper in total darkness and harvested 24 h after imbibition.

Chloroplast Preparation and Immunoblotting

Chloroplasts were isolated as described by Downs et al. (1998). Total detergent-soluble chloroplast proteins were extracted with Laemmli buffer and heated to 85°C for 5 min (Laemmli 1970). Protein concentrations of samples were determined based on Coomassie blue staining according to Ghosh et al. (1988). Equal soluble protein per lane was fractionated by SDS-PAGE using 12% acrylamide mini gels as in Coligan et al. (1997). Proteins were electrophoretically transferred to nitrocellulose. The blots were incubated in Tris-buffered nonfat milk (pH 7.0) for 60 min and then incubated with dehydrin antiserum for 2 h. Primary antibody was revealed with goat antirabbit IgG conjugated to alkaline phosphatase (BioRad), and the protein-antibody complexes were visualized by chemiluminescence (BioRad). Relative amounts of protein-antibody complexes were estimated using a desktop scanner (Agfa Duoscan T1200) and NIH imaging software.

Microscopy Tissue Preparation

Leaf pieces (1 × 1 mm) were fixed in 3% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) overnight at 4°C. The leaf samples were postfixed in 1% OsO_4 for 2 h at room temperature and dehydrated with a graded ethanol series, which also extracted the chlorophyll from the samples, preventing autofluorescence (see "Results"). To completely dehydrate the tissue, samples were transferred into 100% propylene oxide. Tissues were infiltrated, embedded, and cured with Embed 812 (E.M.S, Fort Washington, Pa.). Ultrathin sections (0.2–0.3 μm) for immunofluorescence microscopy were cut with glass knives and prepared for antibody incubation.

Pisum sativum leaf samples for electron microscopy were prepared as above with the omission of the OsO_4 postfixation. Ultra thin sections (60–80 nm) were made with a Reichert-Jung Ultracut E Ultramicrotome and mounted on nickel grids.

Immunofluorescence Microscopy

Ultrathin sections were incubated in 1% nonfat milk in 0.1 M Tris-buffered saline (TBS; pH 7.5) and 0.01% Tween-20 for 60 min, washed in TBS, and then incubated in an antidehydrin-antibody TBS solution (1 : 10) for 90 min. Sections were washed and then incubated in goat antirabbit Rhoda-

mine-red conjugate fluorescent antibody for 45 min. Sections were washed and viewed under a fluorescence microscope (Leica DMLB) with 545 nm excitation and 610 emission filters.

Electron Microscopy

Ultrathin sections on nickel grids were incubated in 1% nonfat milk in 0.1 M TBS (TBS; pH 7.5) and 0.01% Tween-20 for 60 min. The sections were then incubated in a purified antidehydrin-antibody TBS solution (1 : 40) for 3 h. The sections were washed and incubated with goat antirabbit colloidial gold (10-nm particles) TBS solution (1 : 25) for 2 h and subsequently stained with uranyl acetate and Reynold's lead citrate. Sections were then examined with a JEOL 1200 EX TEM at 60 kV.

Results

Leaf Dehydrin Identification in Pisum sativum

To confirm the presence of dehydrins in leaves of *Pisum sativum*, total protein extracts were probed with the antidehydrin antibody. Immunoblot analysis showed the presence of an ca. 31-kD and a 35-kD dehydrin under all treatments (fig. 1*B*). These bands were seen with less intensity in seed protein extracts (fig. 1*A*). Drought-stressed tissue showed the highest production of the 31-kD dehydrin, per equal total leaf protein, compared with other treatments.

Identification of Chloroplast Dehydrins in Pisum sativum *and* Zea mays

To determine if dehydrins are present in chloroplasts, we first isolated chloroplasts from leaves of control, drought-stressed, cold-stressed, NaCl-stressed, and ABA-treated *P. sativum* plants. Immunoblots of the detergent-soluble proteins from these chloroplasts showed the detection of a dehydrin of ca. 31 kD in control leaf tissue (fig. 2*A*), as did the whole-leaf protein extract immunoblot (fig. 1*B*). Higher levels of the chloroplast dehydrin (per equal total protein) were found in the thylakoid fraction, which indicates that the dehydrin was mostly associated with the thylakoid membrane. Densitometry analysis of four immunoblots showed no change in chloroplast dehydrin levels with cold and NaCl treatments but a slight decrease with drought (fig. 2*B*). To determine if the presence of the chloroplast dehydrins can be generalized to other species, chloroplast proteins were also extracted from *Zea mays* choroplasts. As with *P. sativum*, immunoblotting indicated the presence of a chloroplast dehydrin in *Z. mays*, with a molecular weight of ca. 37 kD (fig. 3). The thylakoid fraction also showed higher levels of expression compared with the whole chloroplasts.

Immunofluorescence and Histological Analyses of Dehydrins in Leaves

To confirm the presence of chloroplast dehydrins and investigate cell-type specificity of leaf dehydrins, leaf tissue was fixed, embedded, and sectioned for immunofluorescence analysis. Following immunolabeling, dehydrin proteins were detected constitutively within the chloroplasts of *P. sativum* (fig. 4*A*, 4*F*).

The detection of leaf dehydrins varied among treatments and cell types. In the drought-stressed leaf cells, the number of fluorescing chloroplasts and the intensity of expression of the chloroplast dehydrin was substantially lower compared with other stress treatments (fig. 4*B*, 4*G*). The microscope field of view reflects leaf area and not total leaf protein, which decreases during drought. This may account for the drought treatment difference seen in immunoblot analysis and immunofluorescence. Also, the guard cells of drought-stressed leaves showed the expression of dehydrin, which was not observed in control guard cells. A similar pattern was seen in cold-stressed leaves as in control leaves (fig. 4*C*, 4*H*). NaCl-stressed leaves showed a noticeable decline in chloroplast dehydrin expression (fig. 4*D*). However, there appeared to be fewer chloroplasts present within these cells (fig. 4*I*). ABA-treated leaves showed the expression of the chloroplast dehydrin at levels similar to the control leaves. However, the intensity of the chloroplast signal is much greater in ABA-treated leaves than in control leaves.

The pattern of expression observed in *P. sativum* leaves was similar to that of *Z. mays* leaves (fig. 5). The expression of dehydrins in chloroplasts is constitutive and decreases in drought-stressed leaves. In contrast, the expression decreases with ABA-treated leaves and increases with cold-stressed and NaCl-treated leaves. Interestingly, the chloroplast dehydrin localized to the bundle sheath chloroplasts only.

Fluorescence observed in pea leaves was dehydrin specific. There was no significant fluorescence detected in samples treated with preimmune serum, secondary antiserum only, or buffer only (autofluorescence) (fig. 6). Similar results were seen for *Z. mays* (data not shown).

Electron Microscopy Analysis

Ultrathin sections of *P. sativum* leaf samples were treated with the purified antidehydrin antibodies, and subsequent gold-particle labeling showed the presence of a chloroplast dehydrin in chloroplasts of control tissues (fig. 7). Gold labels were found in all chloroplasts examined and appeared to be attached to both the thylakoid, grana, and stromal regions of the chloroplast. Very little background was observed, and most gold particles appeared to be specifically attached to cellular structures. As with immunofluorescence and immunoblotting, sections incubated with the preimmune serum were examined and showed no appreciable level of gold labeling.

Discussion

Using cell fractionation, immunofluorescence microscopy, and immunogold TEM techniques, this study revealed the presence of chloroplast dehydrins in mature leaves of *Pisum sativum* and *Zea mays*, to our knowledge, the first detailed evidence for chloroplast-localized dehydrins in herbaceous plants. Moreover, the chloroplast dehydrin was found to have a unique expression pattern compared with previously studied dehydrins (Close 1996; Close et al. 1997; Egerton-Warburton et al. 1997). This unique pattern indicates that the chloroplast dehydrin is not regulated by the same mechanisms as most other dehydrins. The constitutive expression of the chloroplast dehydrin infers that it fulfills an as yet unidentified house-

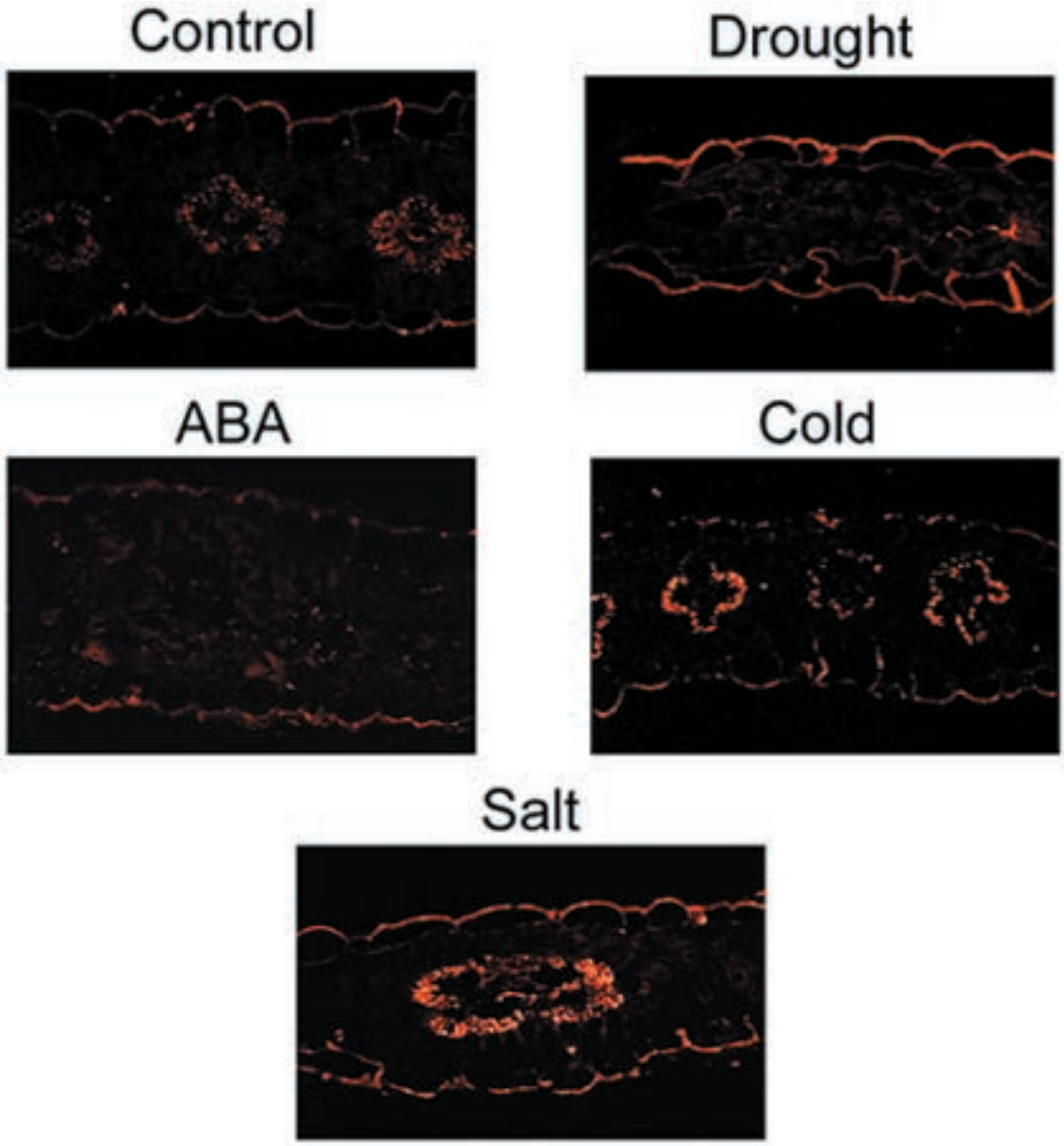

Fig. 5 Cross section of *Zea mays* leaves viewed under a fluorescence microscope. Experimental plants were exposed to drought, low temperature (6°C), 150 mM NaCl (soil application), and exogenous application of 100 μM ABA. All cross sections were exposed for 0.25 s at ×400 magnification.

keeping role in the chloroplast. Robertson and Chandler (1994) also found evidence for a constitutive dehydrin that was not responsive to ABA, which they referred to as a dehydrin cognate, and mitochondria dehydrins appear to be constitutive (Borovskii et al. 2000). We have not yet isolated and sequenced our chloroplast protein, so we can only confirm that the chloroplast dehydrin is immunologically related to the dehydrin family.

The results of chloroplast fractionation indicated that much of the dehydrin was associated with the thylakoids in both *P. sativum* and *Z. mays*. TEM results confirmed this, which indicates that the dehydrin was associating with both thylakoid and stromal chloroplast fractions. Initial results from *Z. mays* indicate that the dehydrin protein is easily washed from thylakoid membranes with high concentrations of NaCl and thus is bound to the thylakoids as a peripheral protein (Mueller 2001).

The localization of the chloroplast dehydrin was further supported by whole-leaf immunofluorescence microscopy. The fluorescent microscopy revealed high dehydrin signal within the chloroplasts, which agreed with chloroplast and whole-leaf protein extract immunoblots that showed the most prominent leaf dehydrin is found within the chloroplast. The immunofluorescence microscopy also revealed unique cell- and stress-specific patterns of dehydrin expression in leaf tissue. Thylakoid-rich chloroplasts contain higher levels of thylakoid

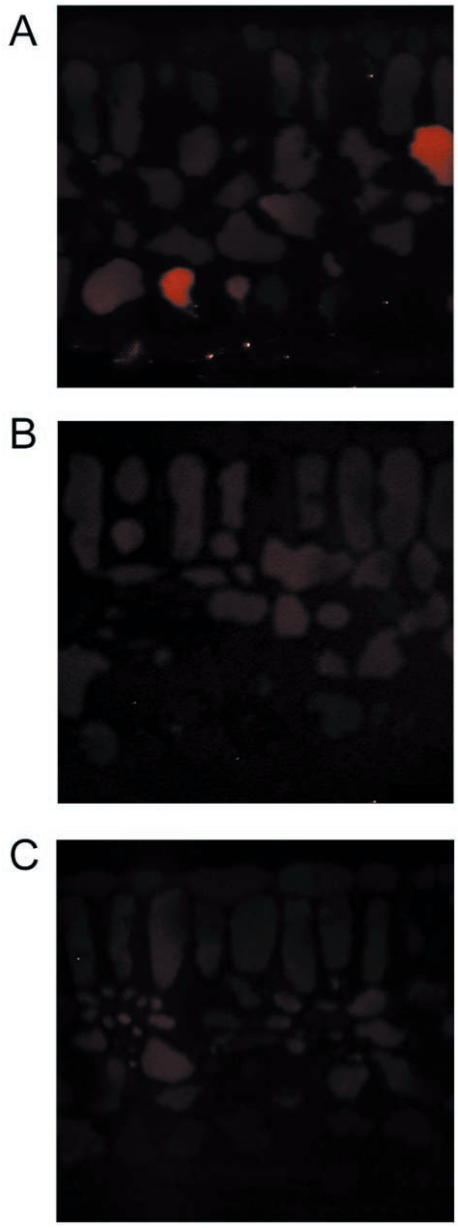

Fig. 6 Cross sections of *Pisum sativum* leaves viewed under a fluorescence microscope after incubation in (*A*) preimmune antiserum, (*B*) secondary antibody only, and (*C*) buffer only (autofluorescence). All cross sections exposed for 1 s and magnified to ×400.

membranes, and this increase in thylakoid membranes is visually detected by the increased density or darkness of many of the chloroplasts. In *P. sativum*, the chloroplasts that showed the greatest amount of dehydrin expression were the thylakoid-rich chloroplasts. This agreed with the chloroplast protein extract immunoblot that detected higher amounts of dehydrin in the thylakoid fractions. Guard cells of the drought-stressed leaf also showed the expression of a dehydrin. Guard cells are the only cells of the epidermis that contain chloroplasts. Immunofluorescence microscopy of *Z. mays* leaf tissue revealed that the expression of the chloroplast dehydrin was specific to bundle-sheath cells. *Zea mays* is a C_4 plant, and thus the photosynthetic light reactions occur in the mesophyll cells, while the carbon assimilation reactions occur in the bundle-sheath chloroplasts (*Z. mays* belongs to a C_4 subtype that has some thylakoids in the bundle-sheath chloroplasts). The expression of the chloroplast dehydrin in the bundle sheath may provide insight into the role of the protein.

Currently we are investigating the function of the chloroplast dehydrin. Using an *in vitro* assay, we have obtained preliminary results suggesting that purified dehydrins can stabilize thylakoid membranes (Mueller 2001). Steponkus et al. (1998) showed that COR15a, a nondehydrin cold-stress protein in *Arabidopsis thaliana*, can increase the cryostability of the chloroplast inner membrane during low temperature. Perhaps the chloroplast dehydrin functions in a similar way.

Acknowledgments

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Fig. 7 Ultrathin sections (60–80 nm) of embedded *Pisum sativum* leaf samples examined by TEM after immunogold labeling. Sections were labeled with colloidial gold conjugated antibody after incubation in affinity purified antidehydrin antiserum.

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