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Isolation and Characterization of *Panax ginseng* 14-3-3 Gene Family

Hwa-Jee Chung[1,3], Joo Young Park[3], In Sook Cho[1], Cheol Goo Hur[2], Dong-Woog Choi[1], and Jang Ryol Liu[3]*

[1]Laboratory of Functional Genomics for Plant Secondary Metabolism, Eugentech Inc., Daejeon 305-333, Korea
[2]National Center for Genome Information, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea
[3]Plant Cell Biotechnology Laboratory, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea

14-3-3 proteins are a family of conserved proteins expressed in all eukaryotes. They function as regulators in signaling pathways through protein-protein interaction. These proteins have been implicated in plant metabolism, development, and responses to abiotic and biotic stresses. Here, we isolated six cDNAs encoding 14-3-3 proteins of *Panax ginseng*, designated as *Pg14-3-3*, by searching the ginseng EST database. Their degree of amino acid identity ranged from 53 to 84%. Phylogenetic analysis showed that the *Pg14-3-3* isoforms can be divided into two groups by early duplication, with one group being further split into three subgroups because of subsequent duplication events. *Pg14-3-3s* are differentially expressed in various root tissues and leaves, and their transcript levels are up- or down-regulated by either cold or drought stresses. These results suggest a possible role for Pg14-3-3 proteins in abiotic stress responses.

Keywords: 14-3-3 protein, abiotic stress, *Panax ginseng*, phylogenetic analysis

The 14-3-3 proteins were first characterized as 30-kDa, acidic, soluble proteins that are present in mammalian brain tissue (Moore and Perez, 1967). Since then, researchers have identified seven isoforms in mammals and two in a couple of yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Wang and Shakes, 1996). Furthermore, recent completion of the *Arabidopsis* genome has revealed 15 such genes in that plant system (DeLille et al., 2001; Rosenquist et al., 2001). Phylogenic analysis based on amino acid sequence data categorizes the *Arabidopsis 14-3-3* family members into two major groups -- epsilon and non-epsilon--which are then split into two and three subgroups, respectively (DeLille et al., 2001). A number of *14-3-3* members have been isolated from other plant species as well (Rosenquist et al., 2000; Zuk et al., 2003).

Expression of the 14-3-3 proteins is apparently tissue- and organelle-specific. For example, the *Arabidopsis GF14 iota* gene is expressed only in flowers, whereas transcript of *GF14 chi* can be detected in petals, sepals, and siliques, but not in leaves or cotyledons (Daugherty et al., 1996; Rosenquist et al., 2001). Three isoforms of the barley *14-3-3* gene are expressed in embryos, with transcript levels being differentially regulated during germination (Testerink et al., 1999). These 14-3-3 proteins have also been reported in the nucleus, mitochondrial matrix, and chloroplasts (Bihn et al., 1997; Sehnke et al., 2000; Bunney et al., 2001). Such observations support the theory that plant 14-3-3 proteins may help regulate diverse biological processes, including development and stress responses, by interacting with partner proteins in different tissues or organelles (Roberts et al., 2002).

Expressed sequence tags (ESTs), which are unedited and single-pass sequence collections, have been used to identify genes involved in reproductive organ development in watermelon, anther development in tobacco, cellulose biosynthesis in cotton, as well as wood and xylem formation in woody species (Pear et al., 1996; Allona et al., 1998; Sterky et al., 1998; Choi and Hong, 2000; Kim et al., 2001). Our laboratory previously generated 12,000 ESTs from various ginseng tissue cDNA libraries, and we have now identified over 6,000 unique expressed sequences (Jung et al., 2003). Here, we report the isolation of ginseng *14-3-3* gene family members and describe the characterization of their tissue-specific expression and means for induction.

*Corresponding author; fax +82-42-860-4608
e-mail jrliu@kribb.re.kr

MATERIALS AND METHODS

Plant Materials and Hormone Treatments

Primary and lateral roots, rhizomes, and leaves were harvested from soil-grown, four-year-old ginseng (*P. ginseng*) plants. In addition, two-week-old seedlings were cultured in the laboratory. To produce the latter, seeds were surface-sterilized with a 20% (v/v) household bleach solution and rinsed with sterilized water. They were then placed on MS basal media in Petri dishes, and grown in the dark. Afterward, the seedlings were transferred to an MS solution containing one of the following: 10 μM methyl jasmonate (MeJA), 100 μM salicylic acid (SA), 50 μM abscisic acid (ABA), or 100 mM NH_4NO_3, and then incubated for 24 h. For the drought treatment, seedlings were left in the open air on a clean laboratory bench until the MS solution had evaporated to 30% of the initial volume. The cold treatment involved holding the seedlings at 4°C under lights for 24 h.

Cloning of *P. ginseng* 14-3-3 cDNAs

Because EST sequence information had been obtained via single-pass sequencing from the 5-end of each clone, *Pg14-3-3b*, *Pg14-3-3c*, and *Pg14-3-3d* were isolated by performing PCR, using T7 and T3 primers and with each EST clone serving as template. For partial clones, 5 RACE-PCR was conducted according to manufacturers instructions for the 5 RACE System (Clontech, USA). The sequences for our gene-specific primers were: *Pg14-3-3a* GSP1, 5-ATC ACC TTT CAT CTT TAG AT-3; *Pg14-3-3a* GSP2, 5-GTA GTC GCG GAT AGT GGC CAC GTG T-3; *Pg14-3-3b* GSP1, 5-ACT CAG CCA AAT AAC GAT GAT-3; *Pg14-3-3b* GSP2, 5-GTC CCC AGC CGA AGC CGA AGG AAT-3; *Pg14-3-3f* GSP1, 5-GCA AG TTA CAA GCA CGA TCA-3; and *Pg14-3-3f* GSP2, 5-CTG GGC TGC CTT ATA GGC GTT GA-3. The resulting PCR products were cloned into pCR4 Blunt-TOPO (Invitrogen, USA) and sequenced.

Quantitative Reverse Transcription-PCR Analysis

Total RNA was extracted from various plant tissues with a TRIZOL, and first-strand cDNA was synthesized with a random hexamer and M-MuLV reverse transcriptase (Finnzymes, USA) at 42°C for 1 h. PCR was conducted with gene-specific primers and Quantum RNA 18S Internal Standards (Ambion, USA), following the manufacturer's protocol. The ratio of 18S rRNA primer to competimer mix was 2:8. Amplified bands, stained with ethidium bromide, were quantified with BIO-CAPT software, and the amount of mRNA was represented as relative intensity. Each amplification included 3 min of pre-denaturation at 95°C, followed by varying cycles of denaturation at 95°C for 30 s, annealing for 30 s, and extension at 72°C for 30 s. The number of cycles was 29 for *Pg14-3-3a* and *Pg14-3-3-c*, and 27 for the rest of the *Pg14-3-3* genes. Annealing temperatures for *Pg14-3-3a*, *Pg14-3-3c*, *Pg14-3-3e*, and *Pg14-3-3f* were 55°C, 60°C for *Pg14-3-3d*, and 62°C for *Pg14-3-3b*. Sequences for the gene-specific primers included: *Pg14-3-3a*, 5-CCA AGC CTG ACA ATG AAT AGT GAA GT-3 and 5-AAA ACT ACA ATG AAG AAG ATA CTG AAA A-3; *Pg14-3-3b*, 5-CAC GAC GGG GCA GAT GAG ATT AAG G-3 and 5-CCA AAA GAA AGG GCA ATG AAA TCA AAG A; *Pg14-3-3c*, 5-ATG CAG GAG CAG ATG GAT GAA G-3 and 5-TAC AGA TAA CAC TAA CTT TAG AA-3; *Pg14-3-3d*, 5-ATG ATT TGT AAG CTG AAC TGC A-3 and 5-TTG GGG GTA CTA CAT CCT CTT GA-3; *Pg14-3-3e*, 5-GCA GAG TAA GTG GAG TGG AAG TC-3 and 5-AGA ATT AAA CGT GGA AAA AAG GAT-3; and finally, *Pg14-3-3f*, 5-GCT GTT TTG ATT GAA GAA GTT GCT-3 and 5-TTT TTG GTG GTG AAG GCT ATA TTT T-3.

Sequence Alignment and Phylogenetic Analysis

We used ClustalX with default gap penalties to perform multiple alignment of our *Pg14-3-3* genes as well as others in the plant 14-3-3 family. Based on this alignment, a phylogenetic tree was constructed according to the neighbor-joining method, using the PhyloDraw and MEGA2 programs (Choi et al., 2000; Kumar et al., 2001).

RESULTS

Isolation of *P. ginseng 14-3-3* genes

Searching the ginseng EST database enabled us to obtain the 5-end sequences of six *14-3-3* gene homologs. Three of these were full-length clones; the rest, partials. To isolate full-length cDNAs of those partial clones, we performed 5RACE-PCR using gene-specific primers that had been designed based on sequence information for the partial clones. The *14-3-3* cDNAs were designated as *Pg14-3-3* (*P. ginseng* 14-3-3).

Pg14-3-3 genes encode proteins of 251 to 276

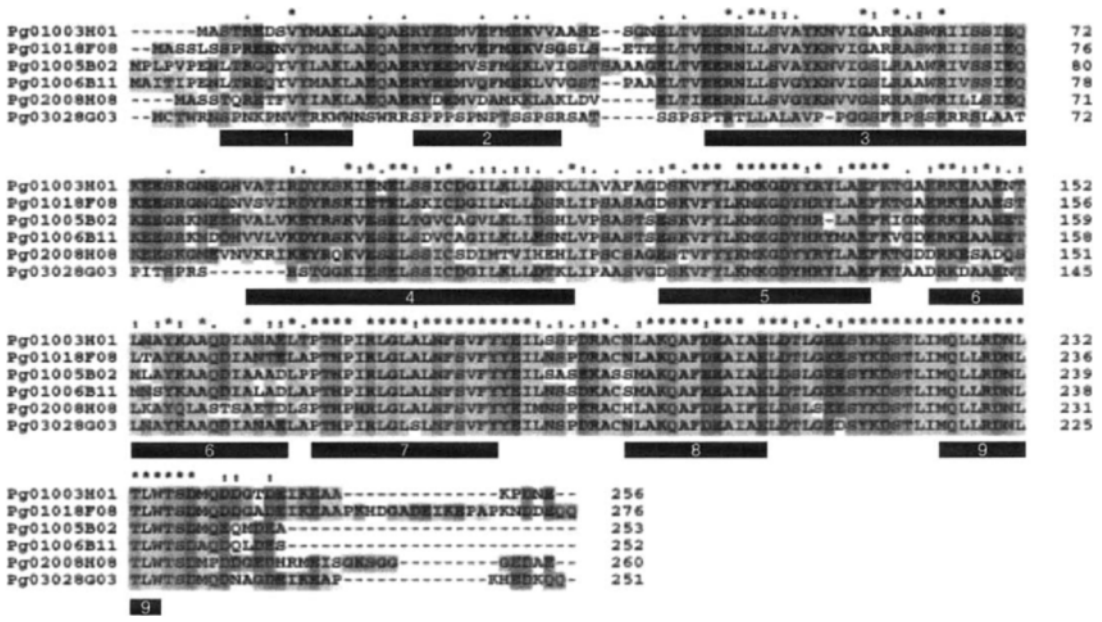

Figure 1. Amino acid alignment of *P. ginseng 14-3-3* isoforms. Black boxes with number inside highlight nine conserved alpha helical regions.

amino acid residues, and show high sequence similarity (Fig. 1). As with other 14-3-3 proteins identified from various plant species, their middle region is conserved while the 5 and 3 ends exhibit sequence diversity. Nine alpha helical regions, which are involved in stabilizing monomer and dimer formation, or other functions, are also conserved, although the first three helices of Pg14-3-3f are less so.

Phylogenetic Relationship of Other Plant *14-3-3* Genes

We first compared the phylogenetic relationships of the *Pg14-3-3* genes with those of the *Arabidopsis 14-3-3* genes (Fig. 2). Two isoforms of the latter--*grf14* and *grf15*--were excluded in that analysis because they were truncated. Based on the unrooted phylogenetic tree, only *Pg14-3-3e* was closely related to *GF14 omicron*, a member of the epsilon group. The remaining *Pg14-3-3* genes could be segregated into three classes within the non-epsilon group: 1) *Pg14-3-3a* and *Pg14-3-3b*, in the *GF14 omega* subgroup: 2) *Pg14-3-3c* and *Pg14-3-3d*, in the *GF14 kappa* subgroup; and 3) *Pg14-3-3f*, placed between the *GF14 omega* and *GF14 psi* subgroups.

To determine the evolutionary relationships among plant *14-3-3* genes (Fig. 3), we used the neighbor-joining (NJ) method to investigate the sequences of 63 *14-3-3* gene family members from other plant species, including two truncated *Arabidopsis* isoforms.

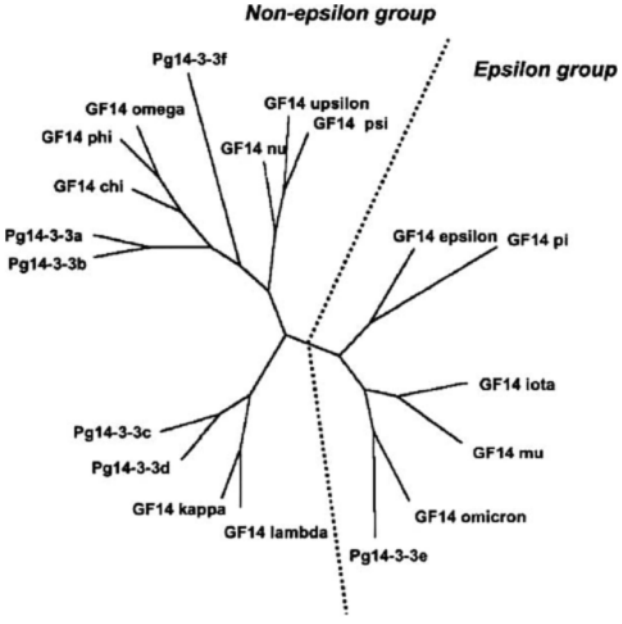

Figure 2. Unrooted phylogenetic analysis of Pg14-3-3 and *Arabidopsis* 14-3-3 gene families. Neighbor-joining tree was obtained from alignment using ClustalX and PhyloDraw programs. Two isoforms of *Arabidopsis*, *grf14* and *grf15*, are not included in analysis. Tree branches are proportional to genetic distances.

This NJ analysis, with bootstrap support, was unable to place *Arabidopsis grf15*, perhaps because it was badly truncated. The rooted phylogenetic tree revealed an early duplication event for the *Pg14-3-3f* isoform in the plant lineage. Furthermore, two isoforms--

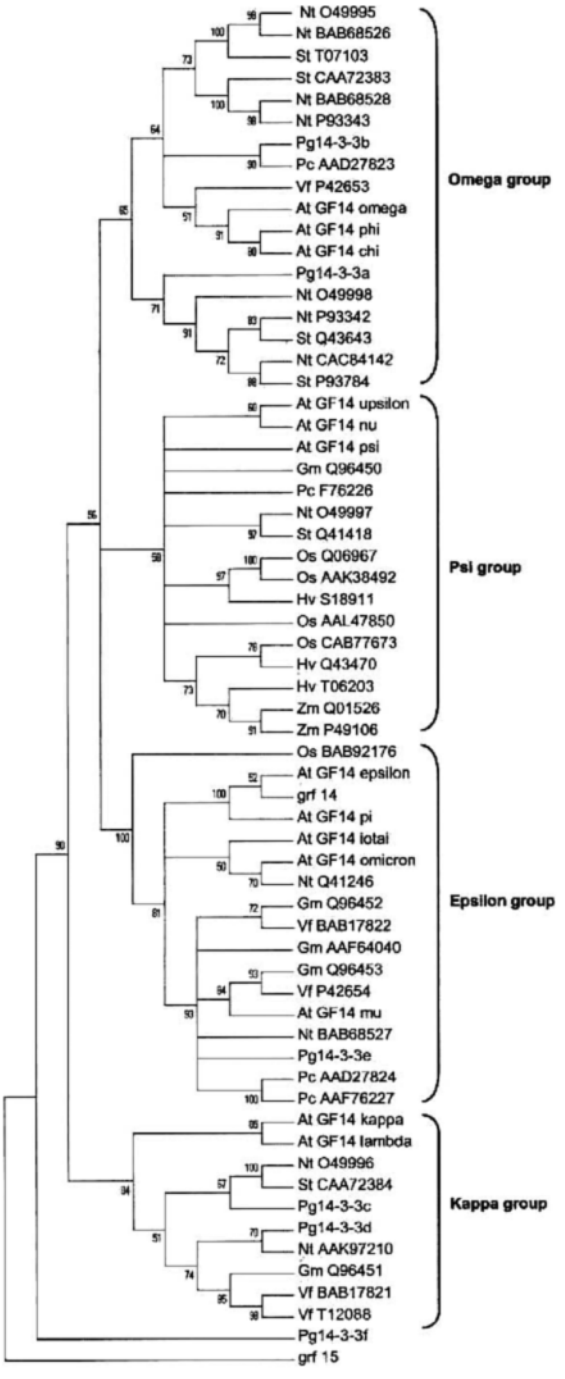

Figure 3. Phylogenetic relationship of *Pg14-3-3* isoforms with plant 14-3-3 family. Sequences were downloaded from GenBank and aligned with ClustalX; tree was produced using MEGA2 program. Bootstrap value for each cluster is shown at nodes. Isoforms are labeled by accession number, using abbreviations for plant species: Nt, tobacco (*Nicotiana tabacum*); St, potato (*Solanum tuberosum*); Pg, ginseng (*P. ginseng*); Pc, hybrid poplar (*Populus x canescens*); Vf, bean (*Vicia faba*); Gm, soybean (*Glycine max*); Zm, maize (*Zea mays*); Hv, barley (*Hordeum vulgare*); Os, rice (*Oryza sativa*); At, *Arabidopsis* (*Arabidopsis thaliana*).

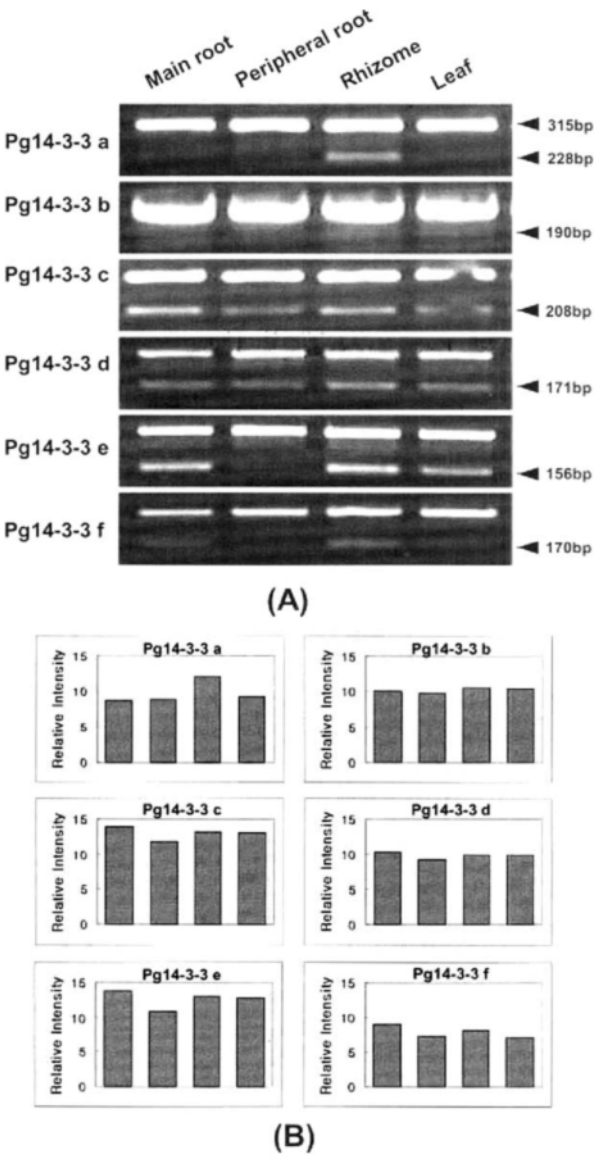

Figure 4. Tissue expression patterns of six *Pg14-3-3* isoforms. **A,** Profiles from RT-PCR, as performed with gene-specific primers and Quantum RNA 18S Internal Standards (Ambion). Total RNA used for RT-PCR was 50 ng for *Pg14-3-3a*, *Pg14-3-3b*, and *Pg14-3-3c*; 25 ng for *Pg14-3-3e* and *Pg14-3-3f*; and 15 ng for *Pg14-3-3d*. Ratio of 18S rRNA primer to competimer mix was 2:8. Gene-specific PCR products are indicated by arrow head below 315 bp on the 18S rRNA band. **B,** Relative transcript levels. Amplified bands stained with ethidium bromide were quantified with BIO-CAPT software; amount of mRNA is represented as relative intensity. Similar expression patterns were obtained in at least three independent experiments.

Pg14-3-3a and *Pg14-3-3b*--could be split into two classes within the GF14 *omega* group. In particular, *Pg14-3-3b* was clustered with the *Populus x canescens*

14-3-3 isoform. Finally, *Pg14-3-3d*, which belonged to the GF14 *kappa* group, was separated from *Pg14-3-3c* and clustered with a tobacco isoform.

Tissue Expression Patterns of *Pg14-3-3* Genes

To study the physiological role of Pg14-3-3s, we conducted reverse transcription-PCR (RT-PCR) to obtain a semi-quantitative assessment of transcript levels in order to evaluate the tissue-expression patterns of our *Pg14-3-3* genes (Fig. 4). To do this analysis, we used gene-specific primers based on the 3'-UTR of each isoform, due to high sequence identity in the coding regions. By swapping their primers, specificity of the primer sets for the six *Pg14-3-3* genes could be verified by PCR. None showed any cross-reactivity here.

Transcripts of both *Pg14-3-3c* and *Pg14-3-3d* were abundant and found at similar levels in all tissue types. However, the latter appeared to have higher expression because it required fewer PCR cycles and three-fold less total RNA. In contrast, the transcript level of *Pg14-3-3b* was barely detectable in any tissue. *Pg14-3-3a* was expressed only in the rhizomes, whereas a low transcript level for *Pg14-3-3f* was detected only in the main roots and rhizomes. Finally, *Pg14-3-3e* was expressed in the main roots, rhizomes, leaves, and total seedlings, but at only much lower levels in the lateral roots.

Stress and Hormone Induction of *Pg14-3-3* Genes in Seedlings

To investigate if the *Pg14-3-3* genes are involved in biotic or abiotic stress responses, we treated *in vitro*-cultured seedlings with various stresses or hormones (Fig. 5). Overall, the transcript amounts of *Pg14-3-3a, Pg14-3-3b,* and *Pg14-3-3d* were unchanged, whereas those of *Pg14-3-3c, Pg14-3-3e,* and *Pg14-3-3f* had varying degrees of fluctuation in their expression levels after exposure to drought or cold stresses. For example, *Pg14-3-3a* showed no difference in its abundant mRNA expression level for either the controls or the treated seedlings. *Pg14-3-3b* also was not induced by stress regardless of tissue type (Fig. 4). In response to cold stress, the transcript level of *Pg14-3-3e* was reduced to 20%, while that of *Pg14-3-3f* showed only slight induction under such treatment. Drought stress reduced expression of the *Pg14-3-3c* isoform to 25%. Finally, ABA, MeJA, and SA also seemed to change the expression levels of our *Pg14-3-3* genes, although the degree of induction or reduction was negligible.

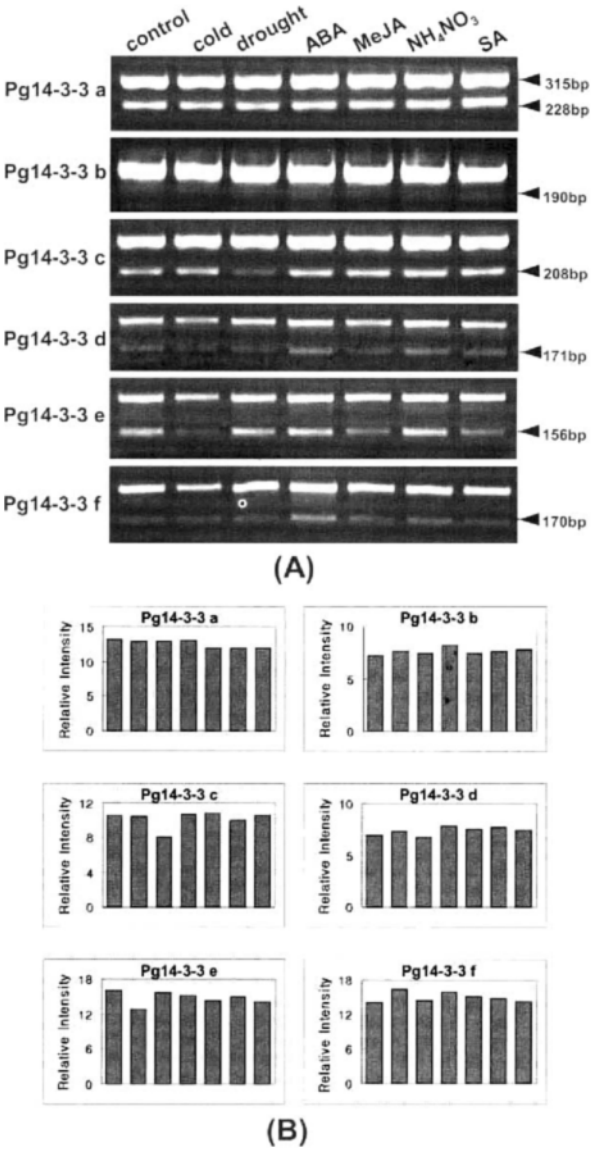

Figure 5. Stress induction of six Pg14-3-3 isoforms. **A,** RT-PCR profiles for seedlings treated with cold, drought, ABA, MeJA, SA, or NH_4NO_3. **B,** Changes in transcript level were calculated using BIO-CAPT software and are represented as relative intensity. Similar expression patterns were obtained in at least three independent experiments.

We observed similar results with ammonium-treated seedlings. Overall, differences in stress-induced expression were noticeable among all *Pg14-3-3* isoforms.

DISCUSSION

In this study, we searched the ginseng EST database to identify six 14-3-3 genes from *P. ginseng*. Members of this gene family share highly conserved amino acid

sequences, with homologies ranging from 53 to 84%. These values are similar to those reported with other plant species (Wu et al., 1997; Piotrowski and Oecking, 1998; Zuk et al., 2003). Our phylogenetic analysis indicated that an early divergence event in the *Pg14-3-3* isoforms resulted in the existence of a single *Pg14-3-3f* isoform, and that the rest of the *Pg14-3-3* isoforms originated from a common ancestor gene.

RNA analysis of 14-3-3 isoforms identified from *Arabidopsis* and *V. faba* has shown that some isoforms are expressed in a tissue-specific manner (Sehnke et al., 2002). Likewise, our RT-PCR results (Fig. 4) clearly demonstrated that *Pg14-3-3* isoforms are differentially expressed. For example, the *Pg14-3-3c* and *Pg14-3-3d* isoforms, clustered together with *GF14 kappa* and *GF14 lambda*, are ubiquitously expressed in the roots, rhizomes, and leaves. This contrasts with transcript levels for *Pg14-3-3b*, which are nearly undetectable in all tested tissues. *Pg14-3-3a* also is expressed only in the rhizome, i.e., the portion of the roots on which a new terminal bud is initiated during mid-summer. This bud eventually produces new stems and flowers. In tomato, the over-expression of 14-3-3 proteins compensates for the loss of function of the *SP* gene that controls inflorescence architecture, probably through direct interaction with SP and SPAK (Pnueli et al., 2001). Based on all these results, we suggest that *Pg14-3-3a* may have a possible role in the development of new organs-- stem, floral, or both--in the rhizomes. Further investigation is necessary to determine any sub-organelle specificity among the *Pg14-3-3* isoforms that show similar tissue-expression patterns.

14-3-3 proteins participate in a wide range of signaling pathways, including stress responses, via direct protein-protein interactions with specific partners (Roberts, 2003). *Pg14-3-3c* seems to be involved in the drought response, whereas *Pg14-3-3e* and *Pg14-3-3f* are induced by low temperatures. In particular, *Pg14-3-3e* transcript is somewhat down-regulated under cold stress, in contrast to *Pg14-3-3f* and two isoforms from *Arabidopsis* that are up-regulated under such conditions (Jarillo et al., 1994). Furthermore, the role of *Pg14-3-3e* in the cold response appears to be associated with ABA- and water deficit-independent pathways. Here, we are the first to demonstrate that transcript of *Pg14-3-3c* is also down-regulated by water deficit, although researchers have previously reported a few other genes whose expression is down-regulated by water deficit, e.g., those that encode cell wall-localization proteins or plasma membrane-intrinsic proteins (Yu et al., 1996; Harrak et al., 1999; Smart et al., 2001).

Unfortunately, only a limited number of partners of 14-3-3 proteins have been identified that have possible roles in abiotic and biotic stress responses (Roberts et al., 2002). For example, the H^+-ATPase genes in plants might be activated by various abiotic and biotic environmental factors and hormones. This enzymatic activity is modulated through interaction with the 14-3-3 protein (Palmgren, 2001; Bunney et al., 2002). Reported changes in levels of those proteins because of cold or drought imply that Pg14-3-3s participate in a protective system against adverse environments that occur during the long period required for ginseng cultivation. Therefore, a biological role for these proteins in abiotic stress responses will be further elucidated when the potential partners for each isoform are identified.

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