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Expression of a Dehydrin-Like Protein in Maize Seedlings Germinated from Seed Exposed to Freezing

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Dehydrins are a family of heat-soluble, hydrophilic proteins that share a considerable degree of sequence homology. Their expression has been reported in numerous plant species in response to a multitude of environmental stresses including low temperature, freezing, and desiccation. It has also been established that exposing plant tissues to freezing temperatures generates desiccation stress. We observed differential accumulation of a dehydrin-like protein and corresponding mRNA in three-day-old maize (*Zea mays* L.) seedlings germinated under favorable environmental conditions from seed that had been exposed to freezing temperatures during maturation. This represents the first documented situation in which a dehydrin-like protein differentially accumulates under favorable environmental conditions. We believe that the dehydrin-like protein and corresponding mRNA are synthesized de novo in seedlings that are germinated from seed that have been exposed to freezing in response to desiccation-like stress that persists under favorable environmental conditions resulting from freeze-induced damage sustained by the ungerminated embryo.

Keywords: dehydrin, freezing, maize, seed

Dehydrin (LEA D11) proteins are extremely hydrophilic, remain soluble at temperatures greater than 100°C, and are found in the nucleus and cytoplasm (Close et al., 1995). They are glycine-rich and free of cysteine and tryptophan. They contain repeated units in a conserved linear order including a lysine-rich repeating unit that occurs twice in each protein, once at the carboxy terminus, and once midway through the polypeptide (Close et al., 1989). This repeating dehydrin consensus peptide sequence, or K segment, is highly conserved among all dehydrins and may play an important role in stabilizing macromolecules during environmental stress (Close, 1997). The majority of the dehydrins contain a tract of serine residues which can be phosphorylated (Close et al., 1995). Phosphorylation of the serine tract may be involved in binding of nuclear localizing signal peptides (Goday et al., 1994). Expression and function of dehydrins have been studied in many plant species under various environmental conditions.

Close et al. (1989) found that expression of dehydrins is inducible by low temperature stress in barley (*Hordeum vulgare* L.) and maize. The dehydrins expressed in these two species were biochemically very similar. The authors concluded that not only were dehydrins expressed in response to low temperature stress, but that these dehydrins were closely related to dehydrins expressed in other plant species in response to desiccation stress, all of which contained the consensus sequence EKKGIMDKIKEKLPG near the carboxy terminus. Van Zee et al. (1995) demonstrated the cold-specific induction of dehydrins in two lines of barley. Germination and incubation of seedlings at 2°C for three weeks resulted in the expression of a protein that was recognized by antiserum against the dehydrin consensus sequence. The authors concluded that although there is good evidence that dehydrins may increase low temperature tolerance, it is unlikely that dehydrins alone can confer winter tolerance in barley. Guo et al. (1992) characterized a polypeptide that contained a lysine rich and a glycine rich domain similar to those found in dehydrins. Corresponding mRNAs were found to accumulate rapidly in response to low temperature and were quickly degraded when the seedlings were returned to normal growing temperatures. Transcripts were also found in relatively high concentrations in seeds and seedlings under water stress and in response to application of exogenous ABA. Lin and Thomashow (1992) have demonstrated the ability of dehydrins to confer resistance to denaturation of lactate dehydrogenase during freezing and proposed several possible functions in dehydrating cells associated with extracellular freezing.

It has been shown that extracellular ice formation during freezing induces a dehydrative stress on cells. It has also been established that expression of dehydrins is induced in maize seedlings subject to low temperature, freezing, and desiccation stress (Close et al., 1989; Close, 1997). We were interested in determining if

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exposing immature maize (*Zea mays* L.) seed to freezing temperatures would elicit the accumulation of dehydrin-like protein(s) and corresponding mRNA(s) in seedlings upon subsequent germination under favorable environmental conditions. We have previously observed that in immature maize seed, similar amounts of dehydrin-like protein and corresponding mRNA are detectable irrespective of freeze treatment. Upon maturation, the dehydrin-like protein is retained while the corresponding mRNA is no longer detectable then, subsequent to germination, the dehydrin-like protein becomes undetectable (unpublished data). The main objective of this project was to concretely establish the first evidence that dehydrin-like protein and corresponding mRNA accumulate in seedlings germinated under favorable environmental conditions from seed exposed to freezing during maturation.

MATERIALS AND METHODS

Plant Material

Hybrid maize seed B73 × (H99 × H95) was harvested at 55%, 45%, and 35% total moisture content (approximately 30, 33, and 36 days after pollination, respectively) as determined by oven drying at 105°C for 24 h. Seed (intact ears with husks) was immediately taken to the lab and exposed to one of three freeze treatments. Treatments included 0 h at −5°C (control), 6 h at −5°C (short exposure), and 24 h at −5°C (prolonged exposure). All treatments were carried out in a growth chamber in the dark. Upon completion of the respective treatments, seeds from each treatment were allowed to recover at 22°C for 24 h, dried to storage moisture (12%), and placed in cold storage (10°C) prior to germination. Lots of 50 seed were taken from each treatment and germinated in towels moistened with distilled water. Towels were placed upright in a wire grid and held in a growth chamber at 22°C under a 12 h photoperiod for 3 d.

RNA Extraction

Total RNA was extracted from three-day-old seedlings from all treatments using the ToTALLY RNA™ isolation system (Ambion, Inc. Austin, TX). Seedlings were separated from seed remnants and ground in liquid nitrogen to a fine powder. Tissue was then suspended in 250 µL of denaturation solution (provided with the ToTALLY RNA™ isolation system). Nucleic acids were isolated from the homogenate by extraction with phenol: chloroform:isoamyl alcohol (25:24:1(v:v:v); pH 8.0). Total RNA was then isolated by extraction with acid phenol (pH 4.5). Finally, total RNA was precipitated from solution with isopropanol, pelleted, resuspended in RNAse-free water, and treated with DNase to remove contaminating genomic DNA. Polyadenylated mRNA was isolated from the total RNA fraction using the MicroPoly(A) Purist™ mRNA isolation system (Ambion, Inc. Austin, TX). Integrity of purified mRNA was verified by agarose gel electrophoresis. Purified mRNA was quantified using UV spectrophotometry.

RT-PCR

Reverse transcriptase-mediated PCR was carried out using the OneStep™ RT-PCR system (Qiagen, Inc. Valencia, CA). Gene specific primers were designed to amplify a 525 bp region containing the coding sequence for the dehydrin consensus peptide using sequence data presented by Close et al. (1989) (forward, 5'-TAC GGTCAGCAGGGGCAGCA-3'; reverse, 5'-GGCAGCT TCTCTTTGATCTTGTCCA-3').

Approximately 0.1 µg of purified mRNA from each treatment was used in each RT-PCR reaction. Additionally, 0.5 µg of RNase-treated, genomic DNA was used in a separate (standard PCR) positive control reaction. After 30 reaction cycles, PCR products were loaded on an agarose gel (2%) along with 100 bp DNA size standards. Electrophoresis was run at 200V for 45 min. Gels were visualized with ethidum bromide under ultraviolet light. RT-PCR products were cloned and sequenced to verify target identity.

Extraction and Quantification of Heat-Soluble Protein

Seedlings were removed from the growth chamber after 3 d and separated from seed remnants. Protein was extracted from seedlings by grinding 10 seedlings from each treatment in ice cold extraction buffer containing 0.1 M sodium phosphate (pH 7.8), 1 mM EDTA, and 1 mM PMSF. Homogenate was centrifuged at 20,000g and the supernatant decanted into clean tubes that were immediately placed on a heating block at 100°C for 5 min. Tubes were again centrifuged at 20,000g and the supernatant decanted into clean tubes. Total protein concentration of each sample was determined spectrophotometrically based on the method of Bradford (1976).

SDS-PAGE

Five micrograms of heat soluble-protein from each

treatment was diluted with 5 µL of sample buffer (62.5 mM Tris, pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 0.5% (w/v) bromophenol blue) and loaded into SDS mini-gels (4% stacking, 12% separating). Additionally, protein molecular weight standards were loaded into the first lane of each gel. Electrophoresis was carried out at 60V for 5 h.

Immunoblotting

Subsequent to electrophoresis, gels, electroblotting paper, and nitrocellulose membranes were soaked in electroblotting buffer containing 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol for 30 min at room temperature. Electroblotting was conducted at 5°C for 5 h at 15V. Membranes were then rinsed briefly in 1X TBS (50 mM Tris, 150 m*M* NaCl, pH 7.5) before incubation at room temperature in 25 mL blocking solution containing 5% non-fat dry milk dissolved in 1X TBS. After 2 h, 12.5 µL of polyclonal anti-dehydrin consensus peptide antiserum (kindly donated by Dr. Timothy J. Close, University of California, Riverside) was added giving a dilution of 1:2000. Following 2 h of incubation in primary antibody, membranes were subject to 3 × 5 min washes in 1X TBST (TBS + 1% (v/v) Tween 20), rinsed with 1X TBS, and placed in secondary antibody solution containing horseradish peroxidase (HRP) conjugated, goat anti-rabbit antiserum diluted 1: 3000 in 50 mL 1X TBS containing 5% non-fat dry milk. After 2 h of incubation in secondary antibody, membranes were subject to 3 × 10 min washes in 1X TBST and rinsed with 1X TBS. Colorimetric detection was carried out by incubation in solution containing 100 mL 1X TBS, 60 µL hydrogen peroxide (30%), 65 mg 4-chloro-1-naphthol, and 20 mL methanol. The reaction was stopped after approximately 10 min by rinsing membranes with distilled water. To verify specificity of the dehydrin antibody to the dehydrin consensus peptide sequence, an identical membrane was prepared and processed as described above but included the addition of synthetic consensus peptide sequence to the primary antibody dilution. Additionally, a third identical membrane was probed with secondary antibody alone to verify specificity to the dehydrin antibody.

All experiments described in this section were independently replicated in triplicate to verify consistency of results.

RESULTS AND DISCUSSION

Analysis of RT-PCR products revealed that mRNA corresponding to the dehydrin consensus peptide coding sequence had accumulated in seedlings germinated from seed exposed to freezing (Fig. 1). The mRNA was represented by a single band that was approximately 525 bp in length. Likewise, the products from the genomic DNA-based positive control reaction were also approximately 525 bp in length while the negative control reaction did not yield any products. Sequencing confirmed that all PCR products were the targeted 525 bp region containing the coding sequence for the dehydrin consensus peptide sequence reported by Close et al. (1989).

Specifically, seedlings germinated from seed harvested at 55% and 45% moisture contained corresponding transcripts when seed was exposed to 6 h or to 24 h of freezing, while in seedlings germinated from seed harvested at 35%, corresponding transcripts were found only in the 24 h treatment (Fig. 1). In seedlings germinated from seed harvested at 55% and 45% moisture, corresponding transcripts appeared to be several-fold more abundant in the 24 h treatments than in the 6 h treatments, while in seedlings germinated from seed harvested at 35% moisture, the extent to which corresponding transcripts accumulated in the 24 h treatment appeared to be similar to that of seedlings geminated from seed harvested at 55% and 45% moisture which had been exposed to 6 h of freezing (Fig. 1).

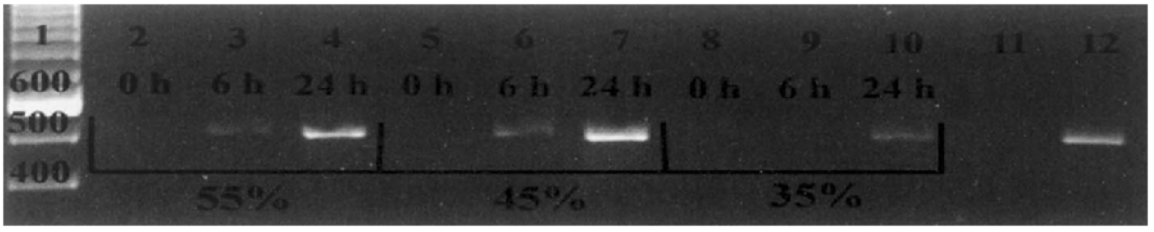

Figure 1. Agarose (2%) gel electrophoresis of RT-PCR products indicating the presence of transcripts corresponding to the dehydrin consensus peptide sequence in three-day-old maize seedlings germinated from seed exposed to freezing. Lane 1 contains 100 bp size standards, while lanes 2-4, 5-7, and 8-10 indicate 0, 6, and 24 h of freezing exposure to seed harvested at 55%, 45%, and 35% moisture, respectively. Lane 11 contains a negative control (no RNA) and Lane 12 contains a positive control (genomic DNA).

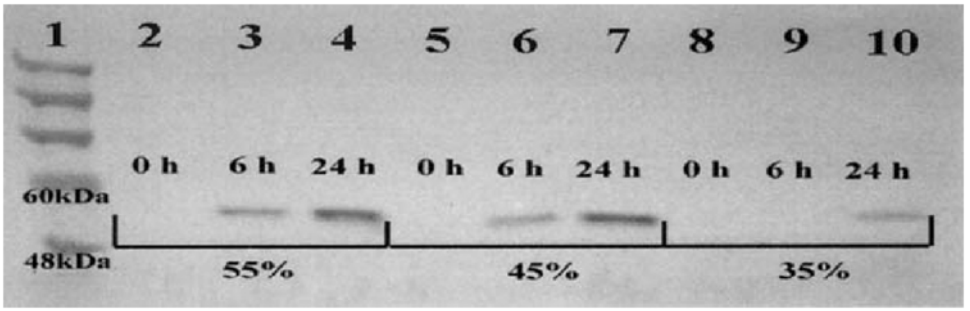

Figure 2. Western blot indicating the differential accumulation of a maize dehydrin-like protein as recognized by an anti-dehydrin consensus peptide sequence antibody in three-day-old maize seedlings. Lane 1 contains protein size standards while lanes 2-4, 5-7, and 8-10 contain heat soluble protein extracted from seedlings germinated from seed exposed to 0, 6, or 24 h of freezing harvested at 55%, 45%, and 35% moisture, respectively.

The anti-dehydrin consensus peptide sequence antibody indicated differential accumulation of a dehydrin-like, heat-soluble protein of approximately 56 kDa (Fig. 2). Expression of the protein was detected only in seedlings germinated from seed exposed to freezing. Accumulation in seedlings germinated from seed harvested at 55% and 45% moisture was detected in both the short (6 h) and prolonged (24 h) freezing treatments, while in seedlings germinated from seed harvested at 35% moisture, accumulation was limited to the prolonged freeze treatment (Fig. 2). Accumulation of dehydrin-like protein with respect to freeze duration was similar to that of the corresponding transcript in that accumulation appeared to be greater in the 24 h treatments than in the 6 h treatments in seedlings germinated from seed harvested at 55% and 45% moisture, while in seedlings germinated from seed harvested at 35% moisture, accumulation in the 24 h treatment was similar to that detected in seedlings germinated from seed harvested at 55% and 45% moisture which had been exposed to 6 h of freezing (Fig. 2). Incubation in primary antibody to which dehydrin consensus peptide had been added inhibited detection in all treatments, thus demonstrating specificity of the anti-dehydrin antibody to the consensus peptide sequence (data not shown). Likewise, incubation in secondary antibody alone yielded no signal, demonstrating specificity to the anti-dehydrin antibody (data not shown).

Differential accumulation of the dehydrin-like protein and corresponding mRNA appear to occur post-germination. Previously, we have observed that in immature seed, similar amounts of dehydrin-like protein and corresponding mRNA are present irrespective of freeze treatment (unpublished data). Upon maturation, the dehydrin-like protein is retained while the corresponding mRNA is no longer detectable then, subsequent to germination, the dehydrin-like protein becomes undetectable (unpublished data). In light of the fact that the dehydrin-like protein and corresponding mRNA are not detected in seedlings germinated from seed that was not subject to freezing, it seems likely that their presence in seedlings germinated from seed exposed to freezing is the result of post-germination de novo synthesis. Due to the fact that the corresponding mRNA is no longer detectable in seed exposed to freezing after drying to storage moisture, it seems unlikely that transcriptional activation of mRNA corresponding to dehydrin-like protein in immature seed exposed to freezing is involved in low temperature acclimation of seedlings germinated from these seed. These responses may be attributable to a stress signal, which remains present even as seeds exposed to freezing germinate under favorable conditions, that activates transcription of genes containing the coding region for the dehydrin consensus peptide sequence as demonstrated by Guo et al. (1992).

Numerous authors have demonstrated the cause and effect relationship between freezing injury and desiccation stress (Steponkus, 1984; Hincha et al., 1989). We have previously demonstrated, through the use of light and electron microscopy, that exposure to freezing temperatures causes physical injury to the embryo in immature maize seed and that seed exposed to freezing at greater moisture content is more susceptible to reduced germination percentage and increased electrolyte leakage (Hartwigsen and Burris, manuscript in review). This, along with the demonstrated accumulation pattern of dehydrin-like protein and corresponding mRNA with respect to moisture content and freeze duration, suggests that not only does exposure to freezing cause physical injury to the embryo in immature seed, but also that as these injured seed germinate, a stress signal is present that triggers a desiccation-like response in the seedlings leading to the expression of dehydrin-like

protein. The possibility that dehydrin-like protein and corresponding mRNA accumulation in three-day-old seedlings germinated from seed exposed to freezing is induced by desiccation stress is further substantiated by the findings of Baker et al. (1995), who reported that dehydrins were no longer detectable after germination, but that expression could be induced in seedlings by desiccation stress. This possibility is also consistent with the findings of Guo et al. (1992) who reported that corresponding transcripts were found in high concentrations in seedlings under desiccation stress.

Dehydrins containing the consensus peptide sequence have been found in many plant species in response to a variety of environmental stresses including low temperature, freezing, desiccation, and salinity. In the present study, we have established a unique set of conditions under which dehydrin-like protein and corresponding mRNA accumulate in seedlings that are grown under optimal temperature and moisture, thus suggesting a link between freeze injury in immature seed and a desiccation-like response in seedlings. These findings warrant future research into the exact mechanism(s) by which seedlings germinating from seed that has been exposed to freezing are prompted to accumulate a stress-related protein despite favorable environmental conditions. The fact that seedlings germinated from seed exposed to freezing differentially accumulate a readily detectable mRNA and protein may prove useful in future development of biological assays that could rapidly detect the presence of frost injury in commercial hybrid seed.

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