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Expression of "Dehydrin-Like" Proteins in Embryos and Seedlings of *Zizania palustris* and *Oryza sativa* during Dehydration[1]

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ABSTRACT

Proteins inducible by dehydration and abscisic acid (ABA), termed dehydrins or RAB (Responsive to ABA) proteins, have been identified in a number of species and have been suggested to play a role in desiccation tolerance, particularly during seed development. Seeds (caryopses) of North American wild rice (*Zizania palustris* var *interior* [Fassett] Dore) are tolerant of dehydration to <10% moisture content (fresh weight basis) only under restricted dehydration and rehydration conditions. In comparison, seeds of paddy rice (*Oryza sativa* L.) readily tolerate desiccation to <5% water content. Expression of "dehydrin-like" proteins in *Zizania* and *Oryza* seedlings and embryos was examined to investigate the relationship between the presence of such proteins and desiccation tolerance. [^{35}S]Methionine labeling of newly synthesized proteins showed that seedlings (first leaf stage) of both *Zizania* and *Oryza* synthesized a novel "heat-stable" protein of apparent molecular weight = 20,000 when dehydrated to <75% of their initial fresh weight. ABA (100 micromolar) induced synthesis of a protein with similar electrophoretic mobility in both species. Western blots using antiserum raised against maize (*Zea mays* L.) dehydrin detected a protein band from dehydrated *Zizania* shoots and mature embryonic axes that comigrated with the labeled 20-kilodalton polypeptide. Northern blots using a cDNA for an ABA-responsive protein from *Oryza* (*rab16a*) showed that both seedlings and excised embryonic axes of *Zizania* accumulated RNA similar in sequence to *rab16a* in response to water loss. *Zizania* seedlings and embryonic axes were also capable of ABA accumulation during dehydration. The intolerance of *Zizania* seeds to dehydration at low temperature is apparently not due to an absence of dehydrin-like proteins or an inability to accumulate ABA.

When plant tissues experience water loss, a number of metabolic changes occur, including accumulation of ABA and synthesis of new mRNAs and polypeptides (see ref. 27 for review). Among these ABA-inducible (Responsive to ABA, or RAB proteins) (22) and water stress-inducible (Water Stress Proteins, or WSP) (7) proteins is a family whose members share extensive amino acid sequence similarity. This family has been designated as "dehydrins" in barley (*Hordeum vulgare*) and maize (*Zea mays*) (6) and it shows sequence similarity to RAB17 in maize (29), RAB16 in rice (*Oryza sativa*) (22), and D11 from cotton (*Gossypium hirsutum*) (2). These proteins are highly hydrophilic and remain soluble after boiling (6), a characteristic that has been termed "heat stability" (12). Nucleotide sequences of dehydrins from a number of plant species (2, 5, 7, 22, 29) indicate conservation of a lysine-rich region in the deduced amino acid sequence that ischaracteristic of the dehydrin family. The conservation of dehydrin sequences across species and their induction by dehydration have led to the suggestion that they may play a role in preventing cellular damage during water stress (2, 5–7, 11, 27).

Most seeds dehydrate at the termination of development, and dehydrin transcripts have been identified among the *l*ate-*e*mbryogenesis-*a*bundant (*lea*) mRNAs synthesized during embryo maturation prior to and during dehydration (refs. 7 and 11, and P.M. Chandler [unpublished results] for maturing maize kernels and barley grains). It is possible that dehydrins or other LEA proteins are involved in the development of desiccation tolerance as seeds mature (7, 11) or in the maintenance of dormancy (21). Seeds from *Arabidopsis thaliana* plants homozygous for both ABA-deficiency and ABA-insensitivity mutations exhibited vivipary, reduced protein accumulation, and intolerance of desiccation (13). Supplying the double mutant plants or developing seeds with ABA or an active ABA analog increased both the synthesis of proteins associated with seed maturation and the tolerance of the seeds to desiccation (13, 20). Other studies have indicated that seed maturation proteins may contribute to the development of desiccation tolerance, but also concluded that their presence alone is not sufficient to confer tolerance (3).

Another approach to test whether dehydrins are involved in desiccation tolerance is to determine whether they are absent from seeds that are not tolerant of desiccation. Although the majority of angiosperm seeds desiccate at maturity, the seeds of a large number of plants are not able to dehydrate without damage. Such seeds, termed "recalcitrant" because of difficulties in their long-term storage, suffer loss of viability at relatively high water contents and do not desiccate at the termination of seed maturation (9, 25). In the most extreme cases, recalcitrant seeds exhibit little or no quiescent period and begin to germinate immediately at the completion of development (9). Although recalcitrance is rare among

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grasses, the seeds (caryopses) of *Zizania palustris* var *interior* (Fassett) Dore, cultivated North American wild rice, have been reported to be intolerant of desiccation (24). *Zizania* is an aquatic plant, and the seeds normally abscise and fall into the water at approximately 30% moisture content (fresh weight basis), remain dormant and submerged during winter, and germinate in the spring. Contrary to earlier reports, it has recently been shown that *Zizania* seeds can be successfully dehydrated and rehydrated, but only under restricted conditions (14). Dehydration to embryo water contents below 10% (fresh weight basis) must occur at temperatures above 25°C, and must be followed by extended slow rehydration at temperatures above 10°C, if viability is to be retained. By comparison, other mature cereal grains readily tolerate dehydration to water contents below 5% without exhibiting such temperature sensitivity (8). *Zizania* seeds are therefore not strictly recalcitrant or "homoiohydrous" (9), but are intolerant of desiccation under some conditions (14).

The objective of the present work was to determine whether *Zizania* seeds and seedlings contain "dehydrin-like" proteins or can synthesize them in response to dehydration or ABA application, as occurs in other cereals. We also determined the capacity of *Zizania* embryonic axes and seedlings to accumulate ABA, as an inability to synthesize ABA in response to dehydration might prevent dehydrin expression.

MATERIALS AND METHODS

Plant Material

Zizania palustris var *interior* (Fassett) Dore seeds were harvested by hand from plants grown in the field near Davis, CA. Seeds were at 30% moisture content (fresh weight basis) at harvest and were quickly cleaned of debris and submerged in water. The seeds were stored at 15°C for the first 5 months, which maintains viability but does not break dormancy (15), then transferred to 5°C for 3 months prior to the start of these experiments to break dormancy. For seedling experiments, seeds were dehulled by hand and sterilized for 30 min in 1% NaOCl, then rinsed extensively in sterile 10 mM HCl and water prior to incubation in sterile water at 20°C under continuous fluorescent illumination (~100 $\mu Em^{-1}\ s^{-1}$). The seeds germinated in beakers under 1.5 cm of water and were used for experiments when the first leaves of most seedlings were approximately 5 cm long (about 9 d). In some experiments, dormancy was broken by removing the pericarp/testa from above the embryo. For experiments with embryonic axes, intact seeds were sterilized as above and the hull and pericarp/testa were removed above the embryo with a scalpel. The embryonic axis was then excised from the scutellar tissue enclosing it.

Seeds of paddy rice (*Oryza sativa* L. cv Calrose) obtained from the CSIRO Plant Quarantine Unit were sterilized as described above and germinated on moist filter papers at 27°C. For embryo experiments, the embryos were excised from the seeds at the scutellum after imbibition overnight.

For seedling dehydration, seedlings were selected for size uniformity and placed inside desiccators containing 1 L of glycerol-water solutions (10–30% glycerol [v/v], or 97–87% [v/v]) to maintain a desired RH. Groups of seedlings (with the caryopses attached) were weighed at intervals to determine the water loss relative to the initial fresh weight. For embryo dehydration, 10 excised embryos or embryonic axes per replicate were placed in an aluminum foil cup and incubated at either 5 or 20°C inside a closed microfuge tube containing 200 μL of either 25% glycerol (90% RH) or a saturated salt solution: K_2SO_4 (97% RH), KNO_3 (93% RH), KCl (86% RH), or $(NH_4)_2SO_4$ (81% RH). The average fresh and dry (1 h at 130°C) weights were determined for fully imbibed embryos or axes and were used to convert weight loss to percent water content values.

For treatment with ABA, seedlings were partially submerged (approximately half of the shoot extending above the solution) in beakers containing 100 μM ABA. The beakers were incubated at 20°C under fluorescent light. The solutions were replaced daily to maintain high ABA levels throughout the 4-d incubation.

Protein Labeling, Extraction, and Electrophoresis

For protein labeling, 5 to 10 seedling shoots or embryos were diced into 1 mm pieces and incubated at approximately 23°C for 1.5 h in 50 μL of water containing 150 μCi of [^{35}S] methionine. The samples were then stored in liquid N_2. For protein extraction, tissues were ground in a mortar with sand in 1 mL ice-cold extraction buffer (20 mM Tes-KOH, 500 mM NaCl, pH 8.0) per 50 mg of tissue and insoluble material was removed by centrifugation. Protein concentration in the supernatant was determined by the Lowry *et al.* (18) method using BSA as a standard. Incorporation of label into protein was determined by scintillation counting after precipitation in 10% TCA. Samples to be heated were immersed in a water bath at 100°C for 10 min, kept on ice for 15 min, then centrifuged to remove precipitated material. Supernatant proteins were precipitated by the addition of four volumes of acetone, centrifuged at 10,000*g* for 5 min, the pellet air-dried, and then redissolved in SDS-dye buffer (28). Sample treatments, discontinuous gel electrophoresis, and processing of gels were carried out essentially as described previously (28). Gels of both total and "heat-stable" proteins were run, but only results for the "heat-stable" fraction will be shown. Lanes were loaded for equal counts (100,000–150,000 cpm) in total protein (prior to boiling).

Extraction and Hybridization of RNA

Total RNA was extracted, electrophoresed on methylmercury hydroxide agarose gels, and visualized with ethidium bromide to test for equal RNA loading and to assess RNA integrity (4). Formaldehyde agarose gels were then run and the RNA transferred to nitrocellulose as described previously (4, 6). A plasmid (pEMBL 12) containing cDNA of the *rab16a* gene of paddy rice was kindly provided by Dr. N.-H. Chua, Rockefeller University, New York (22). The plasmid was amplified in HB101 *Escherichia coli* cells and the entire coding region was excised with *Nhe*I and purified from low melt agarose gels using NACS columns (Bethesda Research Laboratories, Gaithersburg, MD). The purified cDNA fragment was radioactively labeled with [α-^{32}P]dCTP using random primers (10) included in an "oligolabeling" kit (BRESA,

Adelaide, South Australia). Hybridization conditions were standard hybridization solution (19) at 42°C with 40% formamide for 16 h, followed by washes in 2 × SSC, 0.1% SDS at 70°C for 1 h, then 0.1 × SSC, 0.1% SDS at 50°C for 20 min (SSC = 0.15 M NaCl + 0.015 M sodium citrate, pH 7.0). Autoradiography was carried out at −80°C with X-ray film using an intensifying screen.

Western Blots

Proteins were extracted and processed for western blots as described previously using antiserum prepared against maize dehydrin (5).

ABA Assays

For ABA analysis, tissues were powdered in liquid N_2 and extracted with boiling water (17). The water extracts were evaporated under vacuum, and the residue was dissolved in 50% methanol, loaded on a Sep-Pak C_{18} column (Waters Associates, Milford, MA), and eluted with 50% methanol. ABA in the extracts was measured by an indirect ELISA procedure utilizing monoclonal antibody to ABA (Idetek Inc., San Bruno, CA). Reported values were corrected for extraction losses based upon a ^{3}H-ABA internal standard added to each sample before extraction (average recovery = 89%).

RESULTS

Because the majority of previous work on dehydrins in cereals has utilized seedlings, we first sought to establish whether *Zizania* seedlings possessed the capacity to synthesize dehydrin-like proteins in response to dehydration. Seedlings (first leaf just emerging) of both *Zizania* and *Oryza* were held for 4 d at various RH to give different extents of water loss. Samples of the shoots were taken for protein labeling and RNA extraction. Dehydration of *Oryza* seedlings induced the synthesis of several major heat-stable proteins with apparent molecular masses of 17 to 23 kD (Fig. 1). The major protein band, migrating at M_r = 20,000 under our conditions, may correspond to the RAB21 protein described by Mundy and Chua (22). Increased synthesis of this protein occurred when water loss from intact seedlings exceeded 25% of their initial fresh weight. Northern blots showed that RNA hybridizing to *rab16a* (formerly called *rab21*) (22, 23) became detectable after the same extent of water loss (Fig. 1).

A similar response to dehydration was evident in *Zizania* seedlings. Loss of 27 and 39% of the initial fresh weight led to new synthesis of "heat-stable" proteins from 17 to 23 kD, with the major labeled band having an apparent molecular mass of 20 kD (Fig. 1). The *rab16a* cDNA from *Oryza* also hybridized to RNA species in total RNA extracts from *Zizania* seedlings (Fig. 1), as did dehydrin cDNA clones from maize and barley (data not shown). Essentially identical results were obtained with seedling roots as well as shoots (data not shown). The induced protein was recognized by polyclonal antibodies raised against purified dehydrin from maize (5) (Fig. 2, lanes 1, 2). A broad band or doublet was evident in western blots, and the leading band contained the heavily labeled 20 kD protein (Fig. 2, lanes 2, 3). The broader band

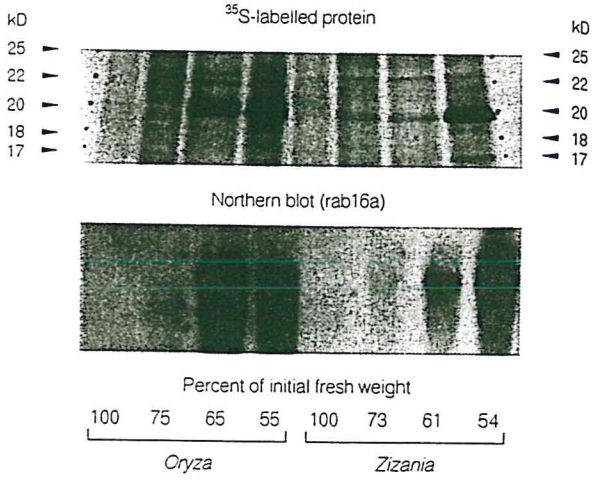

Figure 1. Upper panel, Fluorograph of SDS-PAGE gel of ^{35}S-labeled heat-stable proteins extracted from *Oryza* (left) or *Zizania* (right) seedling shoots after varying degrees of water loss. Seedlings at the first leaf stage were incubated at different RH for 4 d to result in the indicated percent fresh weight relative to the initial seedling weight (100% = d 0 control). The arrows at each side indicate the molecular masses in kilodaltons of pea seed marker proteins (28). Lower panel, northern blot of total RNA extracted from shoots treated identically to those described in the upper panel and hybridized with *rab16a*. Each lane contained 10 μg RNA for *Oryza* samples and 20 μg RNA for *Zizania* samples.

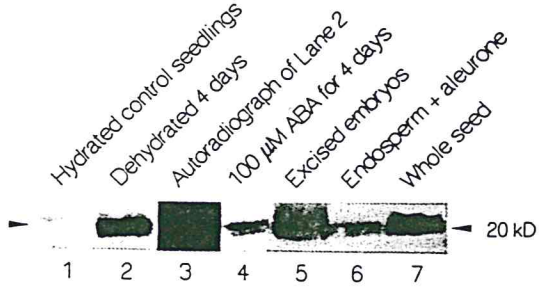

Figure 2. Western blots of heat-stable proteins from *Zizania* using antiserum against maize dehydrin. Lane 1, hydrated control shoots; lane 2, shoots from seedlings dehydrated for 4 d to 54% of their initial fresh weight; lane 3, autoradiograph of the nitrocellulose membrane shown in lane 2 (the protein extract used for the western blot had been labeled with [^{35}S]methionine); lane 4, shoots from seedlings exposed to 100 μM ABA for 4 d; lane 5, embryonic axes from hydrated stored seeds; lane 6, the remainder of the seed after embryonic axis extraction, including the endosperm, scutellum, and aleurone; lane 7, whole intact seeds. Approximately 400 μg protein were loaded per lane.

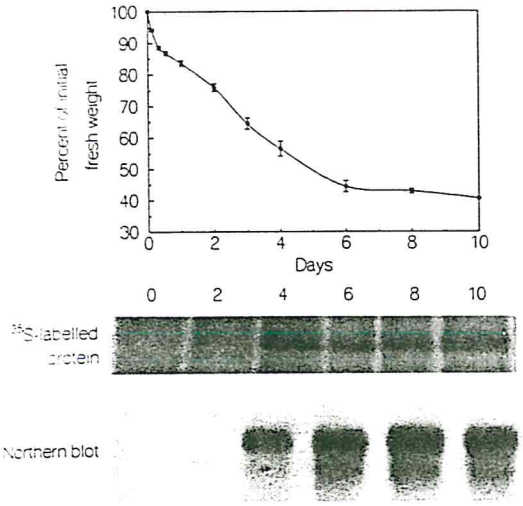

Figure 3. Time course of dehydration of *Zizania* seedlings (top), appearance of radioactivity in heat-stable protein (center; 18 to 22 kD region of the gel shown), and hybridization of total RNA with *rab16a* (bottom). Each lane of the northern blot contained 20 μg of total RNA.

detected by the dehydrin antibody relative to the band labeled during short-term exposure to [^{35}S]methionine may indicate that the protein is first synthesized in a more rapidly migrating form that is subsequently modified posttranslationally to a slower migrating form. In addition, several higher molecular mass proteins (40–45 kD) were also recognized by the antiserum, particularly in extracts from seeds and embryos, even though there was little labeling of heat-stable proteins in this size range. Whether these represent posttranslational modifications or multimeric forms of the synthesized protein is a subject for further experimentation. Taken together, the hybridization and serological data, along with the induction by water loss and heat stability of the 20 kD *Zizania* polypeptide, support the conclusion that it represents a *Zizania* homolog to the maize and barley dehydrins and the closely related RAB proteins from *Oryza*. Despite some minor quantitative differences, it is clear that qualitatively the responses of *Zizania* and *Oryza* shoots to dehydration are similar with respect to the induction of dehydrin-like mRNA and protein (Fig. 1).

To more precisely define the relationship of water loss to synthesis of dehydrin-like proteins in *Zizania* seedlings, seedlings were slowly dehydrated and protein synthesis and mRNA levels were assayed at intervals. Induction of dehydrin-like protein synthesis and the appearance of RNA complementary to *rab16a* again occurred only after the seedlings had lost >25% of their initial fresh weight (Fig. 3). Synthesis of dehydrin-like protein and levels of RNA hybridizing to *rab16a* were subsequently maintained for up to 10 d, when 60% of the initial fresh weight had been lost. In this experiment, a second hybridization band was detected on the northern blot (Fig. 3) that was not evident in most experiments. This could be due to several factors: partial degradation of the mRNA as stress became more severe; the synthesis of multiple transcripts within the gene family, as in barley and *Oryza* (6, 23); or slight differences in washing and blotting stringencies among experiments.

The dehydrins characterized to date are inducible by ABA (27). To determine whether this was also the case for *Zizania*, seedlings were treated for up to 4 d in 100 μM ABA, then assayed for dehydrin-like protein synthesis and transcript levels. In *Oryza*, ABA induced the synthesis of proteins of M_r identical to those induced by dehydration (Figs. 1, 4), and the presence of ABA-responsive transcripts was detected by hybridization to *rab16a* (Fig. 4), in agreement with the results of Mundy and Chua (22). Maximal protein synthesis and mRNA levels occurred within 1 d and remained constant for up to 4 d. Dehydrin-like protein synthesis was also induced by ABA in *Zizania* seedlings, although a longer duration of exposure to ABA was required to achieve maximum synthetic rates as compared to *Oryza* (Fig. 4). The identity of the induced protein as a dehydrin was supported by a positive signal on western blots (Fig. 2, lane 4). Hybridization of *rab16a* to RNA from ABA-treated *Zizania* seedlings was detected, but even using a 10-fold greater mass of RNA per lane, the extent of hybridization seen in *Zizania* RNA samples was consistently less than that for *Oryza* (Fig. 4). This result contrasts with that for seedling dehydration (Fig. 1), where only a twofold difference in RNA loading resulted in similar hybridization signals for the two species.

The weaker induction of dehydrin-like protein by ABA in *Zizania* seedlings compared to *Oryza* (Fig. 4), yet the similar responses of the two species to dehydration (Fig. 1), raised the question of what their endogenous ABA levels were. ABA contents were determined in seedling shoots extracted at various times during dehydration. *Zizania* seedlings possessed an equal or greater capacity to accumulate ABA during dehydration as compared to *Oryza* seedlings (Fig. 5). Since these data include various durations of stress as well as different extents of dehydration, it is inappropriate to make a direct

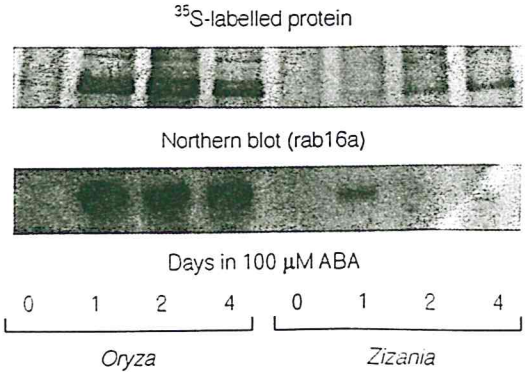

Figure 4. Time course of appearance of heat-stable protein (top; 18 to 22 kD region of the gel shown) and RNA hybridizing to *rab16a* (bottom) in *Oryza* (left) and *Zizania* (right) seedlings during continuous exposure to 100 μM ABA. Each lane of the northern blot contained 2 μg of total RNA for *Oryza* samples and 20 μg RNA for *Zizania* samples.

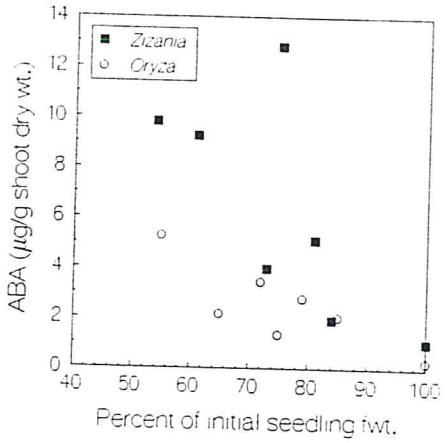

Figure 5. Shoot ABA contents of *Oryza* and *Zizania* seedlings during dehydration. Seedlings were dehydrated for 2 or 4 d at three RH to give the indicated percentages of the initial fresh weights. Data presented are means of duplicate extractions of single tissue samples, each assayed in duplicate dilution series. Values for the duplicate extractions differed from the mean by an average of 11%.

quantitative comparison of the difference in ABA accumulation between the species (Fig. 5) and the differing responses to exogenous ABA application (Fig. 4). Nonetheless, it is clear that *Zizania* seedlings are able to accumulate ABA during dehydration.

The data presented thus far show that *Zizania* seedlings have the capacity to synthesize and accumulate dehydrin-like proteins in response to water loss, in common with the other cereals examined to date (5, 6, 22, 29). We then determined whether dehydrin-like proteins are present in mature *Zizania* embryonic axes and whether their synthesis is induced by dehydration. Dehydrin-like protein was immunologically detectable in both excised embryonic axes and in the remainder of the caryopsis (Fig. 2, lanes 5–7), even though the seeds used in this study had been harvested at a high water content (30%, fresh weight basis) and stored in water for up to 10 months after harvest, the last 5 months at 5°C to break dormancy. The dehydrin-like protein was apparently degraded during germination, as seedlings at the first leaf stage contained only a trace of antibody-reactive protein (Fig. 2, lane 1). No hybridization to *rab16a* was detected in RNA isolated from embryonic axes immediately after excision from stored seeds (Fig. 6, d 0). Whereas dehydrin-like protein persisted during extended hydrated storage, the corresponding mRNA had apparently been degraded. We have subsequently confirmed that RNA hybridizing to *rab16a* is present in *Zizania* embryonic axes during development as the 20 kD protein accumulates (DW Still, DA Kovach, KJ Bradford, unpublished results). Whether the loss of dehydrin-like transcripts corresponds with the loss of dormancy during hydrated cold storage (*c.f.* ref. 21) is currently under investigation. RNA hybridizing to *rab16a* was present, however, after incubation of excised embryonic axes for 5 d at a range of RH, including incubation over water, where less than 6% of the tissue water was lost (Fig. 6).

Because *Oryza* embryos are able to survive dehydration to very low moisture contents, we compared their ability to synthesize dehydrin-like proteins during dehydration with that of *Zizania* embryonic axes. Embryos or axes were excised from water-stored *Zizania* caryopses or from *Oryza* caryopses that had been imbibed overnight. The embryos and axes were then incubated at 20°C in either 100% RH or 90% RH until their moisture contents reached equilibrium at the lower RH. The equilibrium tissue water contents (fresh weight basis) of *Oryza* embryos after 5 d were 60% at 100% RH and 15% at 90% RH. For *Zizania* embryonic axes, the tissue water contents after 7 d were 67% and 27% for 100% and 90% RH, respectively. These values corresponded to losses of 4 and 88% of the initial tissue water for *Oryza* embryos, and 6 and 82% of the initial tissue water for *Zizania* embryonic axes.

After equilibration, embryos and axes were transferred from 90% RH to 100% RH for 1 d to avoid subsequent imbibitional damage, and the water contents increased to 34 and 39% for *Oryza* and *Zizania*, respectively. Samples were then taken for protein labeling and ABA assays. Freshly isolated embryos/axes and embryos/axes that had been isolated and incubated on water-saturated filter paper for 2 d as a control for wounding effects were also sampled. Freshly excised *Oryza* embryos showed only faint labeling of heat-stable proteins in the 19 to 21 kD range, and these disappeared completely when the embryos were incubated for 2 d on water, at which time coleoptiles and roots were emerging (Fig. 7). Incubation in 100% RH, where water loss was slight (due to evaporation during periodic weighing), nonetheless caused some synthesis of heat-stable proteins in *Oryza* embryos, and severe dehydration induced the characteristic 20-kD protein bands (Fig. 7). *Zizania* embryonic axes also exhibited little dehydrin-like protein synthesis either upon isolation or after 2 d further incubation on water, but intense labeling of the 20-kD protein was evident after incubation at either 100% or 90% RH (Fig. 7). Identical results were obtained for *Zizania* embryonic axes incubated at 5°C in either 100% or 90% RH (data not shown).

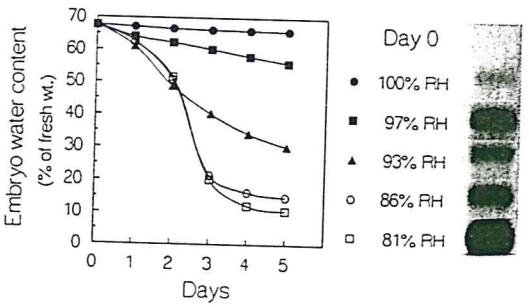

Figure 6. Induction of transcripts hybridizing to *rab16a* in excised *Zizania* embryonic axes by dehydration. Axes either remained at a given RH or were transferred to the next lower RH each day to give similar initial rates of dehydration but differing final water contents (graph at left; values are actual percent tissue water contents in g H_2O/g fresh weight × 100). Total RNA was extracted either immediately after isolation of embryonic axes from hydrated seeds (d 0) or after the 5 d of dehydration. Bands from northern blots hybridized with *rab16a* are shown at right (10 μg total RNA per lane).

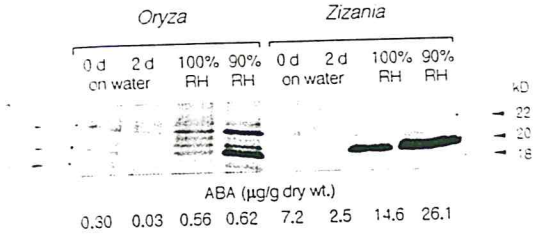

Figure 7. Fluorograph of SDS-PAGE gel of ^{35}S-labeled heat-stable proteins extracted from excised *Oryza* embryos (left) or *Zizania* embryonic axes (right). Within each species, the lanes from left to right are: immediately after excision from imbibed seeds (0 d), after 2 d imbibation on water (2 d), after equilibration at 100% RH (100% RH), and after equilibration at 90% RH (90% RH) at 20°C. The numbers below each lane indicate the ABA contents measured in embryos or axes from the same treatments.

These results, in combination with those in Figure 6, show that *Zizania* embryonic axes are capable of synthesizing dehydrin-like proteins in response to even very slight dehydration.

The ABA content of freshly isolated *Zizania* embryonic axes was 24-fold greater than that of *Oryza* embryos (Fig. 7). The value of 7.2 μg ABA/g dry weight for hydrated *Zizania* embryonic axes determined here using immunological techniques agrees well with a previous report of 6.65 to 4.85 μg ABA/g dry weight measured by HPLC-GLC techniques (1). The low ABA contents of *Oryza* embryos, on the other hand, are consistent with values reported for mature wheat and barley embryos (26, 30). The initial embryonic ABA levels declined by 10- and 3-fold in *Oryza* and *Zizania*, respectively, during 2 d of incubation on water (Fig. 7). Incubation in either 100% or 90% RH resulted in a doubling of ABA content in *Oryza* embryos and a similar or greater relative increase in ABA in *Zizania* embryonic axes (Fig. 7). The relative increase in ABA levels in embryonic tissues during dehydration was not as dramatic as was observed in stressed shoots of either species, and ABA content remained low in *Oryza* embryos after dehydration when compared to the amounts accumulated in dehydrated shoots (Figs. 5, 7). In contrast, *Zizania* embryonic axes contained considerably more ABA per gram dry weight than did shoots (Figs. 5, 7).

DISCUSSION

The evidence presented here supports the conclusion that *Zizania* embryos and seedlings have the capacity to synthesize protein homologs to the dehydrins that have been characterized in other cereals (5, 6, 22, 29). Although absolute verification of the expression of dehydrin proteins in *Zizania* would require isolation of cDNAs corresponding to the mRNAs detected by the northern blots, there is little reason to doubt that *Zizania* embryonic axes and seeds (both dormant and nondormant) contain dehydrin-like proteins at maturity and can synthesize them in response to dehydration.

There was little qualitative difference in the expression of dehydrin-like mRNA or protein between *Oryza* and *Zizania* tissues in response to dehydration or ABA, although quantitatively, the mRNA and protein levels induced in *Oryza* were often somewhat greater than those in *Zizania* at similar extents of water loss or during incubation in ABA, and the timing of appearance sometimes differed between the species. However, the use of heterologous cDNA from *Oryza* for the northern blots of *Zizania* RNA and potential problems in labeling proteins synthesized during rapid rehydration of dehydrated tissues prevent us from putting too much emphasis on the quantitative differences observed. The weaker response of *Zizania* tissues to applied ABA relative to *Oryza* (Fig. 4) was consistent among experiments, and the quantitative difference in dehydrin-like protein and mRNA expression between the species was greater for ABA treatment than for dehydration. This may indicate that *Zizania* is less sensitive to ABA than is *Oryza*, but that this lower sensitivity is compensated by the higher initial levels and greater accumulation of ABA during dehydration in *Zizania*. More detailed work on the sensitivity of dehydrin-like protein expression to ABA and on the accumulation of ABA would be required to support or refute this hypothesis. It is clear, however, that any differences between *Oryza* and *Zizania* in the *in vivo* expression of dehydrin-like proteins during dehydration are relatively minor.

At the time this work was initiated, the evidence was convincing that *Zizania* seeds were recalcitrant and lost viability at embryo water contents below about 45% (24). We subsequently found, however, that this apparent intolerance of dehydration was due to a combination of imbibitional damage upon rehydration and temperature sensitivity during dehydration rather than to an absolute intolerance of dehydration *per se* (14). It is perhaps not surprising, therefore, that dehydrin-like protein expression in *Zizania* embryos is unimpaired. Yet, the presence of dehydrin-like proteins in *Zizania* seeds at maturity and their maintenance during hydrated storage does not prevent loss of viability under unfavorable dehydration or rehydration conditions (14).

In addition, although dehydration of *Zizania* seeds at 5°C is detrimental to survival compared to dehydration at warmer temperatures (14), dehydrin-like proteins were synthesized in embryonic axes during dehydration at this temperature. Our results are therefore in agreement with the conclusion of Blackman and coworkers (3) regarding the role of heat-stable maturation proteins in desiccation tolerance of soybean (*Glycine max* L.) seeds: although such proteins may or may not be necessary for desiccation tolerance, their presence alone is not sufficient to prevent injury under all conditions. Additional mechanisms, including membrane phase transitions, sugar accumulation and composition, "water replacement" and glassy state formation, are undoubtedly involved in anhydrobiosis (16).

On the other hand, the possibility remains that dehydrin-like proteins are essential for desiccation tolerance, even though they are not sufficient, explaining the conservation of their amino acid sequence motifs among widely differing plant species. Examination of more extreme types of recalcitrant seeds or of mutants specifically lacking dehydrin-like or heat-stable maturation protein components is required to critically test this hypothesis. Roberts *et al.* (25) have discussed in detail at least nine reasons why seeds can be misclassified as being recalcitrant when they, in fact, are not. If dehydrin-like pro-

teins can be shown to be necessary for desiccation tolerance, even if they are not sufficient, their presence or absence could provide an additional, less ambiguous criterion for determining whether a seed possesses the minimum requirements for surviving dehydration, as in the case of *Zizania*.

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