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AtHVA22 gene family in *Arabidopsis*: phylogenetic relationship, ABA and stress regulation, and tissue-specific expression

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Abstract

HVA22 is an ABA- and stress-inducible gene first isolated from barley (*Hordeum vulgare* L.). Homologues of *HVA22* have been found in plants, animals, fungi and protozoa, but not in prokaryotes, suggesting that *HVA22* plays a unique role in eukaryotes. Five *HVA22* homologues, designated *AtHVA22a, b, c, d* and *e*, have been identified in *Arabidopsis.* These five *AtHVA22* homologues can be separated into two subfamilies, with *AtHVA22a, b* and *c* grouped in one subfamily and *AtHVA22d* and *e* in the other. Phylogenetic analyses show that *AtHVA22d* and *e* are closer to barley *HVA22* than to *AtHVA22a, b* and *c*, suggesting that the two subfamilies had diverged before the divergence of monocots and dicots. The distribution and size of exons of *AtHVA22* homologues and barley *HVA22* are similar, suggesting that these genes are descendents of a common ancestor. *AtHVA22* homologues are differentially regulated by ABA, cold, dehydration and salt stresses. These four treatments enhance *AtHVA22a, d* and *e* expression, but have little or even suppressive effect on *AtHVA22c* expression. ABA and salt stress induce *AtHVA22b* expression, but cold stress suppresses ABA induction of this gene. Expression of *AtHVA22d* is the most tightly regulated by these four treatments among the five homologues. In general, *AtHVA22* homologues are expressed at a higher level in flower buds and inflorescence stems than in rosette and cauline leaves. The expression level of these homologues in immature siliques is the lowest among all tissues analyzed. It is suggested that some of these *AtHVA22* family members may play a role in stress tolerance, and others are involved in plant reproductive development.

Abbreviations: ABA, abscisic acid; BAC, bacterial artificial chromosome

Introduction

Plants alter their structure and physiology to enhance their tolerance to stressful conditions. Stress-regulated gene expression plays an important role in stress acclimation and tolerance establishment when plants are exposed to unfavorable environmental changes. Many groups of genes induced by environmental stresses have been cloned and characterized, including responsive to desiccation (*RD*) (Yamaguchi-Shinozaki K. *et al.*, 1992), low-temperature-induced (*LTI*) (Nordin *et al.*, 1991, 1993; Welin *et al.*, 1994, 1995), cold-regulated (*COR*) (Gilmour *et al.*, 1992; Horvath *et al.*, 1993; Wilhelm and Thomashow, 1993), kikna indusoidu (Finnish for cold-induced) (*KIN*) (Kurkela and Borg-Frank, 1990, 1993), responsive to ABA (abscisic acid) (*RAB*) (Vilardell *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1990), dehydrins (Roberton and Chandler, 1992 and references therein) and genes en-

The GenBank accession numbers for *AtHVA22a, b, c, d* and *e* cDNA sequences are AF141659, AF141660, AF141661, AF141662 and AF290892, respectively. Accession numbers for *AtHVA22a, b, c, d* and *e* genomic sequences are AF141977, AF141980, AF141978, AF141979 and AF313484, respectively.

coding heat shock proteins (Hong and Vierling, 2000 and references therein). Some of these stress-induced genes share similarities with genes of known functions, but the roles of the majority remain unclear. A great deal of effort has been invested to study how ABA and stress regulate the expression of these genes (Baker *et al.*, 1994; Shen *et al.*, 1996, Shinozaki and Yamaguchi-Shinozaki, 1996; Abe *et al.*, 1997; Ishitani *et al.*, 1997, 1998; Gilmour *et al.*, 1998; Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Lee *et al.*, 1999; Xiong *et al.*, 1999), but little is known about their functions. A major difficulty in studying the function of an unknown gene following a reverse-genetics approach is the existence of homologues and the potential functional redundancy among them. With the completion of the *Arabidopsis* genome project, the complexity of a gene family can be conveniently analyzed. Therefore, studies of the sequence divergence and unique expression patterns among members of a stress-induced gene family would eventually facilitate the understanding of their function.

HVA22 is an ABA- and stress-inducible gene originally isolated from barley (*Hordeum vulgare* L.) (Shen *et al.*, 1993). Its promoter has been studied in a great detail (Shen *et al.*, 1996), but the function of this gene is still unknown. Recently we have identified apparent *HVA22* homologues in a variety of eukaryotes including other plants, mammals, worms, fungi and protozoa but not in prokaryotes by searching GenBank and other databases (Shen *et al.*, 2001), suggesting that the function of *HVA22* is unique to eukaryotes. Taking advantage of the *Arabidopsis* EST and genomic databases, we were able to identify at least five *HVA22* homologues in *Arabidopsis*, designated *AtHVA22a, b, c, d* and *e.* These *AtHVA22* homologues appear to belong to two subfamilies, and they display unique tissue-specific expression patterns and are differentially regulated by ABA treatment and stress conditions. Herein, we report the characterization of this gene family, which lays the foundation for our future efforts in elucidating the function of these genes.

Materials and methods

Plants, growth conditions and stress treatments

All of the plant materials used in this study were *Arabidopsis thaliana* ecotype Columbia. For hydroponic culture, seeds were surface-sterilized and cold-treated for two days. Ten seeds were transferred into a plant growth vessel (Sigma V8380) containing 25 ml of 0.5× MS salt (Gibco-BRL 11117), 1× Gamborg's B5 vitamin (Gibco-BRL 21153), 0.5% sucrose, and 0.5 g/l MES. The pH was adjusted to 5.7 with KOH. The vessels were shaken at 75 rpm at 22 °C under 90 μmol photons m^{-2} s^{-1} light intensity with 16 h/8 h day/night cycle. Fourteen-day old (starting at imbibition) plants were used for ABA and stress treatments. For ABA treatment, ABA (Sigma A2784) stock solution was added into culture medium to bring to 40 μM. To impose cold stress, vessels were moved to a 4 °C cold room, shaken at 75 rpm under 90 μmol m^{-2} s^{-1} light intensity. Dehydration stress was imposed by transferring plants to petri dishes with lids covered after removing excess water with facial tissues. A piece of filter paper was placed underneath the plants to facilitate water evaporation. The relative humidity of the ambient air was 30–40% during the treatment. For salt stress, 5 M NaCl was added into culture medium to bring to 250 mM. For soil-grown plants, seeds were sown on Redi-mix (Scotts)/Germinating mix (Fafard)/vermiculite (1:1:1) in 7.6 cm × 7.6 cm square pots and cold-treated for two days before being moved to a growth chamber set for 22 °C, 50% relative humidity, 150 μmol m^{-2} s^{-1} light intensity, and a 16 h/8 h day/night cycle. The pots were thoroughly watered every two days. Drought stress was imposed to the plants by withholding watering one week after bolting until wilting.

Plant genomic DNA and total RNA isolation

Plant genomic DNA was isolated according to the method of Tai and Tanksley (1990). The plant total RNA isolation procedure was modified from Chomczynski and Sacchi (1987). *Arabidopsis* tissue (1 g) was ground into a fine powder in liquid nitrogen, transferred to a 50 ml Corning tube containing 12 ml of cold extraction buffer (4 M guanidine-HCl, 25 mM sodium citrate, 0.5% lauryl sarcosinate, 0.1 M 2-mercaptoethanol), and mixed by vortexing. Equal volume of cold phenol/chloroform, ca. pH 6, was added to the tube and mixed by inverting frequently for 5 min. The tube was centrifuged at 5000 × *g* at 4 °C for 10 min. Of the upper phase 10 ml was transferred to a new Corning tube. To the new tube 1 ml sodium acetate pH 5.2 and 10 ml of −20 °C isopropanol were added. Mixed briefly, the tube was stored at −20 °C for 1 h and centrifuged at 7000 × *g* at 4 °C for 15 min. The pellet was rinsed with 5 ml of 70% ethanol, dried

in vacuum, dissolved in 0.5 ml H_2O, and stored at −20 °C.

cDNA clones

AtHVA22a, b and *c* cDNA clones were obtained from the *Arabidopsis* Biological Resource Center at Ohio State University, USA. All of them were EST clones with clone ID 159D22T7, YAP206T3 and 131G7T7, respectively. The *AtHVA22d* cDNA clone was kindly provided by Dr Kazuo Shinozaki at RIKEN, Tsukuba, Japan, and has the original clone name FL-49. FL-49 was partially sequenced and deposited in the GenBank under the accession number AB015098. The *AtHVA22e* cDNA clone was purchased from Incyte Genomics in St. Louis, USA, which was also an EST clone (GenBank accession number AI995946). The five clones were sequenced again by the dideoxy method with an automated sequencer.

Genomic DNA sequences

AtHVA22a, *c* and *d* genomic sequences at the coding region were amplified by PCR with pairs of primers: 22aF and 22aR, 22cF and 22cR, and 22dF and 22dR, respectively. The flanking sequence of the *AtHVA22d* genomic coding region was amplified by inverse PCR. *Nde*I digestion and primers 22dI-1 and 22dI-2 were adopted in this procedure. The genomic sequence of *AtHVA22b* and *e* were found in the genomic clones K19B1 (GenBank accession number AB015469) and MFB16 (GenBank accession number AB023037), respectively. All PCR reactions were carried out with the *Taq* DNA polymerase (Gibco-BRL), and the amplified DNA fragments were sequenced by the dideoxy method with an automated sequencer. Sequences of primers are: 22aF, 5′-CGGTACCGTGGTTTTAGTGGAGGAAGA-3′; 22aR, 5′-CGGATCCGGAGCAGAAAGCATCTTTAT-3′; 22cF, 5′-ACACGAGGCATTACACTTTGG-3′; 22cR, 5′-CCCAGTAAGTAAGCCATTGTTC-3′; 22dF, 5′-ATT GATTCGCCTCGAAATTTAC-3′; 22dR, 5′-AATGG AGTGGAGGATGTTGG-3′; 22dI-1, 5′-GAAGAGCA GTGAGGAAAGTCC-3′; 22dI-2, 5′-GTGTTGTTAG AGAACAGTTCAAG-3′.

DNA gel blot analysis

Genomic DNA (5 μg) was digested with *Eco*RV, *Kpn*I, or *Nco*I, separated by 0.8% agarose gel electrophoresis, and transferred onto a piece of nylon membrane (GeneScreen Plus, DuPont). Hybridization was performed according to Church and Gilbert (1984) in 1% BSA, 0.25 M sodium phosphate pH 7.2, 7% SDS, and 1 mM EDTA overnight at 50 °C. The membrane was washed twice in 0.2× SSC and 0.1% SDS for 20 min at 50 °C. The probes used for the hybridizations were cDNAs of *AtHVA22a*, *b*, *c*, and *e*, or genomic DNA fragment of *AtHVA22d* amplified with primers 22dF and 22dR.

RNA gel blot analysis

Total RNA (10 μg) was separated by 1% agarose/1.8% formaldehyde gel electrophoresis, and transferred onto a nylon membrane (GeneScreen Plus) in 10× SSC. The hybridization procedure and *AtHVA22* homologue probes were essentially the same as for DNA gel blot analysis, except the temperature was 63 °C. Templates for *RD29* probe labeling were prepared by PCR amplification of the third exon of *RD29a* (Yamaguchi-Shinozaki and Shinozaki, 1993) with primers 5′-GCGTAACAGGTAAACCTAGAG-3′ and 5′-GTGGGATCAGTAACTTTGGAC-3′. Transcripts of *Arabidopsis* actin 2 gene, *ACT2*, were probed by its cDNA that was kindly provided by Dr Ralph Quatrano of the Biology Department, Washington University, St. Louis, MO.

Amino acid sequences alignment and phylogenetic analysis

Amino acid sequences of the five *Arabidopsis HVA22* homologues were deduced from their cDNA sequences and aligned using the Lasergene software (DNASTAR, Madison, WI). Phylogenetic analysis was performed using cDNA sequences corresponding to a block of amino acid residues between P29 and R103 of AtHVA22a (Figure 1) to ensure the most conserved alignment. Yeast and human (Joslyn *et al.*, 1991) *HVA22* homologues were used as outgroups. Phylogenetic trees were estimated using PAUP* beta version 4.0b1 (Swofford, 1998) with an exhaustive search. Bootstrap re-sampling was used to assess support for individual nodes with 1000 bootstrap replicates using branch and bound searches. Decay indices ('branch support' of Bremer, 1994) were calculated for all internal branches of the tree. Branch and bound searches that retained suboptimal trees were run. The decay index was then tabulated as the number of additional steps required to find a tree that did contain a particular node.

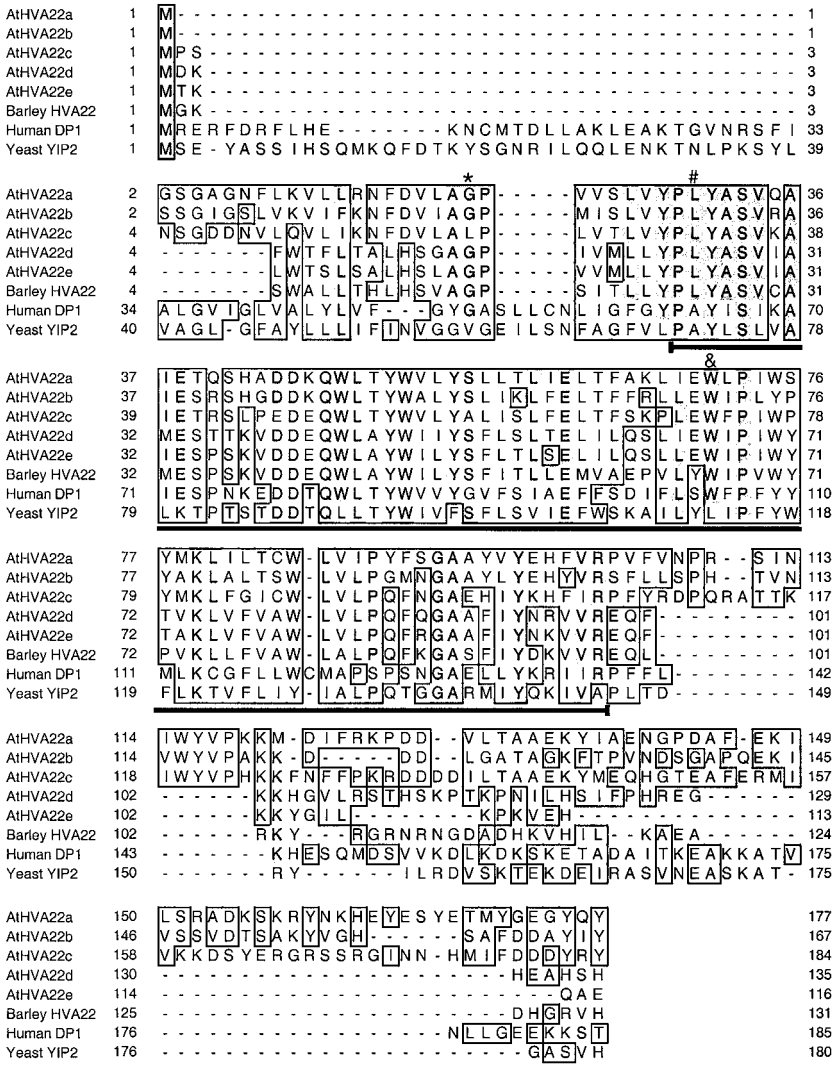

Figure 1. Amino acid sequence alignment of *HVA22* homologues of *Arabidopsis*, barley, yeast and man. The predicted amino acid sequences from cDNAs of *AtHVA22a–e*, barley *HVA22*, yeast *YIP2* and human *DP1* are aligned. The shaded and boxed residues are identical and similar amino acids, respectively. Gaps (dashes) were created to assure maximal alignment. *, #, and & denote the junctions between 1st and 2nd, 2nd and 3rd, and 3rd and 4th exons of *Arabidopsis* genes respectively. The region between amino acid residues P29 and R103 of AtHVA22a used for the phylogenetic analysis in Figure 4 is underlined with a thick bar.

Results

Identification of Arabidopsis HVA22 *homologues*

Initially, the *Arabidopsis* EST database in GenBank was BLAST-searched (Altschul *et al.*, 1990) with the barley *HVA22* amino acid sequence. Homologues found were apparently encoded by three genes based on sequence comparisons. These three genes were designated *AtHVA22a*, *AtHVA22b* and *AtHVA22c*. Another homologue, a partially sequenced cDNA, was later found in the non-redundant database in GenBank deposited by Dr Kazuo Shinozaki's group at RIKEN in Tsukuba, Japan. The corresponding gene was designated *AtHVA22d.* More recently, one more homologue was found in GenBank deposited by Incyte Genomics Company, St. Louis, MO, which was also an EST clone. The gene corresponding to this cDNA was named *AtHVA22e.* The sequences of the five cDNA clones were confirmed and completed. The five complete cDNA sequences were submitted to GenBank, and the accession numbers for *AtHVA22a*, *b*, *c*, *d*

and *e* cDNA sequences are AF141659, AF141660, AF141661, AF141662, and AF290892, respectively. For amino acid sequence comparison and phylogenetic analysis, yeast and human *HVA22* homologues (*YIP2*, GenBank accession number AJ007902; *DP1*, GenBank accession number Q00765) are aligned with the six plant homologues (Figure 1). The amino acid sequence alignment of the eight homologues shows that there are 15 perfectly conserved amino acid residues among these diverse organisms.

The Arabidopsis AtHVA22 *gene family is composed of at least five genes*

Genomic DNA gel blot analysis showed that each *AtHVA22* gene is a single copy in the genome. None of the five hybridization probes could detect other members of this family even at low stringency (Figure 2A). Indeed, similarities in the cDNA sequences of the five *AtHVA22* homologues are low, ranging from 30% to 49%, which makes it unlikely to have cross-hybridizations among these homologues. Searching the *Arabidopsis* genome, which has been completely sequenced recently, we found another potential *AtHVA22* homologue in the BAC clone F14N22 (accession number AC006931) on chromosome II. This gene is designated *AtHVA22f.* However, the expression of this gene is below detection level when using its genomic DNA as a probe (data not shown). Whether *AtHVA22f* is a pseudogene remains to be determined.

Chromosomal positions and structures of the AtHVA22 *genes*

The locations of the *AtHVA22* homologues on chromosomes are presented in Figure 2B. *AtHVA22a* is encompassed in the genomic clone F1M20 that is located at the bottom arm of chromosome I. The sequence of *AtHVA22b* was found in the genomic clone K19B1 located on chromosome V. Three BAC or P1 clones, T6C23, F13M23 and MFB16 were recently found containing the sequences of *AtHVA22c*, *d* and *e*, respectively. Thus the locations of these three genes are on chromosome I, IV and V, respectively. The GenBank accession numbers for *AtHVA22a*, *b*, *c*, *d* and *e* genes are AF141977, AF141980, AF141978, AF141979, and AF313484, respectively.

The structures of all five *AtHVA22* homologues and barley *HVA22* from the translation start to stop are shown in Figure 3. Among the six genes, every gene has five exons scattered in a ca. 1 kb region with the

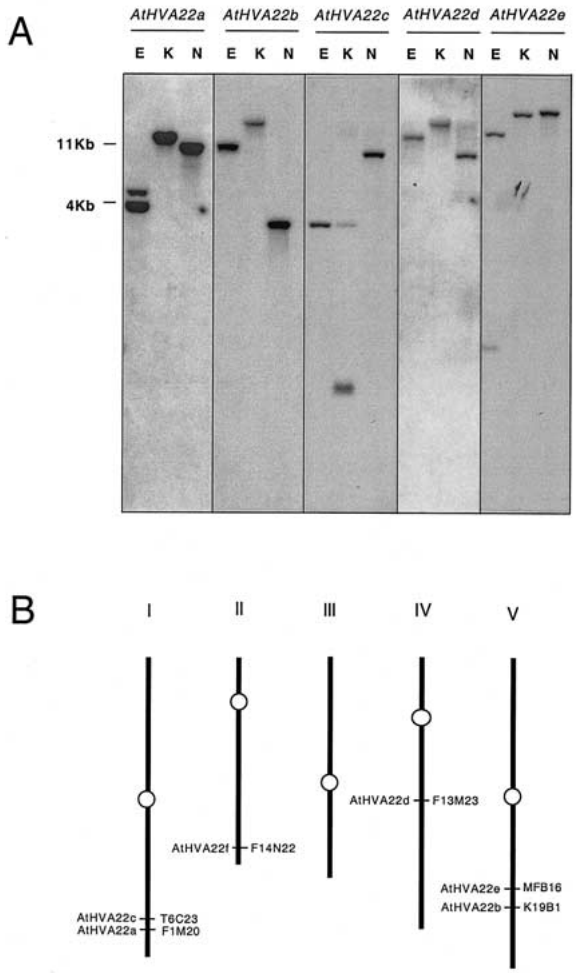

Figure 2. DNA gel blot analysis and distribution of *AtHVA22* genes in the *Arabidopsis* genome. A. Genomic DNA blot analysis indicates that the *Arabidopsis AtHVA22* gene family is composed of five genes. A 5 μg sample of *Arabidopsis thaliana* ecotype Columbia genomic DNA was digested and separated by agarose gel electrophoresis for DNA blot analysis. The membrane was probed at low stringency with labeled cDNA (*AtHVA22a*, *b*, *c* and *e*) or genomic DNA (*AtHVA22d*) separately, as shown on the top of the figure. Note that all the hybridization patterns are different. E, *Eco*RV; K, *Kpn*I; N, *Nco*I. B. Map locations of the *AtHVA22* genes on *Arabidopsis* chromosomes. The positions of *AtHVA22* genes are determined by the positions of the BAC or P1 clones containing these genes. Information about the BAC/P1 clones contigs was obtained from the *Arabidopsis* Information Resources (TAIR) (www.arabidopsis.org). The potential pseudogene, *AtHVA22f*, is also included in this map.

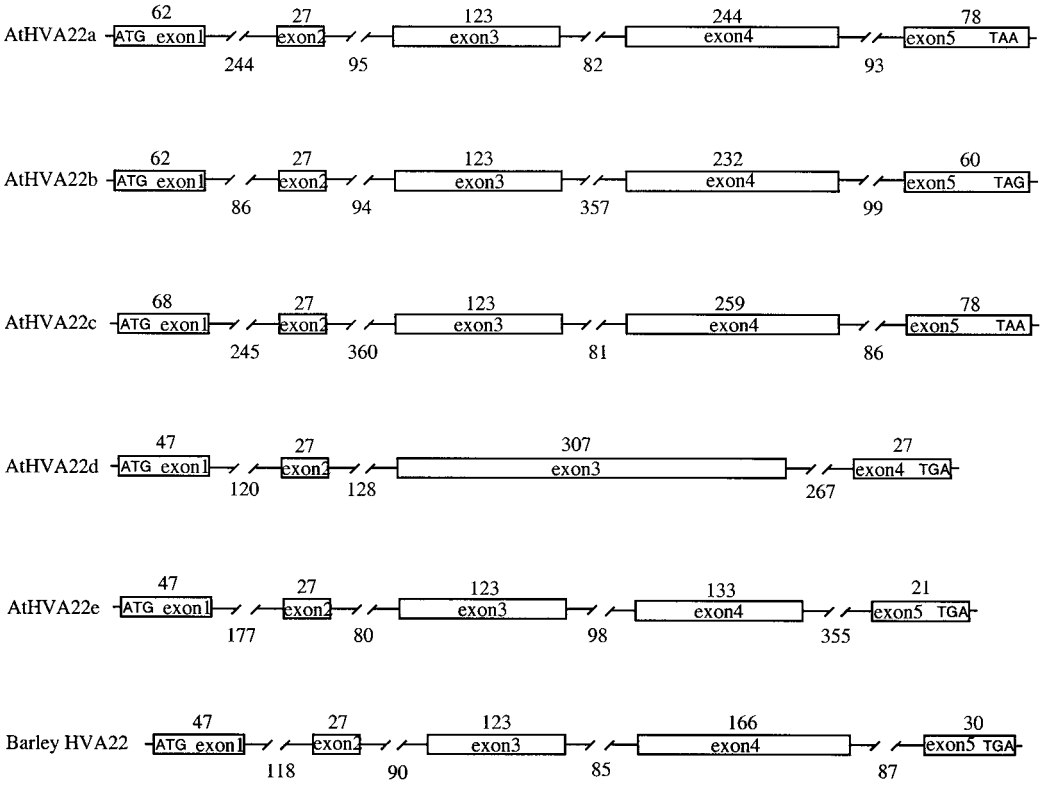

Figure 3. Conservation of gene structures of *AtHVA22a–e* and barley *HVA22*. Sizes of exons and introns of *AtHVA22a–e* and barley *HVA22* genomic DNA from translation start to stop codons are shown. Numbers above the boxes are the base pairs of the corresponding exons. Numbers below the broken lines are the base pairs of the corresponding introns.

exception of *AtHVA22d* which has four exons. The size of the first exon from the translation start is 62 bp for *AtHVA22a* and *b*, 68 bp for *AtHVA22c*, 47 bp for *AtHVA22d* and *e* and barley *HVA22*. The size of the second exon is 27 bp for all of the six *HVA22* homologues. The size of the third exon is 123 bp for *AtHVA22a*, *b*, *c*, *e*, and barley *HVA22*, and 307 bp for *AtHVA22d*, which is likely the result of the fusion of the third and the fourth exons found in other genes. The size of the fourth exon of *AtHVA22a*, *b*, *c* and *e* and barley *HVA22* is also similar, and the similarity also applies to the fifth exon of these genes. The most conserved region in their amino acid alignment corresponds to exons 2 and 3 whose sizes are also the most conserved among the exons of these six genes. The conservation of the distribution and size of exons of the six genes suggests that these genes share a common ancestor.

Phylogenetic relationships of the HVA22 *homologues*

A phylogenetic tree was constructed as shown in Figure 4, which was generated on the basis of parsimony analysis of the 228 aligned DNA bases corresponding to a block of amino acid residues between P29 and R103 of AtHVA22a (Figure 1). Phylogenetic relationships are generally well resolved with monophyly of the *HVA22* homologues from plants and this monophyly receives very good support (bootstrap 99%, decay index 14). Two clades are defined among the plant *HVA22* homologues. Monophyly of the barley *HVA22* and *AtHVA22d* and *e* receives good support (bootstrap 80%, decay index 3) and some support also exists for a clade containing the *AtHVA22a*, *b* and *c* genes (bootstrap 70%, decay index 2). The node among *AtHVA22a*, *b* and *c* is not resolved. The phylogenetic results show that *AtHVA22d* and *e* are closer to barley *HVA22* than to *AtHVA22a*, *b* and *c*. This evidence suggests that the divergence of *AtHVA22* genes occurred before that of monocots and dicots.

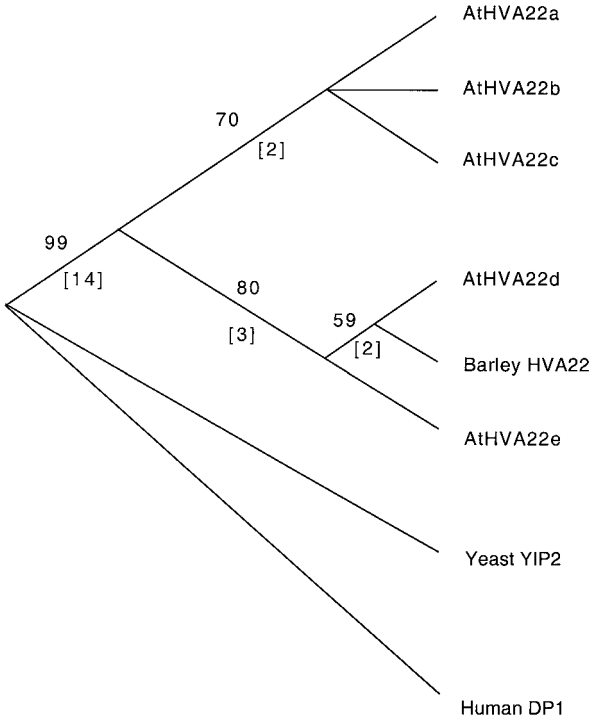

Figure 4. Phylogenetic analysis reveals that *AtHVA22* homologues belong to two subfamilies. Well-aligned cDNA sequences of the eight *HVA22* homologues corresponding to a block in the amino acid sequence alignment, from P29 to R103 of AtHVA22a (Figure 1), were analyzed by the PAUP software with yeast *YIP2* and human *DP1* as outgroups. The single most parsimonious tree was produced from analysis of the 228 aligned DNA sequence positions. The tree has a length of 440 steps. Bootstrap values and decay indices are presented above or below branches, respectively. The results show that *AtHVA22d* and *e* are closer to barley *HVA22* than to *AtHVA22a*, *b* and *c*.

ABA and stress conditions differentially regulate the expression of the AtHVA22 *gene family*

The expression patterns of *AtHVA22* genes in response to ABA and stress treatments were different from each other. RNA gel blot analysis showed that *AtHVA22a*, *c*, *d* and *e* were constitutively expressed at different levels in hydroponically cultured *Arabidopsis* plants. The expression of *AtHVA22a*, *b*, *d* and *e* was enhanced or induced upon ABA treatment, but that of *AtHVA22c* was slightly down-regulated (Figure 5). The expression of *AtHVA22a* and *e* was only slightly enhanced. The expression of *AtHVA22b* was not detectable in the absence of ABA, but induced in 40 min of ABA treatment. The ABA enhancement of the expression of *AtHVA22d* was faster and greater than that of the other genes (*a*, *b* and *e*).

In the cold stress treatment, expression of *AtHVA22a* was slightly enhanced and reached a plateau at 24 h (Figure 5). The expression of *AtHVA22b* was barely detectable throughout the course of the treatment, and this treatment had little effect on the expression of *AtHVA22c*. *AtHVA22d* expression was highly enhanced by cold stress, reaching a peak of transcript level at 8 h, followed by a gradual decline. The expression of *AtHVA22e* showed little change in the first 8 h, but increased afterwards and reached a peak at 24 h.

In the dehydration treatment, the expression of *AtHVA22a* was enhanced slightly and reached a plateau at 3 h (Figure 5). *AtHVA22b* transcripts remained below the limit of detection throughout the treatment. The level of *AtHVA22c* transcripts decreased slightly by dehydration treatment. *AtHVA22d* expression was moderately enhanced throughout the experiment. *AtHVA22e* expression increased gradually and reached a plateau at 3 h.

In the NaCl treatment, the plants lost turgor immediately after the addition of NaCl and remained in that condition for more than 8 h. However, by 24 h the plants had regained their turgor to about the same level as the untreated state. As shown in Figure 5, the expression of *AtHVA22a* increased in 6 min after NaCl treatment, and this increase continued slightly from 6 min to 8 h. By 24 h the *AtHVA22a* transcript level returned to the level of untreated sample, which showed a good correlation with the turgid state of the stressed plants. A peak of expression of *AtHVA22b* was observed at 8 h of the NaCl treatment. *AtHVA22c* expression was suppressed in the first 4 h but increased again at 8 h. The expression of *AtHVA22d* and *e* was induced by NaCl treatment with a peak of transcripts at 2 h and 4 h, respectively. Although the *Arabidopsis* actin gene *ACT2* was believed to be constitutively expressed in vegetative tissue, its expression was surprisingly suppressed by salt stress at 4 h (Figure 5).

AtHVA22b *expression was induced by ABA but suppressed by cold stress*

It is intriguing that the expression of *AtHVA22b* was induced by ABA and salt stress but not by cold and dehydration stresses. Double treatments were performed to further study the effect of ABA and stresses on the expression of *AtHVA22b*. As shown in Figure 6, ABA, but not cold, induced *AtHVA22b* expression in 8 h. *AtHVA22b* transcript level in the ABA + cold double-

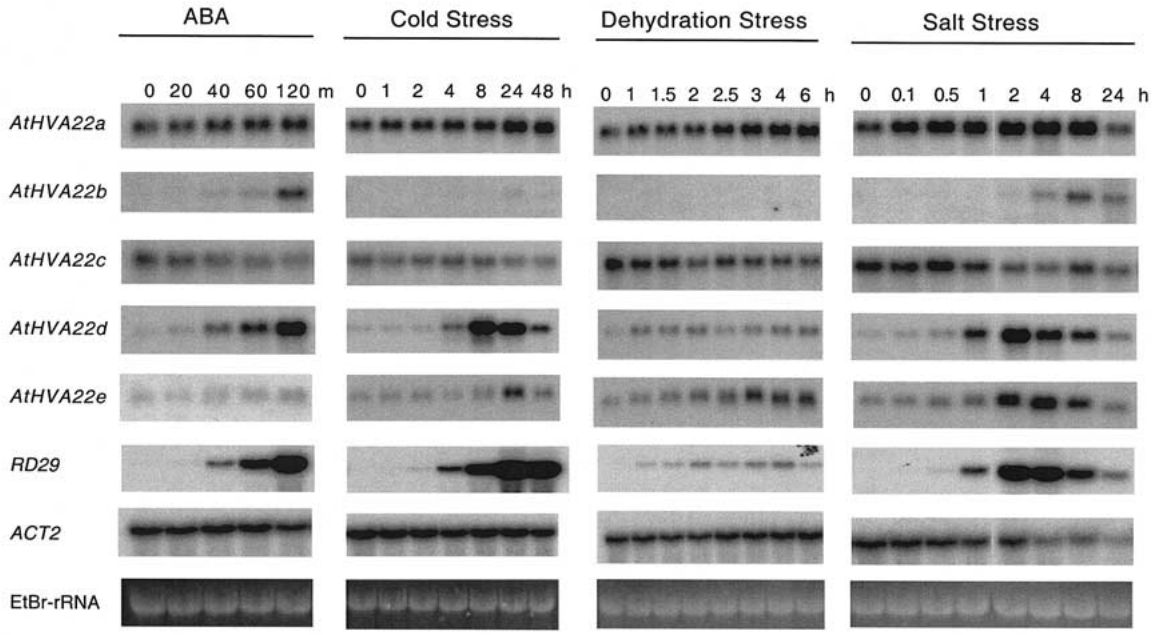

Figure 5. ABA and stresses differentially regulate the expression of *AtHVA22* gene family. Fourteen-day old hydroponically cultured *Arabidopsis* plants were treated with 40 μM ABA, 4 °C, dehydration or 250 mM NaCl, and sampled at times indicated. Total RNA (10 μg) was loaded in each lane. *RD29* was used as a positive control for the ABA treatment. Transcripts of *Arabidopsis* actin 2 gene (*ACT2*) and ethidium bromide-stained rRNA (rRNA-EtBr) were used as loading controls.

treated plants was much lower than in ABA-treated plants. This result showed that cold stress suppressed ABA induction of *AtHVA22b* expression. A different situation was observed in ABA + dehydration double treatments. Although dehydration alone did not induce *AtHVA22b*, its transcript level in double-treated plants was not any lower than that in ABA-treated plants. For the *AtHVA22d* gene, ABA and cold stress apparently had a synergistic effect on its expression. However this effect was not observed in ABA and dehydration double treatment. *ACT2* expression was also suppressed by ABA at 6 h, but not by cold and dehydration.

Expression of the AtHVA22 *gene family is tissue-specifically regulated by drought*

To understand how the expression of *AtHVA22* gene family was regulated in different tissues, total RNA from rosette leaves, cauline leaves, inflorescence stems, flower buds and green immature siliques of soil-grown *Arabidopsis* under normal or drought stress conditions were isolated for RNA gel blot analysis. As shown in Figure 7, *AtHVA22a* transcript level was higher in inflorescence stems and flower buds than in the other tissues in normal and drought stress conditions. The RNA in green immature siliques was degraded in plants subjected to severe drought stress, but the plants were still recoverable if water was provided again. *AtHVA22a* expression was suppressed in drought-stressed rosette and cauline leaves, and inflorescence stems. *AtHVA22b* was expressed at a very low level in normal conditions and slightly enhanced by drought stress in flower buds. In other tissues, transcripts of *AtHVA22b* were barely detectable. Among all tissues analyzed, *AtHVA22c* was expressed at the highest level in flower buds followed by inflorescence stems in normal conditions. Drought stress suppressed *AtHVA22c* expression in rosette and cauline leaves and in inflorescence stems. In normal conditions, *AtHVA22d* was also expressed at the highest level in flower buds followed by inflorescence stems. Drought stress enhanced *AtHVA22d* expression in rosette leaves, cauline leaves, and inflorescence stems, but it had little effect in flower buds. *AtHVA22e* was expressed at a very low level in flower buds, but at the highest level in inflorescence stems. This expression pattern is unique compared to the other homologues. Drought stress also enhanced *AtHVA22e* expression in rosette and cauline leaves but had little effect in influorescence stems. *ACT2* expression was also suppressed in drought stress in the tissues.

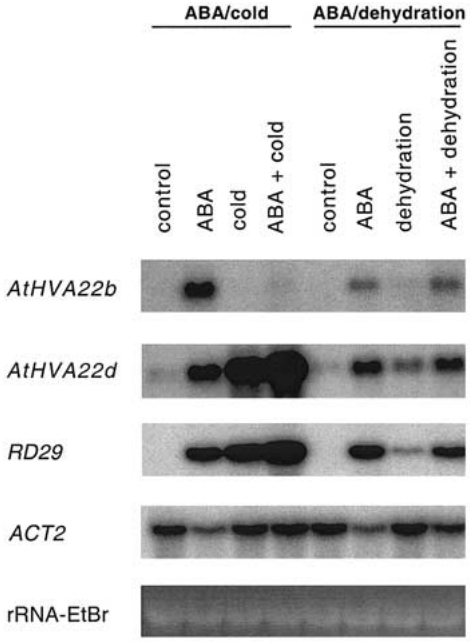

Figure 6. *AtHVA22b* expression is induced by ABA but suppressed by cold stress. Fourteen-day old hydroponically cultured *Arabidopsis* was treated with 40 μM ABA, cold stress, or 40 μM ABA plus cold stress for 8 h on the left panel (ABA/cold); 40 μM ABA, dehydration or 40 μM ABA plus dehydration for 6 h on the right panel (ABA/dehydration). Total RNA (10 μg) was loaded in each lane. *RD29* was used as positive control. Transcripts of *Arabidopsis* actin 2 gene (*ACT2*) and ethidium bromide-stained rRNA (rRNA-EtBr) were used as loading controls.

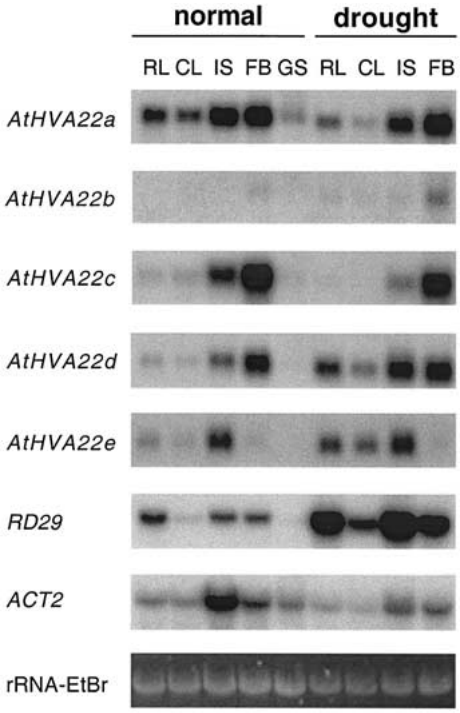

Figure 7. Tissue-specific expression of *AtHVA22* gene family in normal conditions or under drought stress. Tissues of *Arabidopsis* grown in soil at flowering stage were sampled in normal conditions or under severe drought stress. RL, rosette leaves; CL, cauline leaves; IS, inflorescence stems; FB, flower buds; GS, green siliques. Total RNA (10 μg) was loaded in each lane. *RD29* was used as a positive control for the drought stress. Transcripts of *Arabidopsis* actin 2 gene (*ACT2*) and ethidium bromide-stained rRNA (rRNA-EtBr) were used as loading controls.

Discussion

We have characterized a new ABA/stress-regulated gene family consisting of *AtHVA22a*, *b*, *c*, *d* and *e* in *Arabidopsis*, which are homologues of the barley *HVA22*. In a BLAST search we also found a *AtHVA22f* gene located on chromosome II. However, its expression level is beyond detection in any of our RNA samples, including rosette leaves, cauline leaves, inflorescence stems, flower buds, and green siliques. More work is needed to determine whether *AtHVA22f* is indeed a pseudogene.

The conservation of the distribution and size of the exons in the *AtHVA22* and barley *HVA22* genes reflects a close evolutionary relationship. Evidence from phylogenetic analysis (Figure 4), gene structure (Figure 3) and gaps in the amino acid sequence alignment (Figure 1) of the five *AtHVA22* and barley *HVA22* genes leads to the same conclusion, i.e., *AtHVA22d* and *e* are closer to barley *HVA22* than to *AtHVA22a*, *b*, and *c.* Thus, the *Arabidopsis HVA22* homologues appear to belong to two subfamilies. This conclusion implies that separate ancestors of *AtHVA22a*, *b*, and *c*, and *AtHVA22d* and *e* already existed before the divergence of monocots and dicots. The recent report by Blanc *et al.* (2000) has also provided additional support for this two-subfamily classification of *AtHVA22* genes. They showed that large fragments of *Arabidopsis* genomic DNA were duplicated and reshuffled during evolution. *AtHVA22d* is located in the large fragment of F22K18-T13J8 on chromosome 4. According to Blanc *et al.* (2000), the duplicate of F22K18-T13J8 is K2I5-MJB24 on chromosome 5, in which *AtHVA22e* is located. This suggests that *AtHVA22d* and *e* could be duplicates derived from the same ancestral gene. *AtHVA22a* and *c* are both located in the large fragment T1F15-F18B13 on the bottom arm of chromosome 1. However, it is not clear how another member of this putative subfamily, *AtHVA22b*, was translocated to its current location on chromosome 5.

Some stress-inducible gene homologues are known to exist as tandem repeats in the *Arabidopsis* genome, such as *KIN1* and *KIN2* (Kurkela and Borg-Franck, 1992), *RD29a* and *RD29b* (Yamaguchi-Shinozaki and Shinozaki, 1993), and *COR15a* and *COR15b* (Wilhelm and Thomashow, 1993). These tandem-repeated genes appear to be products of gene duplication because the similarities of these gene pairs are very high. All the *AtHVA22* genes, although not tandem-repeated, have similar structures and exon sizes in addition to sequence homologies. However, the expression patterns of these five genes are differentially regulated suggesting that they may play unique physiological roles in different tissues.

Among these homologues, *AtHVA22d* is most tightly regulated by ABA and stresses in vegetative tissues. *AtHVA22a* and *AtHVA22c* are constitutively expressed; however, the expression of *AtHVA22a* is enhanced but that of *AtHVA22c* is suppressed by ABA treatment or stress conditions. The expression pattern of *AtHVA22b* is interesting because it is induced by ABA and salt stress but not by cold and dehydration stresses in vegetative tissues. Further, cold stress suppresses the ABA induction of this gene (Figure 6). Dehydration stress in hydroponically cultured plants and drought stress in soil-grown plants have little or no effect on *AtHVA22b* expression (Figures 5 and 7). Dehydration does not suppress the ABA induction of *AtHVA22b* either (Figure 6). To our knowledge, this is the first report in which ABA and cold stress have antagonistic effects on the regulation of a gene. The significance of this phenomenon is still not clear, but it is conceivable that cold-stressed tissues have elevated levels of ABA, which is needed to regulate processes other than the expression of *AtHVA22.* The ABA induction of *AtHVA22b* expression is also relatively slow when compared to the other ABA-induced genes, such as *RD29* and *AtHVA22d.* It is possible that *AtHVA22b* is insensitive to the ABA-independent signal in dehydration/drought stress (Shinozaki and Yamaguchi-Shinozaki, 1996), and the endogenous level of ABA in stressed tissues is not sufficient to induce *AtHVA22b.* However, further studies are needed to investigate this notion.

The pattern of ABA induction of the *AtHVA22d* gene is quite similar to that of the *RD29* gene (Yamaguchi-Shinozaki and Shinozaki, 1993). Two classes of *cis* elements have been identified in the *RD29a* promoter: ABA-responsive element (ABRE) is responsible for ABA-dependent expression, and drought-responsive element (DRE/C repeat) responsible for ABA-independent drought or cold induction (Yamaguchi-Shinozaki and Shinozaki, 1994). Similarly, three putative ABREs containing the ACGT core and one DRE/C repeat (CCGAC) are found within 300 bp upstream of TATA box in the *AtHVA22d* promoter.

AtHVA22a expression was enhanced by dehydration stress in 14-day old hydroponically cultured *Arabidopsis*, but suppressed by severe drought stress in vegetative tissues at flowering stage. The dehydration stress imposed on the hydroponically cultured *Arabidopsis* was actually mild. At the end of the treatment, the plants did not show any sign of wilting. For the soil-grown plants, the plants showed both wilting and falling of inflorescences at the end of the drought stress. Thus, the expression of *AtHVA22* appears to be enhanced by mild dehydration, but suppressed by severe dehydration.

The level of actin transcripts has been a widely accepted internal control for RNA blot analysis. The *Arabidopsis ACT2* gene is constitutively expressed in vegetative tissues (McDowell *et al.*, 1996). However, the RNA gel blot analyses in this study showed that *ACT2* expression was suppressed by prolonged ABA treatment and under salt stress in vegetative tissues. Severe drought stress also suppressed its expression in both vegetative and reproductive tissues. These results suggest that caution should be exercised when using *ACT2* transcripts as an internal control for RNA gel blot analysis if plants are under stress.

The fact that *AtHVA22a*, *c* and *d* genes are highly expressed in flower buds seems to suggest that they play a role in reproductive growth. Because no *HVA22* homologue is found in prokaryote genomes, we suggest that the function of these genes is related to unique eukaryotic physiology. In addition to the lack of a defined nucleus in prokaryotes, a major difference between eukaryotes and prokaryotes is that the membrane/vesicle trafficking system is present only in eukaryotes. Preliminary observations have shown that the *HVA22* homologue in yeast is indeed involved in vesicle transport (A.B. Brands and T.H.D. Ho, unpublished data), which may be needed to facilitate membrane turnover in coping with stress conditions. More work is currently underway to address the function of these genes in *Arabidopsis.*

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