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Molecular Cloning and Expression Pattern Analyses of Heat Shock Protein 70 Genes from *Nicotiana tabacum*

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We have isolated three genomic clones that code tobacco HSP70s, using a tobacco hsp70 cDNA clone as a probe. *NtHSP70-1*, *NtHSP70-2*, and *NtHSP70-3* contain full open reading frames of 653, 653, and 648 amino acid residues, respectively. All share three conserved regions, namely the C-terminal substrate binding domain, oligomerization domain, and N-terminal ATPase domain. In a comparison of their amino acid sequences, *NtHSP70-1* and *NtHSP70-2* were very similar to each other, while *NtHSP70-3* showed significant differences, instead being highly homologous to the cytosolic HSP70 members of *Arabidopsis thaliana* and other plant species. Therefore, *NtHSP70-1* and *NtHSP70-3* were chosen for further analyses. RNA blot hybridizations showed typical heat shock-responsive expression patterns, although their signal intensities differed significantly. Transcription of *NtHSP70-1* was also induced by dehydration stress and hormone treatments, such as BA, GA, and IAA, but that of *NtHSP70-3* was not.

Keywords: abiotic stress, expression pattern, genomic clones, heat shock protein 70, hormone response, *Nicotiana tabacum*

Because plants lack the capability of locomotion, they must possess strong mechanisms for responding and adapting to various environmental stresses. One important mechanism responsible for the extra strong ability of plants to cope with environmental stresses has been cited from the heat shock response that accompanies the synthesis of heat shock proteins (HSPs), especially under high temperatures (Vierling, 1991; Queitsch et al., 2000; Park and Hong, 2001; Sun et al., 2002; Sung et al., 2003; Sanmiya et al., 2004). HSPs are grouped into five distinct classes based on molecular mass; their functions are most aptly regarded as molecular chaperones (Buchanan et al., 2000). Among these, the HSP70 family (i.e., proteins of ~70 kD) is active in a wide array of processes, from nascent protein synthesis to the protection of proteins during abiotic-stress exposure and developmental programs (Dix, 1997; Krebs and Feder, 1997; Luft and Dix, 1999; Lee and Vierling, 2000; Efremova et al., 2002)

Members of the HSP70 family have been localized to various cellular compartments, including the cytosol, mitochondria, chloroplast, and endoplasmic reticulum. Cytosolic members include the heat-inducible HSP70 and the constitutively expressed heat shock cognate 70 (HSC70), both of which play key roles in the molecular chaperone machinery. HSP70 can prevent the irreversible aggregation of denaturing proteins by binding to their hydrophobic portions, which normally are not exposed in native conformations. The binding and release of nonnative peptides from HSP70 are ATP-dependent; nucleotide- and peptide-binding domains have been localized at the ~27-kD carboxy-terminal portion. Therefore, denatured proteins can be maintained in a form that may be subsequently refolded to a native conformation through a process that usually requires ATP and co-chaperones (Bukau and Horwich, 1998; Mayer et al., 2001; Caplan, 2003).

Because most cellular proteins are likely to require protection by molecular chaperones sometime during their life span, diverse interactions between HSP70s and cellular proteins are expected. In most organisms, heat shock-inducible HSP70 and constitutively expressed HSC70 are encoded by gene families (DeRocher and Vierling, 1995; Guy and Li, 1998; Luft and Dix, 1999; Sung et al., 2001). The diversity of those HSP70 members may be a significant basis for the various interactions between HSP70 and the protected proteins.

To better understand the diversity of these plant HSP70s, we investigated the nucleotide sequences of three genomic clones that code HSP70s from tobacco. Our objective was to examine the similarities and dif-

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ferences in structure and expression patterns among these clones.

MATERIALS AND METHODS

Plant Material, Bacterial Strain, and Chemicals

Tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) plants were raised in a growth chamber under a 16-h photoperiod, at 24-25°C, 60% humidity, and 200 μE m^{-2} s^{-1} irradiance from white fluorescent lamps. *Escherichia coli* strain DH5α was used for the recombinant DNA process, while *E. coli* strain KW251 was used to host bacteriophage λ. DNA modifying enzymes, Taq DNA polymerase, and DNA ligase were obtained from Promega (USA); other materials were from Sigma (USA) or as otherwise mentioned.

Abiotic Stress and Hormone Treatments

Young plants with 4 to 6 leaves were chosen for treatments involving high temperatures, dehydration, or hormones. To induce heat stress, plants were exposed for 1 or 2 h at 40, 44, or 48°C. Relative humidity in the incubator was maintained at >90%, as measured with a hygrometer (UEI, Korea). For the dehydration treatment, plants were subjected to progressive drought by withholding water for 1, 2, 3, or 4 weeks. In the hormonal experiments, abscisic acid (ABA), gibberellic acid (GA_3), and indoleacetic acid (IAA) were each dissolved in ethanol to 100 mM, whereas benzyl aminopurine (BA) was dissolved in 1 N NaOH to 100 mM. After the hormone solutions were adjusted to pH 5.7 with 1.0 M KOH, they were added to MS media (Murashige and Skoog, 1962), at concentrations of 10 μM, 100 μM, or 1 mM, before being applied to the plants.

Screening of the Genomic Library

The tobacco genomic library in the EMBL3 arms (Promega) was plated on an *E. coli* lawn, as described by Sambrook et al. (1989). Approximately 1×10^5 plaques formed on a 23 cm × 23 cm LB bottom agar plate and were blotted onto a Hybond-N membrane (Amersham, USA) for primary screening. This membrane was prehybridized and hybridized in 5X SSPE, 5X Denhardt's solution, 1% SDS, and 100 μg ml^{-1} denatured salmon sperm DNA at 65°C. For the hybridization, a tobacco cDNA clone coding HSP70 that was isolated from EST analysis (Choi and Hong, 2000) was labeled with α^{32}P-dCTP, using the Prime-a-Gene system (Promega). After washing in 0.5X SSPE and 0.1% SDS at 65°C, the membrane was exposed to X-ray film (Kodak, USA) with two intensifying screens (Dupont, USA) at -75°C (Sambrook et al., 1989). For the secondary screening, plaques at the positive signal in the primary screening were picked and plated out to form well-separated plaques. Prehybridization and hybridization of this blot were carried out as described for the primary screening.

Nucleotide Sequencing and Analysis

Insert of the genomic clone was subcloned into pBluescript II SK(+) (Promega), and a set of unidirectional deletion series was obtained by exonuclease III, using a deletion kit for kilo-sequencing (Takara, Japan). Nucleotide sequencing reactions were performed via Sequenase Version 2.0 (United States Biochemical, USA), based on the dideoxy nucleotide chain termination sequencing method (Sanger et al., 1977). The nucleotide and deduced amino acid sequences were searched for homologous genes and proteins from the current GenBank and Swiss-Prot databases.

RNA Extraction and Blot Hybridization

RNA was extracted from 1 g of tobacco leaves that had been ground with a pestle to fine powder in liquid nitrogen. The powder was transferred to a 40-ml polypropylene tube, and 2 ml of lysis buffer (25 mM Tris-Cl pH 8.0, 50 mM LiCl, 35 mM EDTA, 35 mM EGTA, and 0.5% SDS) was added. After vortexing, 2 ml of phenol was added, and the homogenate was vortexed or shaken vigorously. One ml of chloroform was then added to the homogenate, followed by vortexing and centrifuging at 6000*g* for 15 min at 20°C. Afterward, the supernatant was transferred to a 1.5-ml microcentrifuge tube, and an equal volume of 4 M LiCl was added. The mixture was then held at -20°C for over 3 h, and RNA was collected by centrifugation for 15 min at 4°C in a microcentrifuge at 15,000 rpm. After being washed with 2 M LiCl and then 70% ethanol, the RNA pellet was dissolved in DEPC-treated distilled water. RNA was size-fractioned on an 1% agarose gel with formaldehyde, and blotted onto a nylon membrane (Amersham). The membrane was then prehybridized for 1 h and hybridized for 16 to 22 h in a solution containing 0.5 M sodium phosphate (pH 7.2), 7% (w/v) SDS, and 1 mM EDTA (pH 7.0) at 65°C. α^{32}P-dCTP randomly labeled DNA

probes (the ORF of *NtHSP70-1* or *NtHSP70-3*) was used for the hybridization. The membrane was washed sequentially in 1X SSPE and 0.1% (w/v) SDS at room temperature (RT) for 10 min, 1X SSPE and 0.1% SDS at 65°C for 15 min, and twice in 0.2X SSPE and 0.1% SDS at 65°C for 15 min each before being exposed to X-ray film, as above (Sambrook et al., 1989).

Genomic DNA Isolation and Blot Hybridization

Genomic DNA was extracted as described by Junghans and Metzlaff (1990). Briefly, tobacco leaves were ground in liquid nitrogen and incubated at RT for 15 min in lysis buffer (50 mM Tris-HCl pH 7.6, 100 mM NaCl, 50 mM EDTA, 0.5% SDS, and 10 mM β-mercaptoethanol). To the lysate, 1/3 volume of 1:1 phenol:chloroform was added, mixed, and centrifuged for 5 min at RT in a microcentrifuge at 15,000 rpm. The aqueous phase was then collected, and an equal volume of isopropanol was added. Genomic DNA was precipitated by centrifugation for 15 min in a microcentrifuge at 15,000 rpm. Restriction-digested genomic DNA was subjected to electrophoresis on a 0.7% agarose gel, and blotted onto a nylon membrane. A α^{32}P-dCTP randomly labeled DNA probe (the ORF of *NtHSP70-1*) was used for the hybridization. DNA blot hybridization was carried out according to the procedure described above for the primary screening of the genomic library.

RESULTS

Nucleotide Sequences and Deduced Amino Acid Sequences of Tobacco HSP70 Genomic Clones, *NtHSP70-1*, *NtHSP70-2*, and *NtHSP70-3*

Nucleotide sequences of the three genomic clones -- *NtHSP70-1*, *NtHSP70-2*, and *NtHSP70-3* -- showed ORFs of 653, 653, and 648 amino acids, respectively. All ORFs started with ATG and ended with TAA, and their sizes suggested that they were complete for HSP70s.

NtHSP70-1 had a 537-nucleotide-long untranslated region at the 5′ end of the putative ORF. We also located, within this upstream region, more than 20 putative heat-shock elements (5′nGAAn3′ or its inverse complement, 5′nTTCn3′) (Pelham, 1982) as well as the GAGA sequence (Janet et al., 1997) and repeats of the CA sequence (Martin-Farmer and Janssen, 1999). The putative ORF also comprised two putative intron sequences -- one for 66 nucleotides, the other for 54 nucleotides -- over which the sequence for one complete HSP70 could be defined. Putative introns started with GT and ended with AG, the consensus intron-flanking sequence (Kastury et al., 1997). This ORF included several other features, such as a putative nuclear localization sequence, KRKHKKDLSTNVRALRR (Delelis-Fanien et al., 1997; Tom et al., 1999) and the eukaryotic thiol (cysteine) protease histidine active site (Bercovich et al., 1997; Bogyo et al., 1997), plus a glycine-rich region and the C-terminus EEVD sequence, which have been suggested as the linker and the binding site for HSP40 (Qian et al., 2002). The ORF extended for 653 amino acids, with an estimated molecular mass of 71.01 kDa and a pI value of 5.02. In the 3′-region, the sequence extended for 426 nucleotides, including several putative polyadenylation signal sequences (Fig. 1).

As the second clone, *NtHSP70-2* had an ORF led by 1063 nucleotides in the 5′ upstream sequence, which contained more than 20 consensus heat-shock elements. The GAGA sequence and repeats of CA were also located in this region. The putative ORF extended to its full length without an intron. It also showed a putative nuclear targeting sequence, the eukaryotic thiol protease histidine active site, a glycine-rich region, and the C-terminus EEVD sequence. The NtHSP70-2 protein comprised 653 amino acids, with an estimated molecular mass of 71.28 kDa and a pI value of 5.07. In the 3′-region, the sequence extended for 430 nucleotides, with several putative polyadenylation signal sequences (Fig. 2).

The ORF of *NtHSP70-3* was led by 188 nucleotides which contained several consensus heat-shock elements. A putative CAAT box, CCAAT, was also found in this region. One long intron sequence of 876 nucleotides was located in the putative ORF, which also included a putative nuclear targeting sequence and the eukaryotic thiol protease histidine active site. In addition, *NtHSP70-3* possessed a cytosolic compartment sequence, GPKIEEVD, at the C-terminus (Boorstein et al., 1994; Guy and Li, 1998; Tom et al., 1999). This ORF coded a protein of 648 amino acids, with an estimated molecular mass of 71.05 kDa and a pI value of 5.14. In the 3′-region, the sequence extended for 197 nucleotides, with one putative polyadenylation signal sequence (Fig. 3).

Family Genes of NtHSP70 in *N. tabacum*

Genomic DNA blot hybridization for HSP70 genes in tobacco was done using the *NtHSP70-1* genomic

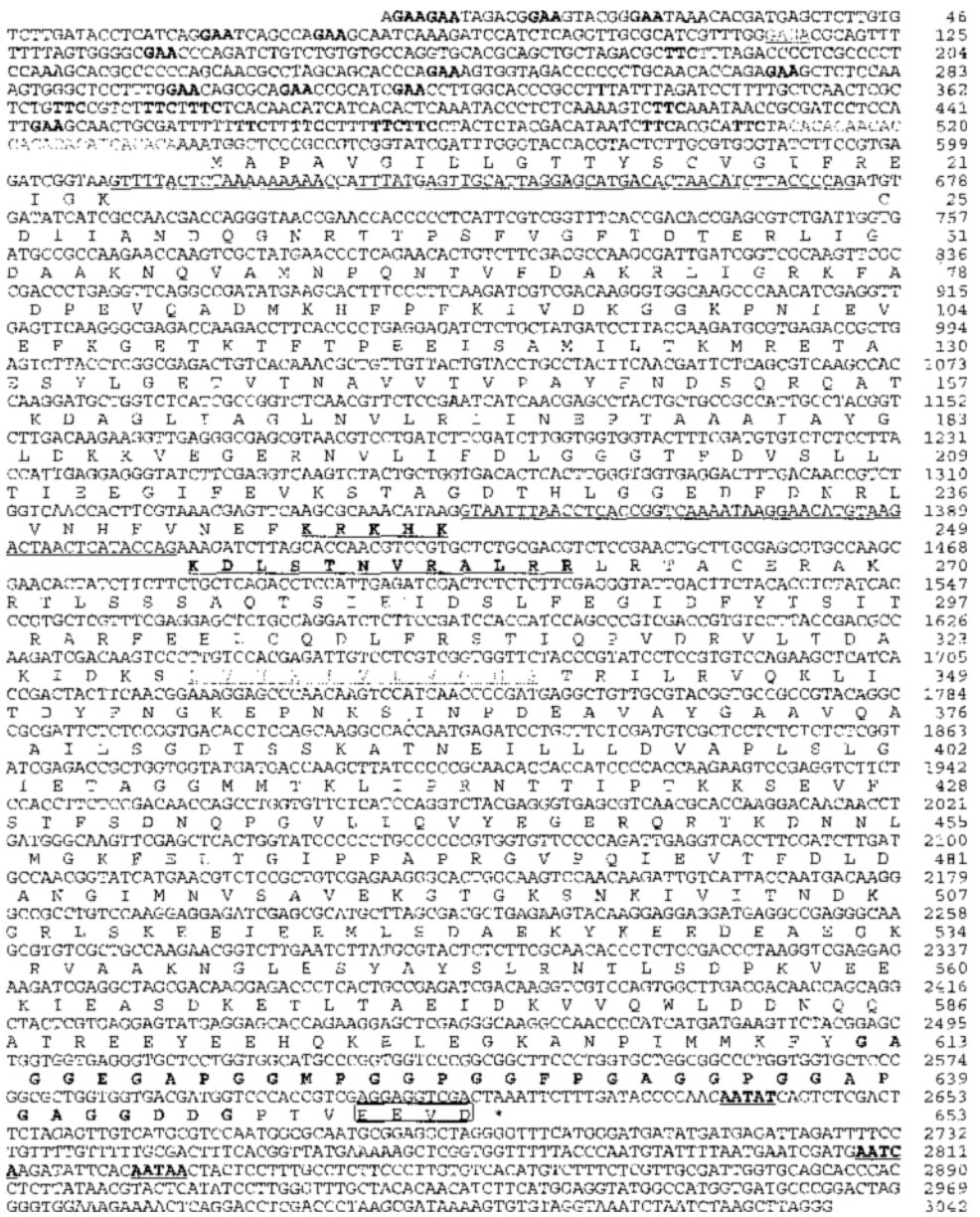

Figure 1. Nucleotide sequence and deduced amino acid sequence for tobacco HSP70 genomic clone, *NtHSP70-1*. A putative open reading frame started with ATG and ended with TAA. Putative intron sequences are underlined. Amino acid sequence is represented by one-letter symbol. In the 5′-untranslated region, putative HSEs are in bold letters, CA-rich region in shaded let - ters, and GAGA-sequence in shaded letters and underlined. In the ORF, underlined bold letters indicate putative nuclear local-ization sequence. Underlined and shaded letters represent putative protease histidine active site. In the C-terminus, glycine ri ch region is in bold letters, and EEVD sequence is boxed. In the 3′-untranslated region, putative polyadenylation sequences are in bold letters and underlined.

clone as a probe. To identify its gene-family members, the hybridization conditions were moderately adjusted. Approximately 10 bands appeared in each of several restriction digests (Fig. 4).

Homology of Proteins Coded by *NtHSP70-1*, *NtHSP70-2*, and *NtHSP70-3*

The deduced amino acid sequences of *NtHSP70-1* and *NtHSP70-2* shared 93.4% identity while those of

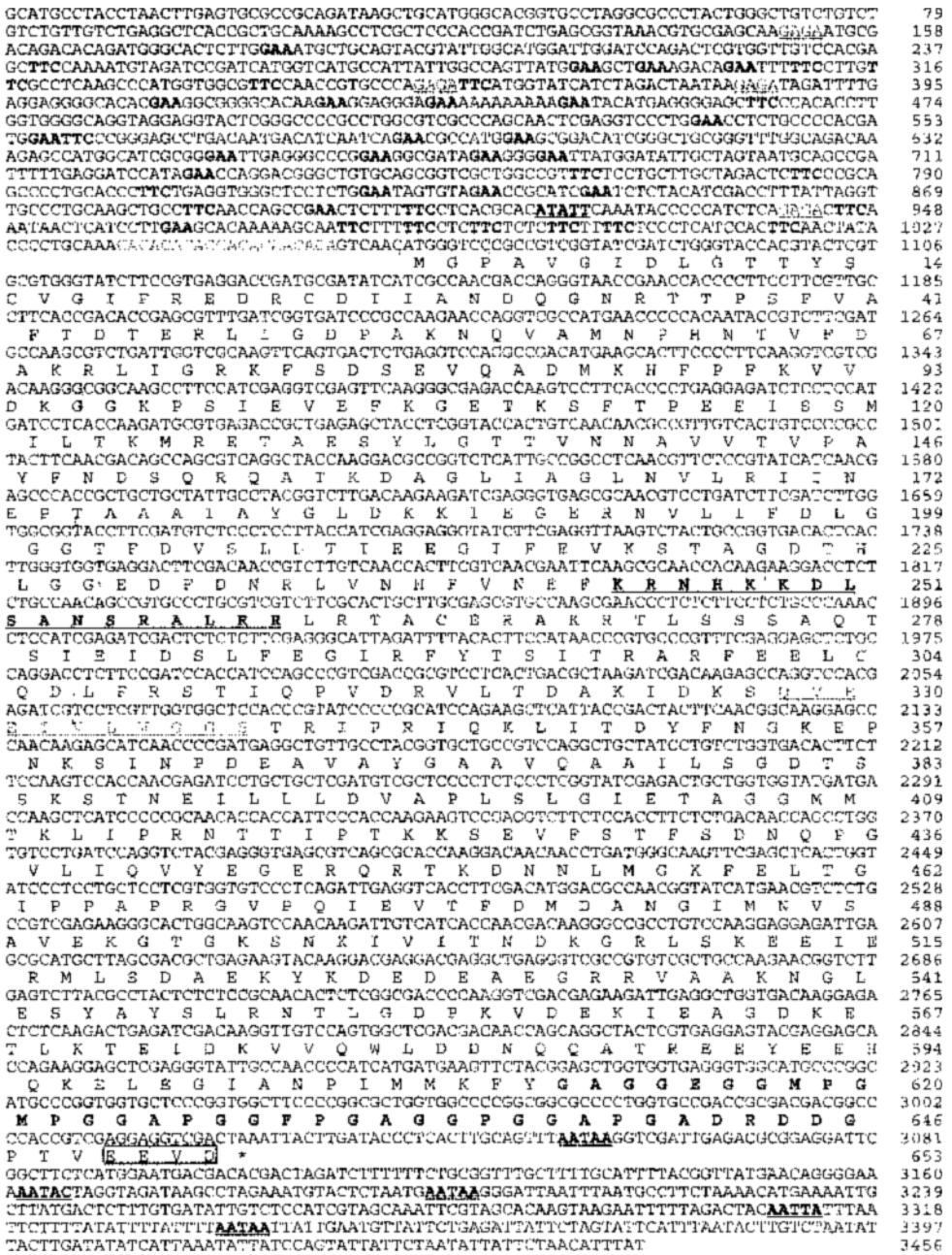

Figure 2. Nucleotide sequence and deduced amino acid sequence for tobacco HSP70 genomic clone, *NtHSP70-2*. Intron sequence could not be located. Amino acid sequence is represented by one-letter symbol. In the 5′-untranslated region, putative HSEs are in bold letters, putative TATA box in underlined bold letters, and CA-rich region in shaded letters. GAGA-sequence is in shaded letters and underlined. In the ORF, underlined bold letters represent putative nuclear localization sequence. Under - lined and shaded letters represent putative protease histidine active site. In the C-terminus, glycine rich region is in bold le tters, and EEVD sequence is boxed. In the 3′-untranslated region, putative polyadenylation sequence is in underlined bold letters.

NtHSP70-1 and *NtHSP70-3* shared 71.6% identity. When the deduced amino acid sequences of these three genes were compared with *Arabidopsis* cytosolic HSP70s (Sung et al., 2001), *NtHSP70-3* showed strong homology, i.e., >83% identity, whereas the range of homologies for *NtHSP70-1* and *NtHSP70-2* were 65 to 71% (Table 1). This discrepancy among our genes in identity to the reported *Arabidopsis* cyto-

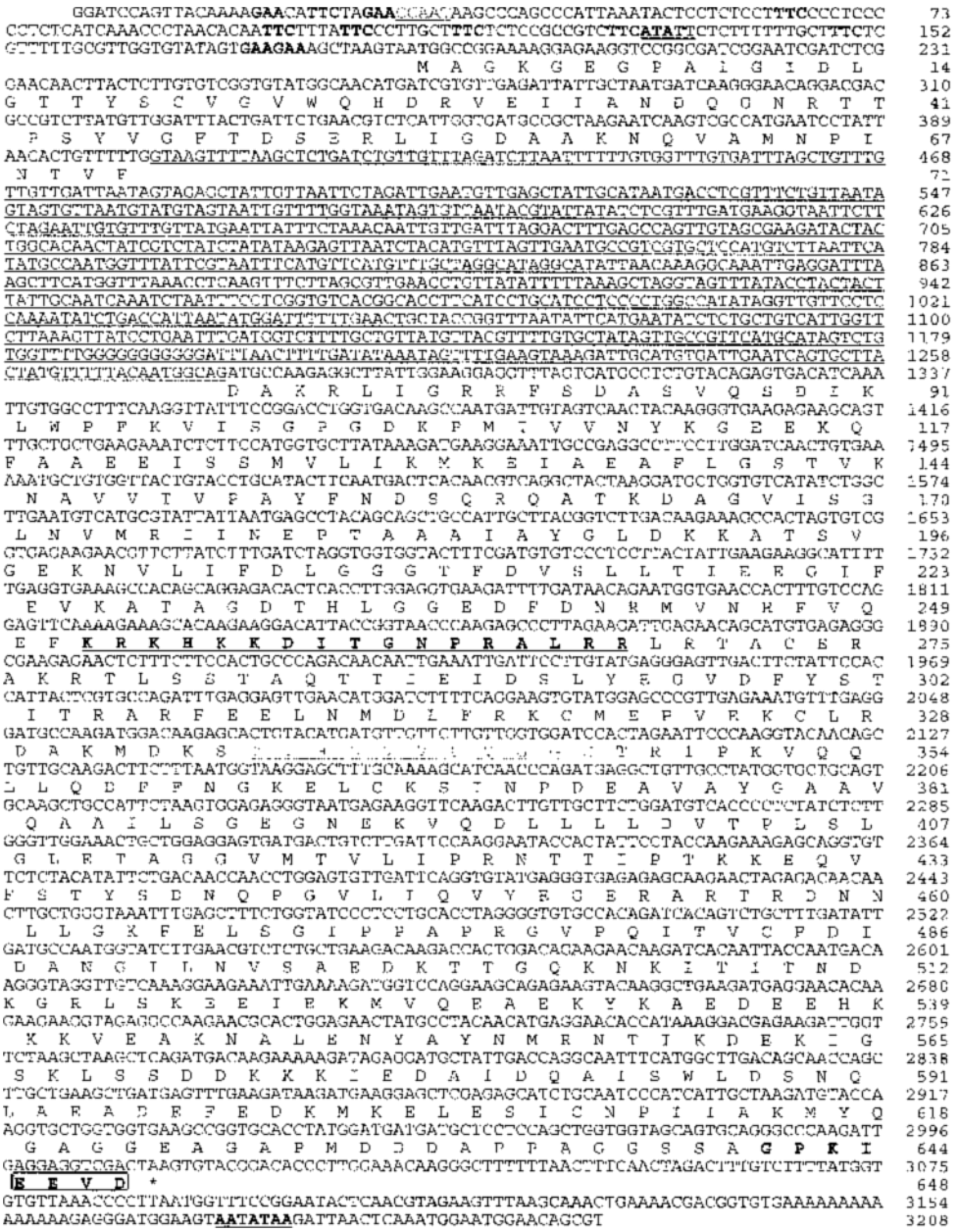

Figure 3. Nucleotide sequence and deduced amino acid sequence for tobacco HSP70 genomic clone, *NtHSP70-3*. Putative intron sequence is underlined. Amino acid sequence is represented by one-letter symbol. In the 5′-untranslated region, putative HSEs are in bold letters, putative TATA box in underlined bold letters, and CCAAT-sequence in underlined and shaded letters. In the ORF, underlined bold letters indicate putative nuclear localization sequence. Underlined and shaded letters represent putative protease histidine active site. In the C-terminus, cytosol compartment sequence, GPKIEEVD, is in bold letters. In the 3′-untranslated region, putative polyadenylation sequence is in underlined bold letters.

solic HSP70 genes, i.e., *NtHSP70-3* showed strong homologies, and *NtHSP70-1* and *NtHSP70-2* showed lower levels of homology, was also found for other reported cytosolic plant HSP70 genes (data not shown).

For all these HSP70s, their ATP-binding domains and substrate binding domains (β-domains) were well conserved, with most of the discrepancies in the amino acid sequences being localized to the C-terminus (Fig. 5).

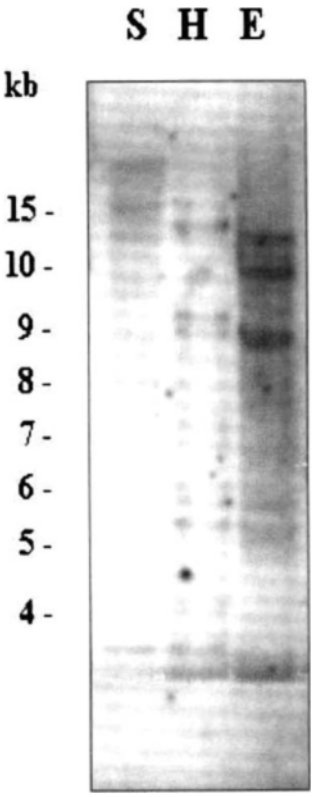

Figure 4. Genomic DNA blot analysis of tobacco for *NtHSP70-1*. Restriction-digested tobacco genomic DNA was hybridized to the ^{32}P-labeled *NtHSP70-1*. S, *Sac*I; H, *Hind*III; E, *Eco*RV.

Expression Patterns of *NtHSP70-1* and *NtHSP70-3* in Response to Heat-Shock, Dehydration Stress, and Hormone Treatments

We selected two genomic clones, *NtHSP70-1* and *NtHSP70-3*, to analyze their expression patterns under abiotic stress or hormonal treatments. RNA levels of these HSP70 members increased in response to heat-shock. The northern bands commonly appeared for both genes were barely detected under non heat-shock condition and strongly detected under heat-shock conditions, however differences in the expression pattern of each HSP70 gene was also strongly apparent. Transcript level of *NtHSP70-3* was much lower than that of *NtHSP70-1* in all the conditions tested. Heat-shock at 40°C for 1 h was sufficient to induce the strong accumulation of *NtHSP70-1* transcript. In contrast, for *NtHSP70-3*, heat-shock at 44°C seemed to trigger stronger expression, although the level of transcript remained low. Finally, heat-shock at 48°C seemed to be too high to induce a significant level of transcription for either gene (Fig. 6A).

Dehydration stress by withholding watering for 2 weeks led to the strong induction of *NtHSP70-1* expression, but not *NtHSP70-3* (Fig. 6B).

When our tobacco plants were treated for 24 h with GA_3, IAA, or BA (10 μM, 100 μM, or 1 mM), transcript was detected for *NtHSP70-1*, but not *NtHSP70-3*. However, ABA apparently was not effective in inducing transcription of either clone. Interestingly, the dose effects for both GA_3 and IAA were quite different for *NtHSP70-1* expression, i.e., 10 μM of GA_3 induced transcription whereas a level of 1 mM IAA was required in order to detect any activity (Fig. 6C).

DISCUSSION

We can classify the three HSP70 genes from *N. tabacum* into two groups, based on their level of homology and putative subcellular locality. *NtHSP70-1* and *NtHSP70-2* are highly homologous to each other and are putatively classified to nuclear forms, whereas *NtHSP70-3* is highly homologous to the cytosolic HSP70s reported from other plant species. The putative ORF in the C-terminus region of *NtHSP70-3*, GPKIEEVD, has been suggested as a tag for cytoplasmic HSP70 (Boorstein et al., 1994; Guy and Li, 1998; Tom et al., 1999). In contrast, *NtHSP70-1* and *NtHSP70-2* lack this cytoplasmic tag, but have their putative nuclear localization sequences in the center (see also Delelis-Fanien et al., 1997; Tom et al., 1999). This difference among tobacco HSP70 genes is paralleled by the variations in their expression patterns. The putative nuclear form, *NtHSP70-1*, is strongly induced under stresses of high temperature and dehydration, as well as by treatments with BA, GA_3, and IAA. Accordingly, while transcription of *NtHSP70-3* is also induced by high temperature, neither dehydration stress nor hormonal treatment seems to affect its pattern of expression in that clone.

These various patterns can be attributed to the structural differences in the promoter regions of each gene. For example, the pentanucleotide heat-shock element (HSE) core, 5′ nGAAn3′, or its inverse complement 5′nTTCn3′ (Pelham, 1982), is common to all three tobacco HSP70 genes. However, only *NtHSP70-1* and *NtHSP70-2*, but not *NtHSP70-3*, contain the GAGA-sequence, which has been suggested as the binding site for the GAGA factor. The promoter regions of those first two clones also comprise another regulatory protein that binds to the heat shock promoter (Janet et al., 1997), as well as the CA repeat,

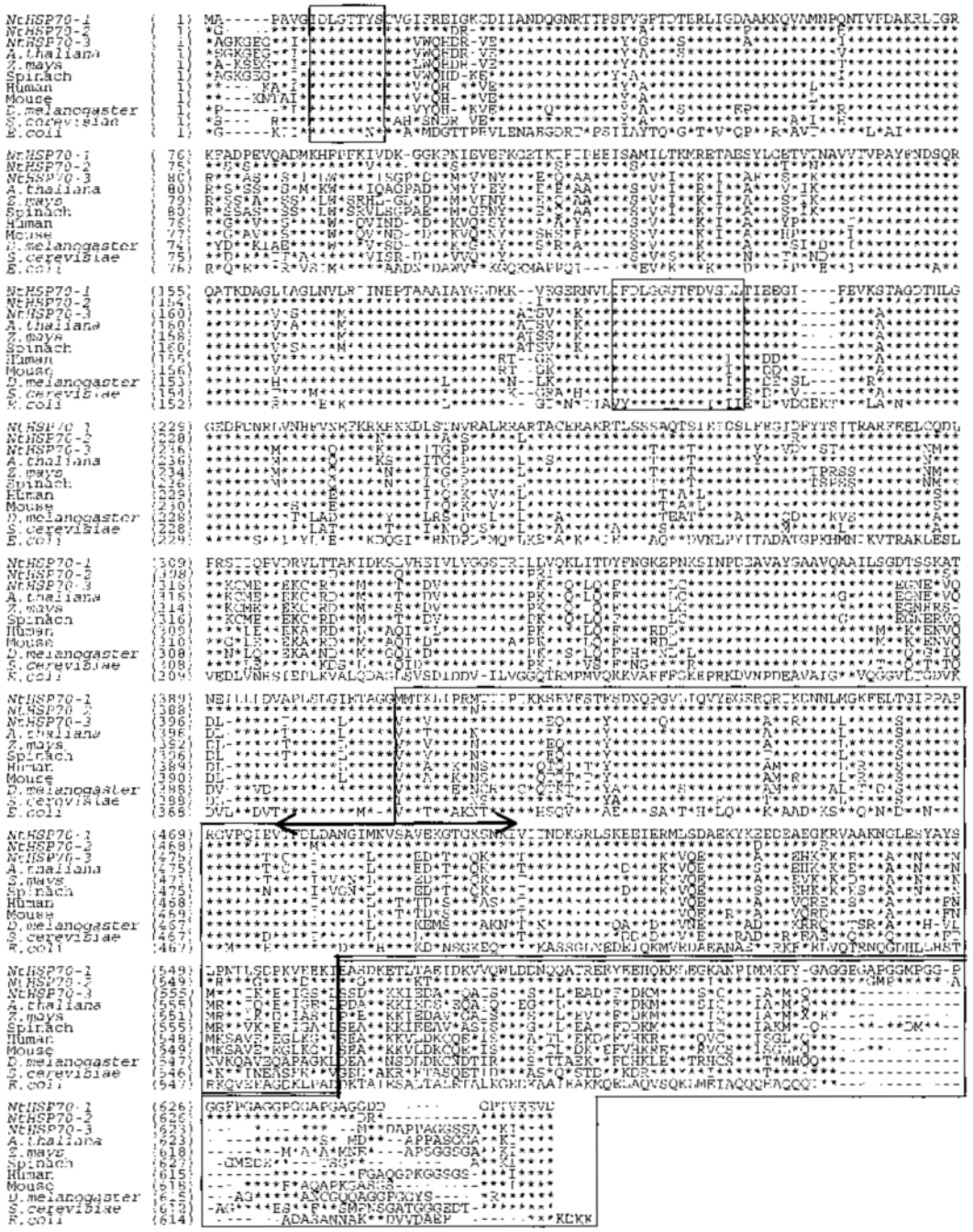

Figure 5. Comparison of deduced amino acid sequences of *NtHSP70-1*, *NtHSP70-2*, and *NtHSP70-3* with other HSP70s. * represents conserved amino acid. A hyphen was inserted within the amino acid sequence to denote a gap. First and second boxes represent HSP70 family signature; third box, the α-domain of substrate binding; and fourth box, the β-domain of substrate binding. Forward arrow indicates ATP-binding domain; backward arrow, the substrate binding domain (Guy and Li, 1998; Database, NCBI).

which has been suggested to stimulate transcription, translation, and mRNA turnover (Martin-Farmer and Janssen, 1999). Although the length of the promoters in the revealed nucleotide sequence also differ among the HSP70 genomic clones, all those elements and sequences mentioned above reside in close proximity to the putative translation initiation codon. Thus, the short promoter described for *NtHSP70-3* cannot be

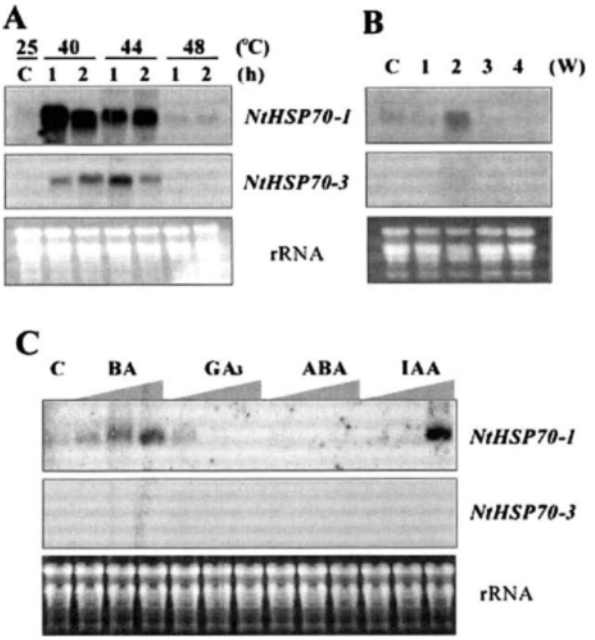

Figure 6. Transcripts of *NtHSP70-1* and *NtHSP70-3* differentially induced by hormones and environmental stresses. Total RNA was isolated from young tobacco plants treated accordingly and blot-hybridized with ^{32}P-labeled genomic clone, which is indicated on right side of each blot. (**A**) Plants were heat-shock treated for 1 or 2 h at 40°C; for 1 or 2 h at 44°C; or for 1 or 2 h at 48°C. (**B**) Plants were dehydration-stressed by withholding watering for 1, 2, 3, or 4 weeks. (**C**) Plants were treated for 24 h with one of the following hormones: BA at 10 μM, 100 μM, or 1 mM; GA_3 at 10 μM, 100 μM, or 1 mM; ABA at 10 μM, 100 μM, or 1 mM; or IAA at 10 μM, 100 μM, or 1 mM. Lane C in (**A**), (**B**), and (**C**) represents untreated control tobacco plants maintained at 24-26°C.

the reason for this difference.

Our tobacco HSP70 genes also vary in their ORFs. For example, although each consists of an ATP-binding domain, substrate binding domain (β-domain), and C-terminal domain (α-domain), these genes show diversity in their proteins. Whereas NtHSP70-1 possesses a glycine-rich region that is a putative interacting site with other co-chaperones, such as HSP40 (Bercovich et al., 1997; Bogyo et al., 1997; Qian et al., 2002) to the C-terminus, NtHSP70-3 does not have this component. Likewise, while NtHSP70-3 has the conserved sequence of cytosolic HSP70 at its C-terminus, NtHSP70-1 is lacking in this.

NtHSP70-1 and *NtHSP70-3* also manifest different expression patterns in response to heat-shock, dehydration, and hormonal treatments. Here, both *NtHSP70-1* and *NtHSP70-3* were expressed upon exposure to high temperature, but only the former responded in like manner to dehydration or hormone applications. This further demonstrates that the functions of NtHSP70 members can be variable and differentiated. ABA has been widely implicated in plant functioning under abiotic stress, but the roles of auxin, cytokinin, and gibberellin in that adaptive response have rarely been reported (Rabbani et al., 2003). Therefore, it is noteworthy that expression of *NtHSP70-1* is rather strongly induced by BA, GA_3, and IAA.

Auxin and cytokinin, in particular, act synergistically to regulate the process of cell division (Riou-Khamlichi et al., 1999), while gibberellin governs the mobilization of soluble sugars from starch in cereal grains and converts genetic dwarfs of corn, pea, and rice into taller plants (Johri and Mitra, 2001). Therefore, our results indicate that nucleocytoplasmic NtHSP70-1 is linked to major physiological or developmental processes. Although the induction of transcription does not necessarily mean higher-level synthesis of the protein, positive regulation of *NtHSP70-1* expression by BA, GA_3, and IAA (but not ABA) suggests that the functional mode of HSP70 in abiotic-stress responses should differ from that of the so-called stress proteins, e.g., super oxide dismutase, late embryogenesis proteins, and small chaperones. Based on the hormonal induction of the HSP70 gene, it is tempting to speculate that HSP70 might be dually active during nonstress conditions and stress conditions that can be added up to stronger tolerance against abiotic stresses.

Our genomic DNA gel blot analyses of the HSP70 family genes revealed ~10 HSP70 members in tobacco. Because of their differences in structure and expression patterns, we should expect very diverse complexity in this family. Therefore, comprehensive studies that include many gene members should be conducted in order to better understand HSP70 functioning *in vivo*.

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