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The stress- and abscisic acid-induced barley gene *HVA22*: developmental regulation and homologues in diverse organisms

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Abstract

Abscisic acid (ABA) induces the expression of a battery of genes in mediating plant responses to environmental stresses. Here we report one of the early ABA-inducible genes in barley (*Hordeum vulgare* L.), *HVA22*, which shares little homology with other ABA-responsive genes such as *LEA* (late embryogenesis-abundant) and *RAB* (responsive to ABA) genes. In grains, the expression of *HVA22* gene appears to be correlated with the dormancy status. The level of HVA22 mRNA increases during grain development, and declines to an undetectable level within 12 h after imbibition of non-dormant grains. In contrast, the HVA22 mRNA level remains high in dormant grains even after five days of imbibition. Treatment of dormant grains with gibberellin (GA) effectively breaks dormancy with a concomitant decline of the level of HVA22 mRNA. The expression of *HVA22* appears to be tissue-specific with the level of its mRNA readily detectable in aleurone layers and embryos, yet undetectable in the starchy endosperm. The expression of *HVA22* in vegetative tissues can be induced by ABA and environmental stresses, such as cold and drought. Apparent homologues of this barley gene are found in phylogenetically divergent eukaryotic organisms, including cereals, *Arabidopsis*, *Caenorhabitis elegans*, man, mouse and yeast, but not in any prokaryotes. Interestingly, similar to barley *HVA22*, the yeast homologue is also stress-inducible. These observations suggest that the *HVA22* and its homologues encode a highly conserved stress-inducible protein which may play an important role in protecting cells from damage under stress conditions in many eukaryotic organisms.

Introduction

Abscisic acid regulates a variety of plant genes in response to developmental cues and environmental stresses. In seed development and germination, ABA appears to be involved in growth and differentiation of the embryo, accumulation of reserves in storage tissues (cotyledon and/or endosperm), and prevention of precocious germination (Crouch and Sussex, 1981; Bray and Beachy, 1985; Vilardell *et al.*, 1990). Studies with ABA biosynthesis inhibitors and genetic mutants further suggest the involvement of ABA in the maintenance of seed dormancy (Fong *et al.*, 1983). Maize and *Arabidopsis* mutants with a reduced level of ABA or sensitivity to ABA are viviparous (Robichaud *et al.*, 1980; Koornneef *et al.*, 1982).

Recently, studies have focused on the role of ABA-regulated genes in the development of desiccation tolerance in seeds. Abscisic acid levels in embryos begin to rise and reach a peak at the late stage of embryogenesis, when specific mRNAs accumulate in the seeds. These mRNAs include those encoding late embryogenesis-abundant (LEA) proteins, which have been described in various plant species (Dure *et al.*, 1989; Hong *et al.*, 1992; Gaubier *et al.*, 1993). Some of these proteins have been suggested to be involved in protecting proteins and membranes from damage during the desiccation process, presumably by forming a dimer with a coiled-coil structure capable of sequestering ions built up due to water loss during the desiccation process (Dure *et al.*, 1989). In fact, the expression of some *LEA* genes is closely corre-

lated with the development of desiccation tolerance in embryos. Treatments of embryos isolated at an earlier developmental stage with ABA result in a precocious accumulation of LEA proteins and other mRNAs, and the acquisition of desiccation tolerance in these young embryos (Bartels *et al.*, 1988).

Not only do seeds have to cope with low water potential. Vegetative tissues also face similar stress under unfavorable growth conditions. The level of ABA in vegetative tissues is known to increase in response to drought, salt and cold stress (Lachno and Baker, 1986; Chen *et al.*, 1983). Many of the genes expressed at the late stage of seed development are also induced by ABA in vegetative tissues (Hong *et al.*, 1988; Cohen and Bray, 1990). The elevated level of ABA induces the expression of *RAB* (responsive to ABA) genes in rice (Mundy and Chua, 1988) and maize (Vilardell *et al.*, 1990). Other ABA- and/or dehydration-regulated genes include those encoding a putative RNA-binding protein (Ludevid *et al.*, 1992), lipid body membrane proteins from maize (Vance and Huang, 1988) and carrot (Harzopoulos *et al.*, 1990), and an aldose reductase involved in the synthesis of sorbitol, a putative osmolyte in plant cells (Bartels *et al.*, 1991). Involvement of ABA in cold acclimation and responses to salt stress has also been reported in quite a few plant species (Bornman and Jansson, 1980; Chen and Gusta, 1983; Singh *et al.*, 1987).

We are committed to studying the mode of the ABA action in barley aleurone tissue, a convenient system for the study of plant hormones. It has been shown that ABA induces more than a dozen polypeptides in barley aleurone layers, and most of them are heat-stable (Lin and Ho, 1986; Jacobsen and Shaw, 1989). Several ABA-inducible barley cDNA and genomic clones have been cloned and sequenced. One of these, *HVA1*, is a *LEA* gene (Hong *et al.*, 1992). Another clone, *HVA22*, represents a unique type of ABA-inducible genes (Shen *et al.*, 1993). Beside ABA, cycloheximide and stress conditions are also strong inducers of *HVA22* (Shen *et al.*, 1993). While the ABA regulation of expression of these genes has been a subject of intensive studies, little is known about their potential functions. As an initial attempt to investigate the *HVA22* gene, we studied its expression pattern in response to developmental and environmental cues. In addition, we show that *HVA22* homologues are present in a variety of organisms including *Arabidopsis*, mammals and yeast. Interestingly, the yeast homologue can also be induced by salt stress. The potential function of the HVA22 protein and its homologues is discussed.

Materials and methods

Chemicals

Guanidine-HCl was purchased from Fisher Scientific, Pittsburgh, PA. Unlabeled deoxynucleotide triphosphates and nucleotide triphosphates were acquired from Boehringer Mannheim Biochemicals, Indianapolis, IN. Deoxycytidine α-[^{32}P]triphosphate (3000 Ci/mmol) and guanidine α-[^{32}P]triphosphate (3000 Ci/mmol) were purchased from NEN Research Products, Boston, MA. T3 RNA polymerase was obtained from Stratagene, La Jolla, CA. Restriction enzymes were obtained from Promega, Madison, WI; New England Biolabs, Beverly, MA; and International Biotechnologies, New Haven, CT. Luciferin was purchased from Analytic Luminescence Laboratory, San Diego, CA. Methyl jasmonate was obtained from Bedoukian Research, Danbury, CT. All other reagents were acquired through Sigma Chemical Co., St. Louis, MO.

Plant material and incubation conditions

Barley grains (*Hordeum vulgare* L. cv. Himalaya), obtained from 1985 or 1988 harvests at the Department of Agronomy and Soils, Washington State University, Pullman, WA, were used throughout this study with the exception of the dormancy experiments which utilized seeds of cv. Steptoe. The sterilization and imbibition of whole seeds (embryo and seedling studies) and embryoless half-seeds (aleurone layer study) and the preparation of barley aleurone layers were done as described (Belanger *et al.*, 1986).

For the development study, barley grains were planted in 15 cm pots in a standard soil mix and grown in a greenhouse under a temperature regime of ca. 15 °C on the day and 10 °C at night. Supplemental lighting was used to increase light intensity and extend daylight to 16 h per day. The time of anthesis was determined by daily opening a floret from a central spikelet on a maturing spike and checking for elongation of the filaments, dehiscence of the anthers, and the coincident pollination event. Anthesis marked day 0 for the time course of seed development. At specified times whole grains were collected and frozen immediately in liquid nitrogen or dissected into embryo, aleurone plus pericarp, and starchy endosperm fractions before freezing and storage at −70 °C.

ABA and stress treatments of seedlings

For ABA treatment, 3-day old seedlings, originally grown aseptically at room temperature on a layer of 3 MM filter paper above vermiculite soaked with H_2O, were transferred to and incubated in a petri dish containing two layers of 3 MM filter paper soaked with 10^{-4} M ABA solution. In dehydration experiments, seedlings were kept on moist paper for non-stress treatment or dehydrated to 85% of original fresh weight on the laboratory bench, then kept in the wilted state in a moist chamber for 24 h. To prepare cold stress-treated plants, beakers containing 3-day old seedlings on two layers of 3 MM paper soaked with H_2O were exposed to a low temperature of ca. 1 °C by sitting on ice in a cold room for 4 days. For heat shock treatment, 3-day old seedlings were put in glass beakers and incubated at a 37 °C water bath for 4 h. The roots were covered with a thin layer of water to avoid water loss during the heat treatment. After treatments, the seedling were dissected into root tips (1 cm), upper roots (the rest of the root), coleoptiles and leaves unless specified otherwise. The tissues were frozen in liquid nitrogen, and stored at −80 °C until further analyses.

Barley RNA preparation

Total RNA was prepared from aleurone layers and other barley tissues using guanidine-HCl as described in Belanger *et al.* (1986) and in Koehler and Ho (1990). Total RNA from developing endosperm was prepared using the method of Rogers and Milliman (1983).

Northern analysis

Northern analysis was performed by electrophoresing RNA (10 μg total RNA for barley and 20 μg for yeast per lane) in formaldehyde-agarose gels and blotting onto GeneScreen nylon membranes (New England Nuclear, Boston, MA) as described in Sambrook *et al.* (1989). Equal RNA loading for all samples was confirmed by monitoring the intensity of rRNAs stained with ethidium bromide. RNA was covalently cross-linked to the membrane by UV exposure according to the manufacturer's instructions. The blots were hybridized and washed according to the method of Church and Gilbert (1984). The autoradiography was quantified with a computing densitometer (Molecular Dynamics, Sunnyvale, CA).

Ribonuclease protection assay

The *HVA22* gene was cloned as two adjacent *Bam*HI fragments, named 5′ and 3′ BHI clones, from a partial genomic DNA library constructed in the polished *Xho*I site of the λZapII vector (Stratagene) (Shen *et al.*, 1993). A 3′ deletion construct was prepared from the 5′ BHI clone with *Eag*I digestion, which deleted the DNA between +95 (related to the second transcription start site) and the *Bam*HI site, as well as the polylinker sequence between the *Xho*I and the *Eag*I sites (Shen *et al.*, 1993). A 1.8 kb *Bgl*I/*Bgl*I fragment was obtained from this deletion construct, including the T3 promoter sequence and the *HVA22* sequence between -282 (*Bgl*I) and +95 (*Eag*I). The *Bgl*I end was polished with T4 DNA polymerase since transcriptions of templates with protruding 3′ termini usually generate significant numbers of long RNA molecules aberrantly initiated at the termini (Sambrook *et al.*, 1989). *In vitro* transcription was conducted with T3 RNA polymerase, α-[^{32}P]GTP and cold ATP, CTP and UTP mix, producing a ^{32}P-labeled 408 base RNA fragment which is complementary to the transcribed strand of the *HVA22* DNA between −282 and +95 plus pieces of polylinker sequence. Total RNA was isolated from aleurone layers treated with or without ABA (10^{-5} M) and cycloheximide (10 μg/ml), which synergistically induce *HVA22* expression (Shen *et al.*, 1993), and from mature embryos imbibed for 3 h. In order to compare the *HVA22* transcript in seeds and vegetative tissues, total RNA was also prepared from 3-day old seedlings cold-stressed for an additional 4 days. A 30 μg portion of total RNA was hybridized overnight at 55 °C with the ^{32}P-labeled RNA probe. *Escherichia coli* tRNA was also included as a negative control. The RNase digestion and further processing were performed as described (Gilman, 1989).

DNA constructions, particle bombardment and enzyme assays

For the studies of methyl jasmonate (MJ) and gibberellin (GA), we used construct PsalIIGU, which contains the 3 kb promoter plus 46 bp untranslated sequence of the *HVA22* gene linked to the HVA22 intron1-exon2-intron2 fragment and the coding sequence for the *E. coli GUS* (encoding β-glucuronidase) gene with a modified ATG initiation codon. The 3′ sequence containing the poly(A)-addition signal of the *HVA22* gene is fused to the 3′ end of the *GUS* gene (Shen *et al.*, 1993). Each testing construct containing the *GUS* reporter gene was

mixed with the internal control construct, pAHC18 (maize ubiquitin promoter/luciferase construct; Bruce and Quail, 1990) at 1:0.5 molar ratio. Half-seeds, after being imbibed for three days, were bombarded with the DNA mixture and incubated with or without 2×10^{-5} M ABA for 24 h. The homogenization of seeds after incubation, quantitative enzyme assays, and data reduction were basically the same as described before (Lanahan *et al.*, 1992; Shen *et al.*, 1993). The normalized GUS activity represents the total number of fluorescent units that would be generated in 20 h from an aliquots of extract that contained 2×10^6 relative light units of luciferase activity.

Yeast cell culture, RNA isolation and single-strand probe labeling

Saccharomyces cerevisiae was raised in 1 liter of 1% yeast extract/2% peptone/2% glucose in a 2-liter flask at 30 °C, 175 rpm to late log phase. The cells were harvested by centrifugation at 5000 rpm for 5 min at room temperature, and resuspended in 15 ml of medium. For salt stress treatment, 10 ml of the resuspension was transferred to a 2-liter flask containing 500 ml of 1% yeast extract/2% peptone/2% glucose/1.5 M NaCl and incubated under the same conditions as above. For the control, conditions were the same as with the stressed condition but without NaCl in the medium.

For RNA isolation, yeast cells at specific stages of culture were harvested at 5000 rpm for 2 min, and frozen in liquid nitrogen immediately after centrifugation and stored at −70 °C. The cells were thawed in 0.5 ml of 0.5 M NaOAc pH 4 and transferred to a 2 ml microfuge tube. After brief pelleting in a benchtop centrifuge, the cells were resuspended in another 0.5 ml of 0.5 M NaOAc pH 4, followed by the addition of 0.1 ml of chloroform/isoamyl alcohol (49:1) and 0.5 ml of phenol prewarmed at 70 °C. The mixture was vortexed immediately for 5 min with a microfuge tube mixer and centrifuged at 17 000 × *g* for 10 min at 4 °C. The aqueous upper phase (0.54 ml) was transferred to a new microcentrifuge tube, and mixed well with 0.5 ml of cold isopropanol. After storage at −20 °C for 30 min, the RNA was recovered by centrifugation at 17 000 × *g* for 10 min at 4 °C, washed with 70% ethanol, dried in a speed vac.

For labeling of single-strand probe, the template was amplified from yeast genomic DNA by PCR. The two primers used were 5′-ccgaatatgcatctagtattc-3′ (yhva5) and 5′-atgaacagaagcacctgtagc-3′ (yhva3). The PCR product was confirmed by sizing and restriction digestion. The single-strand probe was labeled by PCR in the following mixture: 1 μl of template (25 ng), 1 μl of yhav3 primer (25 μM), 1 μl each of dATP, dGTP and dTTP (1 mM), 5 μl of α-[^{32}P]dCTP (50 μCi), 2 μl of buffer, 0.5 μl of *Taq* DNA polymerase (Dynazyme), and H_2O to make up the total volume of 20 μl. Seven PCR cycles were performed in the following parameters: denaturation at 94 °C for 1 min, annealing at 55 °C for 1.5 min, and extension at 72 °C for 5 min. The probe was purified with a G-50 gel filtration column and denatured before hybridization.

Results

To study the mode of action of ABA in regulating gene expression, we have screened several aleurone layer cDNA libraries and obtained dozens of ABA-regulated clones. Among these clones, HVA22 appears to be the least abundant. This gene is mapped to barley chromosome 1 (Shen *et al.*, 1993). The schematic representation of the *HVA22* gene structure is shown in Figure 1A. Two ABA response promoter complexes are present within −300 region of the promoter, each containing an ACGT box (A2 or A3) and a coupling element (CE2 or CE1) (Shen *et al.*, 1993, 1996).

HVA22 cDNA, with a size of 816 bp, has a long (261 bp) CT-rich 5′-untranslated region. The deduced amino acid sequence indicates that *HVA22* encodes a basic protein, with an isoelectric point of 9.2 as calculated with the Isoelectric program in the GCG software package (Devereux *et al.*, 1984). It shares no apparent homology with any of the reported ABA-inducible genes, such as *RAB* and *LEA*.

Northern analyses with the cDNA as probe indicated the expression of *HVA22* can be rapidly induced by either ABA or cycloheximide, a protein synthesis inhibitor (Figure 1B). Addition of both inducers had a synergistic effect on the expression of this gene (Figure 1B). In the absence of cycloheximide, the ABA induction of *HVA22* was transient, with its mRNA level peaking between 4 to 8 h of ABA treatment and declining later to background level (Figure 1B) (Shen *et al.*, 1993).

HVA22 homologues are present in other cereals

Other members of Gramineae were examined for *HVA22* homologues in plants with seed morphology similar to barley. Total RNA was isolated from embryos of wheat (*Triticum aestivum* L.), rye (*Secale*

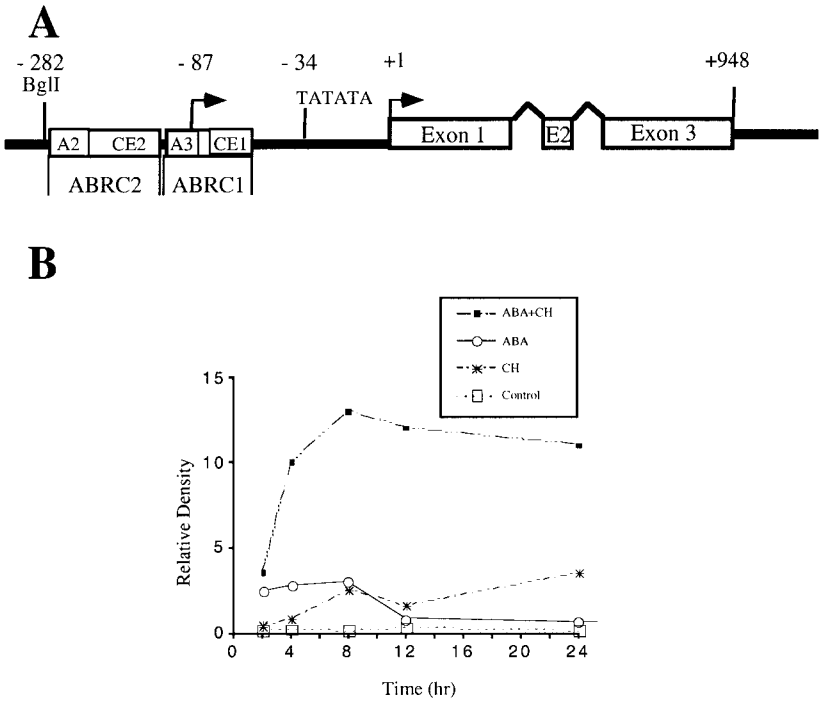

Figure 1. The *HVA22* gene is ABA- and cycloheximide-inducible and can be superinduced by the presence of both inducers. A. Schematic diagram of the *HVA22* gene. The promoter region proximal to the TATA box is shown. ABRC stands for ABA-responsive complex composed of an ACGT-box (A2 or A3) and a coupling element (CE1 or CE2). The positions of the ABRCs and the TATA box relative to the transcription start site are shown. Two transcription start sites, shown as arrows, appear to be present in this gene; the first one (−87) corresponds to the 5′ end of the cDNA sequence and the second one (+1) is the mapped transcription start site. Thin black angled lines represent the introns of this gene. B. The HVA22 gene is ABA- and cycloheximide-superinducible. The expression of *HVA22* is regulated by ABA and cycloheximide as determined by the northern blot analysis. Each lane was loaded with 10 μg of total RNA prepared from the aleurone layers treated with or without 2×10^{-5} M ABA, 10 μg/ml cycloheximide (CH) or combination of ABA and cycloheximide (ABA+CH) for the time indicated (h). The density of each band was quantified with a densitometer.

cereae L.), oat (*Avena sativa* L.), rice (*Oryza sativa* L.) and maize (*Zea mays* L.) and used for northern blot analyses with rRNA as the loading control. In all of the RNA samples, *HVA22* cDNA hybridized to a mRNA species with mobility similar to that of *HVA22* from barley. They are most likely the *HVA22* homologues since the hybridization with the ^{32}P-labeled *HVA22* cDNA was conducted at high stringency (Church and Gilbert, 1984) (Figure 2A).

HVA22 homologues are present beyond the plant kingdom

The presence of *HVA22* homologues is not limited to Gramineae species. When the barley HVA22 amino acid sequence was compared by the BLAST program to the database of GenBank, its homologues were identified in a variety of organisms including yeast (with a BLAST score of 76 and a probability of 9×10^{-13}), mouse (BLAST score 76, probability 5×10^{-13}), and *Caenorhabditis elegans* (BLAST score 65, probability 2×10^{-9}). In addition, as has been reported before (Vance and Huang, 1988), a human HVA22 homologue has been identified (BLAST score 75, probability 2×10^{-12}). When the search was conducted against the dbEST database, an *Arabidopsis* homologue (BLAST score 108, probability 2×10^{-22}) and a rice (BLAST score 110, probability 3×10^{-25}) were found.

HVA22 and its homologues contain a conserved sequence which could be phosphorylated by a casein kinase II

Analyses of the HVA22 amino acid sequence (Figure 2B) with the Motifs program of the GCG software package indicated that this protein contains two consensus casein kinase II (CKII) phosphorylation sites ([S/T]XX[D/E]), with the first one (SKVD) located between positions 36 and 39 and the second between

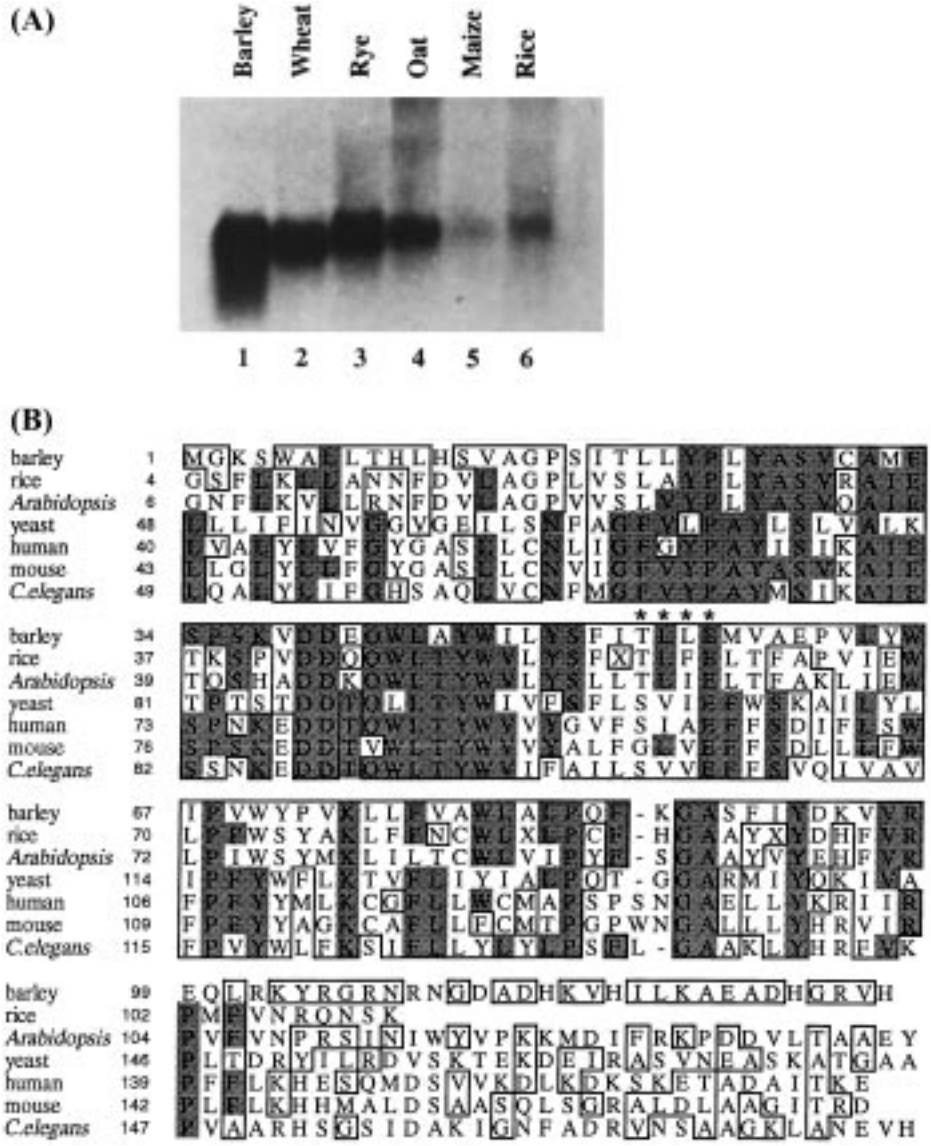

Figure 2. HVA22 gene homologues are ubiquitous among eukaryotes. A. HVA22 homologous mRNA is present in other Gramineae species. Total RNA was isolated from mature embryos of the seeds listed. The northern blot analysis was conducted as described in Materials and methods with the ^{32}P-labeled HVA22 cDNA as the probe. Equal RNA loading for all samples was confirmed by monitoring the intensity of rRNAs stained with ethidium bromide. B. *HVA22* homologues are present in diverse organisms. The barley amino acid sequence was compared to the nr and dbEST databases, homologous sequences were identified and aligned by BLAST. Identical residues are shaded, similar residues, as determined by SeqVu 1.0, 90% on the GES scale, are outlined. The numbers indicate the positions of the amino acids within the individual proteins.

positions 54 and 57 (Dure *et al.*, 1989). Interestingly, the second CKII phosphorylation site is conserved among all homologues except the mouse one as shown in Figure 2. Whether these proteins are actually phosphorylated *in vivo* remains to be determined.

HVA22 expression in seeds is tissue-specific and developmentally regulated

To study the expression pattern of *HVA22* at different stages of seed development, RNA was isolated from different tissues of the developing and mature seeds for northern analyses. The amount of rRNA in each gel lane was monitored to ensure equal RNA loading

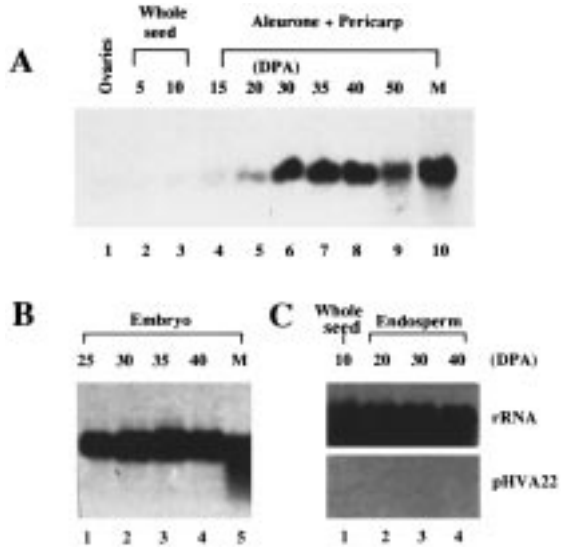

Figure 3. Tissue-specific expression of the *HVA22* gene in developing seeds. Steady-state HVA22 mRNA level in developing aleurone layers (A), embryos (B) and endosperm cells (C, bottom panel) were determined by northern blot analyses. Equal RNA loading for all samples was confirmed by monitoring the intensity of rRNAs stained with ethidium bromide. HVA22 cDNA insert was used as probe. As a control, the filter containing endosperm RNA was probed with a maize 26S rRNA, pZM26R (C, top panel). DPA, days after anthesis.

for all samples. It was shown that in developing seeds HVA22 mRNA was readily detectable in the aleurone layers as early as 15 days (lane 4, Figure 3A) after anthesis and its level remained high throughout the later stages (lanes 5 to 10, Figure 3A). The HVA22 mRNA was also abundant in the embryo through all stages of seed development studied (Figure 3B). However, it was not detectable in the starchy endosperm during seed development, where seed storage proteins are synthesized (Figure 3C). As a control, rRNA in the same endosperm RNA samples was readily detectable.

Turnover rate of HVA22 mRNA is correlated with the seed dormancy status

The HVA22 mRNA in embryos dissected from non-dormant barley (cv. Himalaya) grains started to degrade upon imbibition and dropped to undetectable levels in 12 h (lane 6, Figure 4). However, the mRNA remained at a high level even after 48 h of imbibition when 20 μM ABA was included in the imbibition buffer. Since the mRNA accumulates during seed development and degrades rapidly during imbibition, we reasoned that *HVA22* expression might be related to seed dormancy. We tested this idea with another culti-

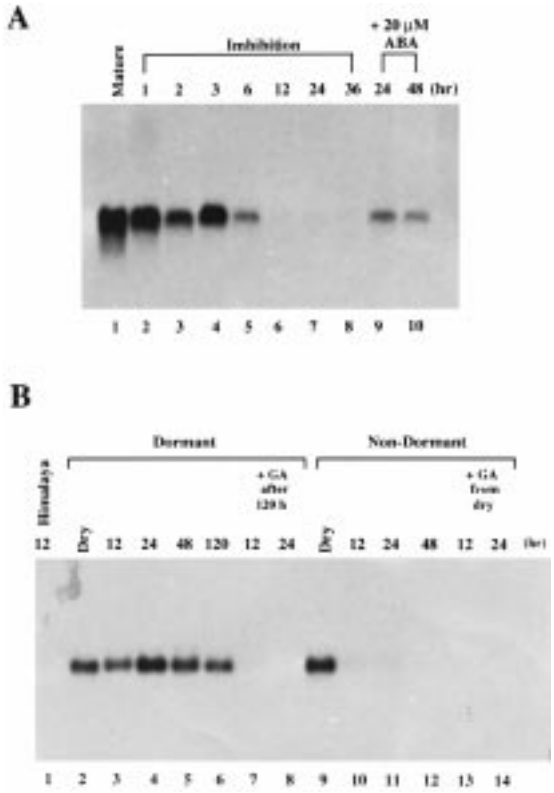

Figure 4. Levels of HVA22 mRNA in embryos are correlated with dormancy status. A. HVA22 mRNA in the mature embryos of cv. Himalaya degrades rapidly upon imbibition. Barley grains were imbibed at 25 °C for the time (h) indicated, in the buffer alone (lanes 1 through 8) or in the presence of 2×10^{-5} ABA. B. Levels of HVA22 mRNA in the embryos are correlated with the dormancy status of the cv. Steptoe seeds. The Steptoe seeds were freshly harvested 3–4 weeks before use and the majority of these seeds showed dormancy, thus designated as dormant seeds (lanes 2–8). The seeds harvested previously (more than 6 months before), which exhibited uniform and immediate germination, were the non-dormant seeds (lanes 9–14). After 120 h of imbibition, two of the dormant seed samples were transferred to a moist plate containing 1 mM GA_3 to induce germination (lanes 7 and 8). As a control, non-dormant seeds were imbibed from dry state in moist plates containing 1 mM GA_3 (lanes 13 and 14). At the end of each treatment, embryos were excised (except in the 48 h non-dormant sample (lane 12), where only the scutellum and the cotyledonary node were used), frozen, and processed for total RNA as described in Materials and methods. Total RNA from 12 h imbibed, non-dormant embryos of cv. Himalaya (lane 1) was included for comparison. Equal RNA loading for all samples was confirmed by monitoring the intensity of rRNAs stained with ethidium bromide. The northern blot analysis was carried out with the HVA22 cDNA as the probe.

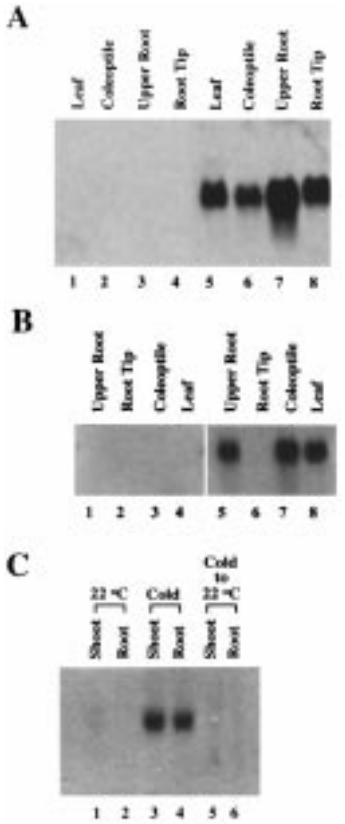

Figure 5. HVA22 expression in the vegetative tissues is induced by ABA, drought and cold. Total RNA was prepared from the following samples for northern analyses. Equal RNA loading for all samples was confirmed by monitoring the intensity of rRNAs stained with ethidium bromide. A. Three-day old seedlings were treated with (lanes 5 and 6) or without (lanes 1–4) 2×10^{-5} ABA. B. Three-day old seedlings were dried to 85% of original fresh weight and kept in the wilted state in a moist chamber for 24 h (lanes 5–8). For the non-stress treatment (lanes 1–4), the seedlings were kept in moist paper for the same period of time as the drought treatment. C. Three-day old seedlings were exposed to 1 °C for 4 days (lanes 3 and 4), kept at 22 °C (lanes 1 and 2) or returned to 22 °C after 1 °C stress. Some of the cold-stressed plants were returned to and kept at 22 °C for 14 h before the total RNA is prepared (lanes 5 an 6). After the treatments, the seedlings were dissected into root tips (1 cm), upper roots (the rest of the roots), coleoptiles and leaves (for the ABA and drought studies) or into root and shoot only (for the cold treatment).

var, Steptoe, whose seeds remain dormant for about three months after harvesting. To confirm that seed dormancy in Steptoe barley is embryonic, twenty embryos were dissected from dormant or non-dormant Steptoe barley grains and incubated in the MS medium at 25 °C for 5 days. All embryos from dormant Steptoe barley grains remained dormant while 100% of embryos from non-dormant grains germinated. The data suggest that the Steptoe barley seeds indeed have true embryonic dormancy.

Total RNA samples were prepared from the dormant and non-dormant Steptoe seeds for northern analyses. The amount of rRNA was monitored to ensure equal RNA loading for all samples. As shown in Figure 5B, the HVA22 mRNA level of the embryos in the non-dormant seeds was slightly higher than that of dormant seeds (compare lane 9 with lane 2 of Figure 4B). Upon imbibition, the HVA22 mRNA level of the non-dormant seed embryos dropped to an undetectable level by 12 h (lane 10), similar to what was observed with Himalaya barley grains (lane 1 of Figure 5B and lane 6 of Figure 4A). In contrast, the level of HVA22 mRNA in dormant embryos remained high even after imbibition for 5 days (lanes 3–6 of Figure 4B). Correspondingly, the dormant seeds did not germinate after incubation for 5 days at 25 °C. Germination of the dormant seeds was triggered by GA treatment, and so was the degradation of the HVA22 mRNA (lanes 7 and 8 in Figure 4B).

HVA22 expression in vegetative tissues is regulated by ABA and environmental stress

We also studied the effect of ABA and stress on the expression of *HVA22* in vegetative tissues. To study the effect of ABA, three-day old barley seedlings were treated with 100 μM ABA for 24 h. RNA samples were prepared from root tips, upper roots, coleoptiles and leaves and northern analysis was conducted. The HVA22 mRNA was detected in all of the organs treated, with the upper root having the highest level (lanes 5–8, Figure 5).

Dehydration also induced the expression of *HVA22* in the three-day old seedlings. Like ABA treatment, dehydration resulted in the accumulation of HVA22 mRNA in both shoots and roots of the treated plants. However, unlike ABA treatment, the induction of *HVA22* in root by dehydration was predominantly in upper root. In contrast, *HVA1*, another ABA-induced gene expresses predominantly at the root tip (Hong *et al.*, 1988).

The HVA22 mRNA was also detected in cold-stressed seedlings. The levels of HVA22 mRNA increased in both shoots and roots of three-day old plants after being exposed to 1 °C cold treatment for 4 days (lanes 3 and 4, Figure 5C). When the cold-stressed seedlings were returned to room temperature, HVA22 mRNA decreased to the pretreatment level within 14 h (lanes 5 and 6). The effect of cold stress appears to be

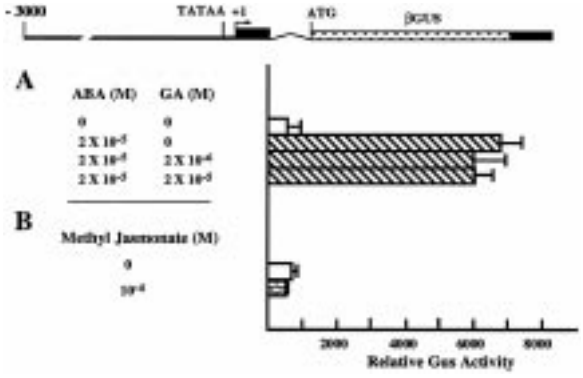

Figure 6. The *HVA22* gene is up-regulated by ABA but unaffected by GA or methyl jasmonate (MJ). A. GA does not antagonize ABA in inducing the expression of the 3 kb *HVA22* promoter sequence. B. The 3 kb *HVA22* promoter is not responsive to MJ. A schematic diagram of the testing construct is shown at the top. The thick black line at left represents the 3 kb *HVA22* promoter sequence. The thin black angled line indicates the position of the intron1-exon2-intron2 fragment of *HVA22* inserted between the 5′-untranslated sequence (solid box) and the GUS coding sequence (box with clusters of dots). The 3′ region (black bar to the right of the GUS coding sequence) was from the *HVA22 Sph*I-*Sph*I genomic fragment including the polyadenylation sequence (AATAAA). Construction of the construct LS-P, containing the 3 kb HVA22 promoter with the *Bgl*I site, has been described before (Shen *et al.*, 1996). The numbering of the sequence is relative to the second transcription start site (Shen *et al.*, 1993). The bombarded seeds were treated with or without 2×10^{-5} M ABA in the presence of GA at the concentrations indicated. The relative GUS activity, calculated as described in Materials and methods, is the mean of four replications. Error bars indicate the standard error of each set of replications.

specific to seedlings. The same cold treatment, for 1 to 4 days, did not induce the expression of *HVA22* in the barley aleurone layers (data not shown). The HVA22 mRNA was not detected in vegetative tissues treated at 37 °C for 4 h (data not shown).

ABA up-regulation of HVA22 promoter activity is not affected by GA or methyl jasmonate (MJ)

In the light of the potential antagonism between ABA and GA, the effect of GA on the ABA-regulated *HVA22* expression was studied with the 3 kb *HVA22* promoter-GUS fusion construct, LS-P. The 3 kb promoter routinely gives a 15–20-fold induction in response to ABA (Shen *et al.*, 1996). As clearly shown in Figure 6, GA had no effect on the ABA up-regulation of *HVA22* promoter activity in our transient expression system. The expression of GA responsive genes, such as those encoding α-amylases, is dramatically induced by GA at concentrations lower than 10^{-6} M (Nolan *et al.*, 1987), yet GA does not suppress the ABA-up-regulated *HVA 22* promoter activity at either 10^{-6} or 10^{-5} M.

In addition to GA and ABA interaction, we are also interested in studying the cross-talk between ABA and jasmonic acid (JA). Several genes regulated by ABA and JA have been reported. For example, *Phaseolus vulgaris* lipoxygenase genes are regulated by JA, its methyl ester (MeJA) or ABA (Porta *et al.*, 1999). In addition, genes encoding leucine aminopeptidase in *Lycopersicon esculentum* (Chao *et al.*, 1999) and storage proteins in *Brassica napus* (Hays *et al.*, 1999) are also regulated by both ABA and JA. However, as shown in Figure 6, MJ at a concentration of 10^{-4} M failed to induce *HVA22* expression.

Transcription of HVA22 in vegetative tissues uses the same start site as detected in seeds

As described before, the *HVA22* gene appears to have two potential transcription start sites (Shen *et al.*, 1993). The upstream one represents the initial nucleotide of the HVA22 cDNA and the downstream one is mapped by three different techniques (Figure 7). It is intriguing to test whether the transcription start site usage is tissue-specific or signal-specific, i.e. ABA vs. cycloheximide or stress. There have been reports of the usage of different transcription start sites in responding to different signals in other systems. For instance, a carrot gene encoding a hydroxyproline-rich glycoprotein has been shown to have two transcription start sites (Chen and Varner, 1985). Wounding stimulates transcription from both sites (Chen and Varner, 1985) while ethylene treatment suppresses transcription from the downstream site yet increases that from the upstream one (Ecker and Davis, 1987). Total RNA was isolated from cold stressed shoots and roots, mature barley embryos and aleurone layers treated with ABA or ABA plus cycloheximide. Our data indicate, however, the transcription start site appeared to be the same in all samples studied (Figure 7). No protected product was detected in the *E. coli* RNA control (data not shown). These results indicate the upstream transcription start site is rarely used.

Expression of the yeast HVA22 homologue is also enhanced by stress

Because the expression of *HVA22* in barley is induced by environmental stress, it would be interesting to study whether the yeast *HVA22* homologue (*yHVA22*) is also stress-inducible. When the yeast cells were cultured for 30 min in the presence of 1.5 M NaCl,

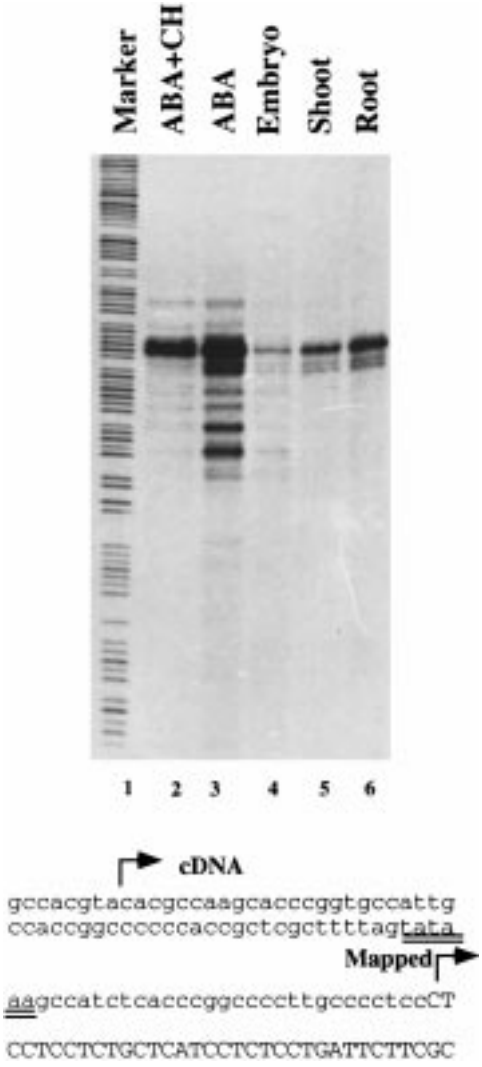

Figure 7. Same transcription start site of *HVA22* is used in seeds and in vegetative tissues. Total RNA was isolated from the aleurone layers treated with 10^{-5} M ABA (lane 3) or ABA plus cycloheximide (10 μg/ml, lane 2), dry embryos (lane 4), and cold-stressed shoots (lane 5) and roots (lane 6). A 30 μg portion of total RNA was hybridized with a ^{32}P-labeled RNA probe at 55 °C for 24 h and digested with RNase T1 and RNase A. The protected RNA fragment was resolved on a 6% polyacrylamide gel. The size marker shown at lane 1 was from the combination of ddA, ddT and ddC sequencing reactions performed with a primer complementary to the promoter region of the *HVA22* gene. The proximal promoter sequence is shown at the bottom panel. The upstream arrow represents the first nucleotide of the longest cDNA sequence obtained and the downstream one marks the mapped transcription start site. The TATA box is double-underlined.

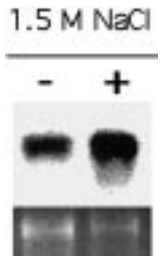

Figure 8. Yeast homologue of *HVA22* is inducible by salt stress. The yeast cells were cultured in the presence or absence of 1.5 M NaCl and RNA was isolated as described in Materials and methods. Northern blot was hybridized with the yeast *HVA22* homologue probe (upper panel). Equal RNA loading was confirmed by monitoring the intensity of rRNAs stained with ethidium bromide (lower panel).

the level of yHVA22 mRNA is 2.5 times higher than that obtained from the control (Figure 8). Interestingly, a significant level of yHVA22 mRNA was detected in the control without 1.5 M NaCl. In contrast, as mentioned above, HVA22 mRNA is not detectable in control tissues of barley. The presence of glucose and other nutrients in the rich yeast culture medium may account for the high level of *yHVA22* expression even in the absence of 1.5 M NaCl. This is supported by the observation that at the stationary phase (absorbance at 600 nm (A_{600}) = 10) the level of the mRNA declined drastically, to about 5% of that obtained from cells at the early log phase (A_{600} = 3). The consumption of the nutrients by the dividing yeast cells may lower the level of osmotic stress and consequently lower the level of induction of *yHVA22* gene expression.

Discussion

Plants in the field often experience unfavorable growth conditions during their development. One way to cope with the environmental stress is the alteration of synthesis of specific polypeptides. Since ABA has been known to mediate the stress responses, studies of ABA-regulated genes should help us understand how plants respond to a stressful environment. The expression of the barley *HVA22* gene is tightly regulated by stress and developmental cues. Its homologues are present in both dicot and monocot plants. Homologues of *HVA22* have been identified in organisms from other kingdoms, including yeast, *C. elegans* and mammals. Interestingly, similarly to barley *HVA22*, the yeast *HVA22* homologue is also induced by environmental stress. In this study, we also show that ABA induction of the *HVA22* promoter is not affected by

GA even though it is well known that the effect of GA on gene expression could be inhibited by ABA.

Studies of *HVA22* expression during seed development and germination indicate that the accumulation or degradation of HVA22 mRNA correlates well with seed dormancy status. This gene is expressed in embryos and aleurone layers, but not in the endosperm. This is in contrast to the barley α-amylase/subtilisin inhibitor which is expressed in the endosperm but not in the aleurone layers during seed development, although its expression can be induced by ABA in mature aleurone layers (Mundy *et al.*, 1986; Leah and Mundy, 1989). The accumulation of HVA22 mRNA starts at 20 days after anthesis (DPA) and peaks at 30 DPA, which is about the time ABA reaches the maximal level in developing barley seeds (S.J. Uknes and T.H.D. Ho, unpublished results). This suggests that *HVA22* expression is part of the ABA regulatory network involved in the maintenance of seed dormancy. Germination studies with dormant and non-dormant grains provide additional support for this suggestion. The level of HVA22 mRNA is a good indicator of seed dormancy status. Its level in non-dormant embryos declines drastically upon imbibition while that in dormant grains remains at a level similar to that in dry grains even after imbibition for 5 days. Treatments of dormant grains with GA_3 break seed dormancy and promote a reduction in HVA22 mRNA. Similar observations have been made with several wheat ABA-inducible genes (Morris *et al.*, 1991) and another barley gene, *HVA1* (Hong *et al.*, 1988). Whether these genes are the determinants of seed dormancy awaits further studies.

It is worthwhile noting that the regulation of *HVA22* expression appears to be complex. The expression of *HVA22* in the aleurone tissue is induced by either ABA or protein synthesis inhibitors, and a synergistic effect is observed when both types of inducers are present (Figure 1B). The timing of ABA induction has a short lag (less than 30 min) and appears to be transient. This type of expression pattern is common among many regulatory molecules such as transcription factors in both mammals (Edwards and Mahadevan, 1992) and plants (Franco *et al.*, 1990; Abel *et al.*, 1995). Although the mechanism of cycloheximide superinduction is not clear, it has been suggested that this chemical superinduces the expression of some transcription factor genes by (1) inhibiting the synthesis of labile repressors acting negatively on diverse superinducible genes, (2) delaying the shutdown of transcription and stabilization their mRNAs due to translational arrest or (3) acting positively as nuclear signaling agonists to stimulate the phosphorylation of transcription factors (Edwards and Mahadevan, 1992). It remains to be determined whether cycloheximide also regulates the HVA22 gene expression at the transcriptional level.

Besides ABA and stress, we also studied whether GA has any effect on the ABA-induced expression of *HVA22*. There have been only a few reports about the effect of GA on ABA induction of gene expression. In the vegetative tissues of tomato, ABA inhibits the GA_3-induced expression of a shoot-specific gene, *GAST1* (Shi *et al.*, 1992). It has been observed that the addition of GA_3 results in the reduction of the ABA-induced mRNA accumulation from an ABA-responsive gene, *HVA1*, in barley (G.H. Heck and T.H.D. Ho, unpublished data). In contrast, the ABA up-regulation of the *HVA22* promoter is unaffected by GA (Figure 6). However, GA treatment does reduce the level of HVA22 mRNA in dormant embryos. Therefore, it is likely that GA exerts its regulatory role in *HVA22* expression at a post-transcriptional level.

The *HVA22* gene may function in other organs/tissues in addition to seeds since its expression is also detected in other parts of the barley plant. Its mRNA has been detected in both shoots and roots of seedlings treated with ABA, cold and drought. When cold-treated seedlings were returned to room temperature, the HVA22 mRNA declined to background levels in 14 h. It remains unanswered whether the expression of this gene is independently induced by stress or by the elevated level of ABA present in stressed tissues. A stress response pathway separated from that of the ABA response appears to be present in plants. A low-temperature-induced gene, *lti 140*, which is also responsive to ABA, responds to cold stress in the ABA-deficient (*aba*) and the ABA-insensitive (*abi*) mutants of *Arabidopsis*. Similarly, drought stress also induces the expression of the *lti* gene in the *abi* mutant. These data have led to the suggestion that there might be three independent but converging signal pathways regulating the expression of this gene, i.e. the ABA, cold stress and drought response pathways (Nordin *et al.*, 1992). Furthermore, Yamaguchi-Shinozaki and Shinozaki (1994) have defined a *cis*-acting element specifically involved in the drought response of an *Arabidopsis* gene, *rd29A*. This element (DRE, TACCGACAT) is clearly different from the G-box and the CE element in the ABA response promoter complex (Shen and Ho, 1995; Shen *et al.*, 1995). Interestingly, the drought-inducible *HVA22* promoter also contains

a sequence homologous to DRE (Shen *et al.*, 1993). However, whether dehydration or cold stress could directly induce the expression of *HVA22* awaits further studies.

The biochemical function of the HVA22 protein is unknown. Although many HVA22 homologues are present in the non-redundant and EST databases, all of them were initially identified as part of sequencing projects, and thus have no known biochemical function associated with them. The yeast homologue has been named YIP2 (Ypt interacting protein 2) at the *Saccharomyces* Genomic Database (http://genome-www.stanford.edu/Saccharomyces/), apparently because of a two-hybrid interaction with YPT1, a rab-like small G protein which plays a role in vesicular transport (Segev, 1991). However, there is no information in the published literature about this interaction or the potential function of YIP*2*. Although homologues of HVA2*2* are present in diverse eukaryotic organisms, so far none have been found in a prokaryote, despite the complete sequencing of several prokaryotic genomes. This suggests that HVA22 and its homologues are involved in processes that are unique to eukaryotic organisms.

Analyses of the deduced amino acid sequence of HVA22 have revealed the presence of two consensus amino acid sequences that could be potentially phosphorylated by casein kinase II (CKII). One of these CKII phosphorylation sequences is conserved among all HVA22 homologues, except the one in mouse (Figure 2). It is interesting to note that another ABA-induced protein, the glycine-rich maize RAB17, is highly phosphorylated *in vivo* and the purified protein can be phosphorylated *in vitro* by both rat and maize CKII (Plana *et al.*, 1991). The maize *RAB-17* belongs to group 2 *LEA* genes, whose products contain a cluster of serine residues in the middle part of their sequences (Dure *et al.*, 1989; Vilardell *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1990). In maize RAB-17, this region with a cluster of eight serine residues has been shown to be phosphorylated (Plana *et al.*, 1991). However, the serine clusters are not determinants of the substrate specificity of CK II (Devereux *et al.*, 1984). Therefore, it is essential to determine whether HVA22 protein is actually phosphorylated *in vivo* by CK II.

With the identification of the *HVA22* homologue in yeast, it will become easier to study the function of the HVA22 protein. Yeast is known as an excellent genetic system for the study of the function of a protein. In fact, we have shown that, similar to the barley *HVA22* gene, the expression of the *yHVA22* gene is also induced by the osmotic stress. It would be interesting to study whether the expression of *yHVA22* is compromised in the yeast mutants, such as *Hog1* (Brewster *et al.*, 1993), which are sensitive to osmotic stress. In addition, knocking out the yeast gene by homologous recombination should facilitate the identification of the function of the *HVA22* homologue if a phenotype of the mutant is observed. The *yHVA22* knock-out mutant has been obtained and the study of phenotype of the *yHVA22* mutant is underway.

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