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Isolation of a wheat cDNA clone for an abscisic acid-inducible transcript with homology to protein kinases

(dehydration/environmental stress/plant hormone/protein phosphorylation/*Triticum aesitivum*)

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ABSTRACT **Increases in the plant hormone abscisic acid (ABA) initiate water-stress responses in plants. We present evidence that a transcript with homology to protein kinases is induced by ABA and dehydration in wheat. A 1.2-kilobase cDNA clone (PKABA1) was isolated from an ABA-treated wheat embryo cDNA library by screening the library with a probe developed by polymerase chain reaction amplification of serine/threonine protein kinase subdomains VIb to VIII. The deduced amino acid sequence of the PKABA1 clone contains the features of serine/threonine protein kinases, including homology with all 12 conserved regions of the catalytic domain. PKABA1 transcript levels are barely detectable in growing seedlings but are induced dramatically when plants are subjected to dehydration stress. The PKABA1 transcript can also be induced by supplying low concentrations of ABA, and coordinate increases in ABA levels and PKABA1 mRNA occur when seedlings are water-stressed. Identification of this ABA-inducible transcript with homology to protein kinases provides a basis for examining the role of protein phosphorylation in plant responses to dehydration.**

Plants must adapt to changing environmental conditions. Drought or extreme temperatures trigger large increases in the plant hormone abscisic acid (ABA) and subsequently induce ABA-responsive genes. Plant mutants lacking the ability to produce or respond to ABA are far less capable of surviving environmental stress (1). The steps in the transduction pathway from ABA increases to stress-responsive gene expression are not known. One possible link in this signal transduction pathway is an ABA-regulated protein kinase. In animals and yeast, protein kinase activity often mediates a cellular adjustment to external stimuli (2), and a similar activity in plants is likely.

While many ABA-inducible genes have now been cloned, the function of these genes in adaptation to environmental changes is not yet clear. A number of ABA-responsive genes encode late embryogenesis abundant (LEA) proteins that accumulate in drying seeds and in water-stressed seedlings (3–7). In wheat the LEA proteins include Em (8, 9), dehydrin (RAB) (9, 10), group 3 LEA (11), and group 3 LEA (II) (9). Many of the LEA proteins are hydrophilic and may bind water tightly to function as protectants against desiccation (4). The maize LEA protein RAB-17 has been found to be highly phosphorylated *in vivo* (12, 13). *In vitro* experiments show that RAB-17 can be phosphorylated by a maize casein kinase II at a serine-cluster region of the protein (14). Whether phosphorylation affects the cellular function of RAB-17 or other LEA proteins has not been determined.

The potential importance of protein kinases in dehydration stress responses has led us to search for ABA-regulated protein kinases. Previously, clones encoding plant protein kinases have been obtained by using degenerate oligonucleotides corresponding to the conserved catalytic regions of eukaryotic protein kinases (15, 16). With these methods, protein kinase homologs have been obtained from maize (17, 18), soybean (19), carrot (20), pea (21), and bean and rice (22). We have employed a similar strategy using the polymerase chain reaction (PCR) and oligonucleotide primers to screen an ABA-treated wheat embryo cDNA library for protein kinase clones. We report here the selection and sequence analysis of a cDNA clone† with sequence homology to serine/threonine-specific protein kinases. The transcript corresponding to the clone is inducible both by dehydration and by ABA.

MATERIALS AND METHODS

Materials and General Methods. *Triticum aestivum* cv. Brevor was used in all experiments. For the seed developmental study, seeds were harvested from plants grown at the Washington State University Spillman Agronomy Farm near Pullman, WA. Seeds were harvested at 25 days postanthesis (dpa) (70% moisture), at 33 dpa (57% moisture), and at maturity (6.7% moisture). For the dehydration experiments, seeds were germinated and grown on moist filter paper in a high-humidity chamber at 20°C. After 3 days the cover of the chamber was removed and the whole seedlings were allowed to dry under low humidity at 20°C. ABA was extracted and assayed with a monoclonal antibody for (*S*)-ABA (23).

Escherichia coli strains XL1-Blue, BB4, and SURE and interference-resistant helper phage R408 were obtained from Stratagene. The cDNA library was prepared from ABA-treated dormant seed embryos of wheat cv. Brevor treated with 5 μM ABA for 12 hr (9). Unless otherwise indicated, protocols, buffers, and media were as described (24, 25).

Protein Kinase Probe Synthesis. Degenerate oligonucleotides (shown in Fig. 1) corresponding to highly conserved regions in almost all serine/threonine protein kinase catalytic subdomains VIb and VIII were custom made (National Biosciences, Hamel, MN) for use in PCR amplification. The 5′ PCR primer was a 23-mer coding for an amino acid sequence homologous to the catalytic subdomain VIb of serine/threonine-specific protein kinases and the 3′ primer was a 26-mer coding for an amino acid sequence homologous to catalytic subdomain VIII of the same protein kinases (15, 16).

To obtain the probe to screen the cDNA library, dormant wheat seeds were imbibed in water for 18 hr. At that time embryos were dissected from the seeds, poly(A)$^+$ RNA was prepared (9), and cDNA was produced by using avian myeloblastosis virus reverse transcriptase. PCR (26) was per-

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Abbreviations: ABA, abscisic acid; dpa, days postanthesis; GCG, Genetics Computer Group; LEA, late embryogenesis abundant.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M94726).

formed on this cDNA in a Coy thermal cycler with a cycle of 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C. The PCR product was cloned into the *Eco*RV site of pBluescript SK(+) (Stratagene) and sequenced. This clone contained both primer sequences of subdomains VIb and VIII and the deduced amino acid sequence Asp-Phe-Gly, which is conserved in subdomain VII of protein kinases (15, 16). Based on sequence results it was concluded that the DNA fragment resulting from PCR amplification was suitable for use as a probe to screen a cDNA library for protein kinase clones.

Isolation of cDNA Clones. A wheat cDNA library prepared from ABA-treated embryos from dormant seed (9) was screened with the PCR-generated protein kinase DNA fragment described above. Approximately 10,000 clones (plaque-forming units) of the primary amplified library were screened. Plaque lifts were made according to manufacturer's instructions (Colony/Plaque Screen; DuPont/New England Nuclear) and screened with the PCR-amplified probe according to published techniques (9). Two plaques hybridized to the probe. Excision rescue according to the manufacturer's protocol (Stratagene) produced derivatives as pBluescript SK plasmids. The plasmids were digested with *Eco*RI to liberate the cloned inserts and analyzed by Southern hybridization using the PCR-generated protein kinase probe. Both cDNA inserts exhibited strong homology.

Preliminary assessment of ABA effects on the transcripts corresponding to these two clones was conducted by Northern analysis. Poly(A)$^+$ RNA (1 μg) was denatured and electrophoresed in 1.2% (wt/vol) agarose gel containing 0.37 M formaldehyde (9). Gels were stained with ethidium bromide and visualized under UV light to confirm that equivalent amounts of RNA were present in each lane. Northern analysis showed that one clone hybridized to ABA-inducible transcripts, and this clone, called PKABA1, was chosen for further analysis.

DNA Sequencing. Both strands of PKABA1 were sequenced by the dideoxy chain-termination method using Sequenase 2.0 (United States Biochemical). Subcloning of PKABA1 and use of custom primers (Genosys, Woodlands, TX) were employed in order to sequence both stands in their entirety. Sequence data were analyzed using the Genetics Computer Group (GCG) sequence-analysis program (version 7.0) (27) with the assistance of the VADMS Computing Center, Washington State University.

RESULTS

Selection of the PKABA1 cDNA Clone. A DNA probe [146 base pairs (bp)] for protein kinases was produced by PCR amplification techniques with cDNA from wheat embryo tissue and degenerate oligonucleotide primers (Fig. 1). The resulting probe corresponded to the consensus amino acid sequences of serine/threonine-specific protein kinase subdomains VIb, VII, and VIII (15). The DNA probe was used to screen a cDNA library from ABA-treated wheat embryos. This library was chosen because it maximized the chances of obtaining cDNA clones for protein kinases functioning in ABA-regulated responses. Preliminary analysis with Northern blotting showed that one selected cDNA clone corresponded to a transcript that was inducible by ABA. This cDNA clone, called PKABA1, for protein kinase responsive to ABA, was sequenced and characterized.

PKABA1 cDNA Sequence Analysis. The sequence of PKABA1 is shown in Fig. 1. This cDNA is 1210 bp in length with a 996-bp open reading frame. The PKABA1 clone lacks the 5′ end of the open reading frame but is judged to be near full-length because the size of the transcript as determined by Northern blot analysis is about 1300 bases. Putative polyadenylylation signals (ATAA) are present at bases 878 and 1140. Potential RNA splice junction dinucleotides are present starting at base 892 (GT) and base 1167 (AG).

The PKABA1 deduced amino acid sequence is highly homologous to the conserved catalytic subdomains of other serine/threonine-specific protein kinases (Figs. 1 and 2).

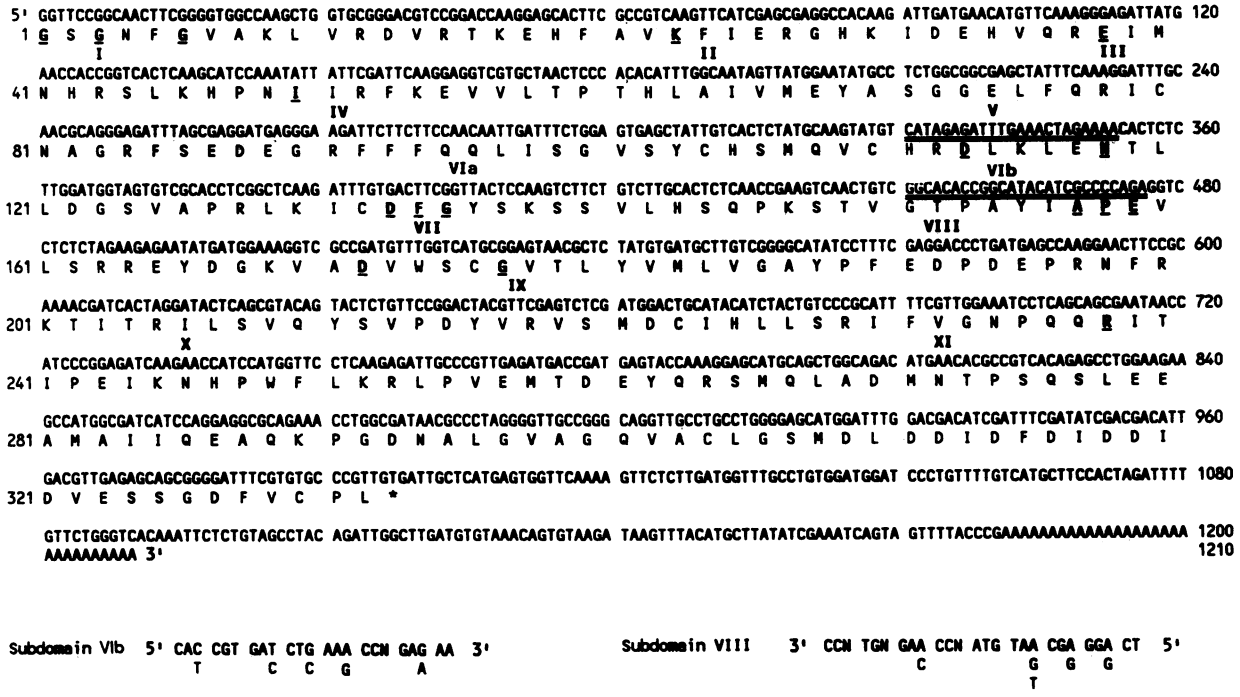

FIG. 1. (*Upper*) Nucleotide sequence and deduced amino acid sequence of cDNA clone PKABA1. Bold underlined amino acids represent residues that exhibit particularly high homology to other serine/threonine protein kinase sequences. Roman numerals below the amino acid sequence indicate catalytic subdomain regions. Doubly underlined nucleotide sequences correspond to the location of oligonucleotide primers used to generate a kinase-specific probe to screen a cDNA library. (*Lower*) Sequence of degenerate oligonuleotides used to synthesize the protein kinase probe by PCR amplification.

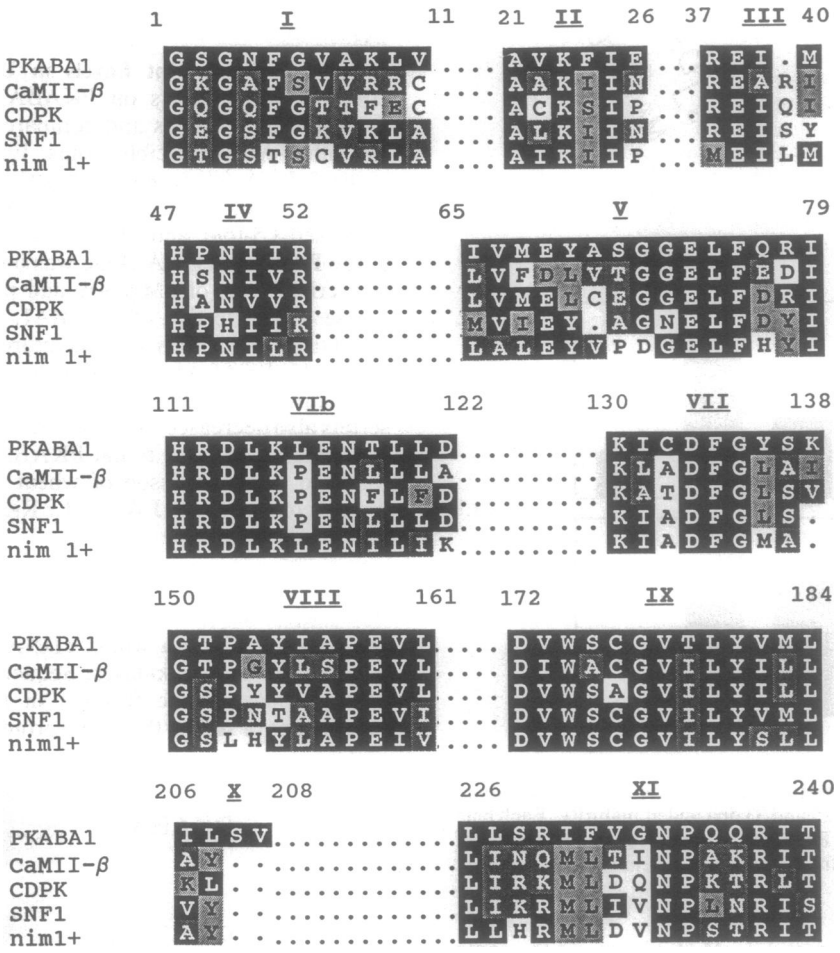

FIG. 2. Sequence identify and similarity of the deduced amino acid sequences within 11 catalytic subdomains. The catalytic subdomains of PKABA1 were compared with the sequences of the β form of the catalytic subunit of rat calcium/calmodulin-dependent protein kinase II (CaMIIβ) (28), soybean calcium-dependent protein kinase (CDPK) (19), yeast SNF1 protein kinase (29), and yeast nim1+ protein kinase (30). Because there is a low degree of homology of subdomain VIa of PKABA1 with these protein kinases, that subdomain is not shown. Identical amino acids are shown in black, while chemically similar (31) amino acids are shaded, with increasing similarity marked by darker shading (PRETTYBOX, GCG version 7.0). Numbers above the boxes refer to amino acid positions in PKABA1.

Conserved amino acids characteristic of each of the 12 catalytic subdomains found in almost all serine/threonine protein kinases (15) are present. The sequence includes all 14 individual amino acids conserved in serine/threonine protein kinases that are considered critical for catalytic function (16). These include Gly^{1}, Gly^{3}, Lys^{23}, Glu^{38}, Val^{101}, Asp^{113}, Asn^{118}, Asp^{133}, Phe^{134}, Gly^{135}, Glu^{159}, Asp^{192}, Gly^{197}, and Arg^{238}. A nucleotide binding site, Gly-Xaa-Gly-Xaa-Xaa-Gly, is in subdomain I, as expected for a protein kinase. The sequence Asp-Leu-Lys-Leu-Glu-Asn beginning at position 113 indicates serine/threonine protein kinase substrate specificity, with the exception of Leu^{116} substituted for Pro. The consensus sequence Ala-Pro-Glu, considered important in protein kinase catalytic function, begins at position 157. A region of ≈20 amino acids upstream of this triplet is a potential site of autophosphorylation because 7 of the 20 amino acids are either serine or threonine residues (15, 16).

A search of the sequence data bases (GenBank, EMBL, and Swiss-Prot, February 1992) using GCG version 7.0 software (27) showed that the PKABA1 sequence has the most similarity with several protein kinases from animals, yeast, and plants. These protein kinases include soybean calcium-dependent protein kinase (19), yeast SNF1 protein kinase (29), rat calcium/calmodulin-dependent protein kinase type II, β subunit (28), and yeast nim1+ protein kinase (30). Comparisons of PKABA1 with these protein kinases showed that there was a high degree of sequence identity and similarity within 11 conserved subdomains of the catalytic domain (BESTFIT, GCG version 7.0). Those results are shown in Fig. 2. As is typical for protein kinases, less sequence identity was found when regions spanning the subdomains were included in the comparison of PKABA1 and the selected protein kinases. The percent sequence identity of amino acids 100–190 of PKABA1 with these protein kinases is as follows: 52% with soybean calcium-dependent protein kinase, 49% with SNF1, 48% with the β subunit of rat calcium/calmodulin-dependent protein kinase II, and 43% identity with yeast nim1+ protein kinase.

PKABA1 sequence homology with other protein kinases does not extend to the region downstream of catalytic subdomain XI (Fig. 1). This region does not contain significant homology to any reported protein kinase regulatory sequences (GCG version 7.0). Interestingly, the amino acid sequence from 309 through 321 does include a near-perfect palindromic sequence, which may be functionally important.

PKABA1 Transcript Levels in Developing Seed Embryos. The relationship between endogenous ABA and PKABA1 transcript levels was assessed in developing wheat seed embryos. During seed development this tissue accumulates ABA and exhibits ABA-responsive gene expression. Steady-state levels of the PKABA1 transcript were measured in maturing seed embryos as the seeds reached the desiccation stage (Fig. 3). Between 25 and 33 dpa the seeds lost moisture and embryonic ABA increased from 0.89 μM at 25 dpa to an

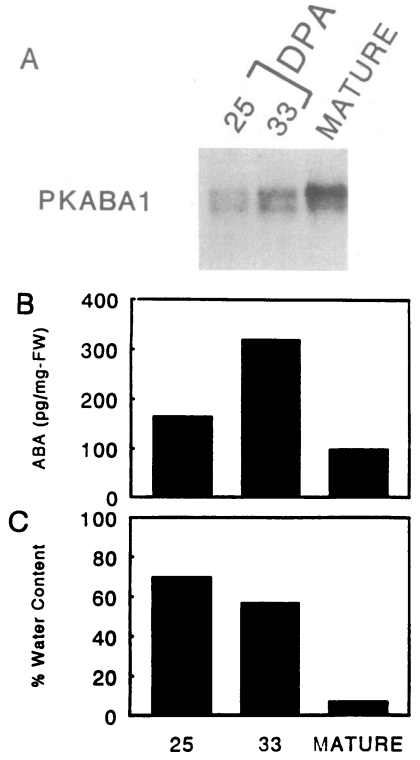

FIG. 3. (*A*) Transcript levels of cDNA clone PKABA1 in immature wheat seed embryos at 25 and 33 dpa and at maturity. Each lane contained 1 μg of poly(A)$^+$ RNA. (*B*) Endogenous concentration of embryonic ABA (pg/mg of fresh weight). (*C*) Relative water content of embryos.

endogenous concentration of 2.2 μM at 33 dpa. At the same time PKABA1 transcript levels increased. PKABA1 transcript levels were highest in mature dry seed embryos, though by that stage ABA had declined. The PKABA1 cDNA clone hybridized to a prominent 1.3-kilobase (kb) band, as would be expected from the size of the cDNA clone, and to a fainter 0.9-kb transcript. The transcript levels of another wheat ABA-responsive gene (cDNA clone pMA2005) (11) showed an increase in steady-state levels with seed maturation similar to that for PKABA1 (data not shown).

PKABA1 Transcript Levels in ABA-Treated Embryos. Supplying ABA to isolated seed embryos has been shown to induce ABA-responsive transcripts (9), and the same result was obtained for PKABA1. Incubation of isolated wheat embryos in ABA as low as 0.1 μM resulted in the accumulation of the PKABA1 transcripts compared to a water control (Fig. 4). The 1.3-kb transcript accumulated to slightly higher levels than the 0.9 kb transcript at 0.1 to 10 μM ABA,

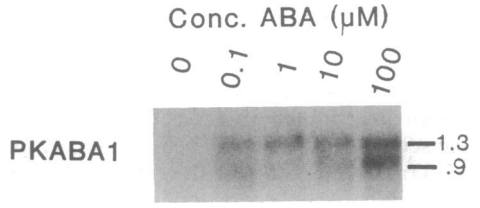

FIG. 4. Transcript levels of pPKABA1 cDNA clone in embryos incubated for 48 hr at 20°C in water or ABA. Seeds were imbibed in water for 3 hr, and then the embryos were dissected from the seeds. The isolated embryos were placed in water, 0.1, 1.0, 10, or 100 μM (*S*)-ABA. At 48 hr embryos were harvested, poly(A)$^+$ RNA was extracted, and Northern blot analysis was performed with 1 μg of poly(A)$^+$ RNA in each lane.

whereas both transcripts were induced to high levels with 100 μM.

PKABA1 Transcript Levels in Dehydrated Tissue. The effect of water stress on PKABA1 transcript levels was measured in seedlings and compared with the induction of two other ABA-inducible genes, Em (clone pMA1959) and group 3 LEA (clone pMA2005) (Fig. 5). By 12 hr, water-stressed shoot tissue lost 3% fresh weight, ABA levels increased 3.5-fold, and a high level of transcripts hybridizing to the PKABA1 cDNA clone was induced. The steady-state transcript levels of pMA1959 and pMA2005 showed an increase similar to that of PKABA1. By 48 hr of drying, the shoot tissue lost almost 40% moisture, ABA levels plateaued, the 1.3-kb PKABA1 transcript disappeared, and the 0.9-kb transcript decreased. Both pMA1959 and pMA2005 transcripts also decreased in the severely stressed tissue. In other experiments we have found PKABA1 mRNA is induced in root and scutellar tissue of wheat seedlings under similar drying conditions (R.J.A., J. L. Ried, and M.K.W.-S., unpublished data).

DISCUSSION

We have identified a wheat cDNA clone, PKABA1, with homology to protein kinases. Sequence analysis reveals that PKABA1 has all the features in the conserved catalytic subdomains of serine/threonine protein kinases. The deduced

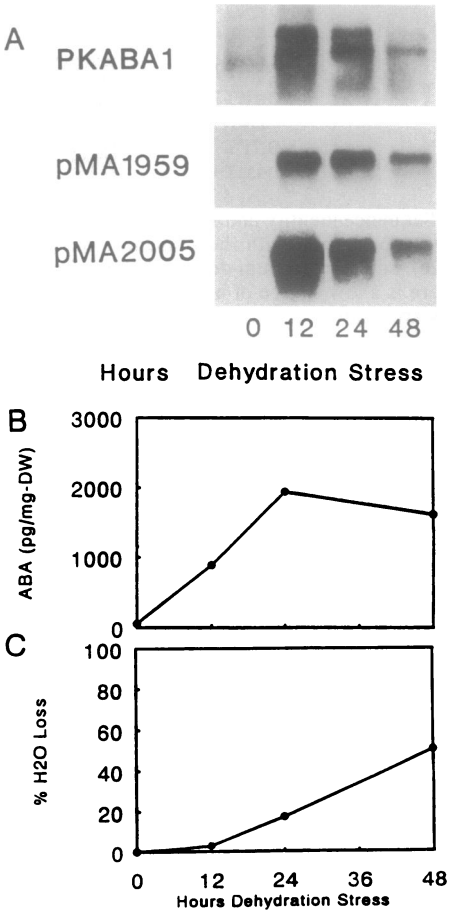

FIG. 5. Effects of water stress on ABA and transcript levels of PKABA1, Em (clone pMA1959), and wheat group 3 LEA (clone pMA2005). (*A*) Seedlings were dried at 20°C. At the indicated times, shoot samples were harvested, poly(A)$^+$ RNA was prepared, and transcript levels were assessed by Northern blot analysis. Each lane contained 1 μg of poly(A)$^+$ RNA. (*B*) ABA levels (pg/mg of dry weight). (*C*) Percent water loss.

amino acid sequence for the catalytic subdomain of PKABA1 is most similar to several protein kinases that respond to external signals: soybean calcium-dependent protein kinase (19), rat calcium/calmodulin protein kinase II β subunit (16, 28), and yeast SNF1 (29), which is responsive to changes in carbon-source availability. The homology of PKABA1 to these and other protein kinases does not extend beyond the catalytic domain. To determine whether PKABA1 represents a new member of an identified protein kinase family or is a novel protein kinase will require characterization of its catalytic activity.

PKABA1 hybridizes to two transcripts, 1.3 and 0.9 kb, that are induced by ABA. Relative levels of the two transcripts differ depending on the tissue source and the conditions of induction (Figs. 4 and 5). A likely interpretation is that the transcripts correspond to related protein kinases from a gene family. Alternatively, the smaller transcript could result from degradation of the 1.3-kb transcript. Another possibility is that the smaller transcript results from RNA splicing. A potential first splice junction at base 892 (GT) is located downstream of a possible polyadenylylation signal (ATAA). A second potential splice junction (AG) is at base 1169 and is followed by an in-frame stop codon (TAG) starting at base 1171. This would allow for removal of a 277-base fragment by splicing of the PKABA1 transcript, resulting in a smaller message complete with polyadenylylation and translation termination signals.

The accumulation rates for PKABA1 and other ABA-responsive transcripts, including Em (pMA1959) and group 3 LEA (pMA2005), are similar in water-stressed wheat seedlings (Fig. 5). The similarity in induction times suggests that some ABA-inducible proteins may be potential substrates for this ABA-inducible protein kinase, though phosphorylation of existing proteins is just as likely. Some ABA-responsive proteins, such as maize LEA protein RAB-17, are found to be highly phosphorylated (13). *In vitro* studies have shown that a serine-cluster region of the RAB-17 protein can be phosphorylated by maize casein kinase II (14). In wheat, potential sites of phosphorylation have been identified in the sequences of cDNA clones including group 3 LEA (pMA2005) and pMA1949 (9, 11), based on the substrate specificity of wheat embryo calcium-dependent protein kinase (32). Preliminary evidence indicates that the number of phosphorylated proteins of similar molecular weight to wheat group 3 LEAs increases in embryos imbibed in ABA compared to water controls (J. L. Ried and M.K.W.-S., unpublished data). Thus, a function of the PKABA1 protein product could be phosphorylation of ABA-responsive proteins.

Improving plant tolerance of environmental stresses is critical for reducing agricultural losses due to weather-related damage. Our findings suggest that an ABA-inducible protein kinase is involved in water-stress responses. Such a protein kinase has the potential to phosphorylate and modify a number of proteins in dehydrated plant tissue. One possible function of this enzyme pertaining to water-stress survival is modulation of gene expression, perhaps by modifying DNA-binding proteins as has been shown for another plant protein kinase (33). Potential targets for the PKABA1 gene product are trans-acting proteins that recognize ABA response elements (8, 34, 35). Alternatively, the PKABA1 gene product could be involved in modifying proteins or enzymes important for specific water-stress responses. Such modifications could regulate ABA levels or enhance properties of structural proteins involved in desiccation tolerance. Determining the function of the PKABA1 gene product will require isolating the protein, determining the substrate specificity, and identifying the regulatory molecules. The protein encoded by the PKABA1 gene may be an important link between ABA increases and other plant responses to water stress.

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