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Genes and Expression Pattern of Tobacco Mitochondrial Small Heat Shock Protein under High-Temperature Stress

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In plants, small heat shock proteins (sHsps) have been localized to the cytosol, mitochondria, chloroplasts, and endoplasmic reticulum. Using *NtHSP24.6*, a cDNA clone for tobacco mitochondrial sHsp, as a probe, we performed genomic DNA blot analysis and identified presence of a small gene family for mitochondrial sHsp in tobacco (*Nicotiana tabacum*). Several putative genomic clones for mitochondrial sHsp were isolated when a tobacco genomic library was screened. After restriction mapping of seven genomic clones, three that had shown different maps were selected, and their nucleotide sequences for the putative coding sequence were determined. From these three independent clones, one identical nucleotide sequence was obtained that had two exons with one intron. RNA blot hybridization of heat-stressed tobacco plants revealed a typical heat-shock-responsive accumulation of *NtHSP24.6* transcript. Under severe heat-shock conditions, an additional band was apparent, but of a larger transcript size. When we compared the amino acid sequence of NtHSP24.6 with mitochondrial sHsps from various other species, we found a high level of homology throughout the ORF, with an almost complete match to the carboxyl terminus. Our comparison of NtHSP24.6 with tobacco cytosolic class I sHsps also resulted in high homology between their α-crystalline domains, but significant divergence in their amino-terminus regions.

Keywords: expression pattern, genomic clones, mitochondrial sHsp, tobacco

Because heat stress is one of the most serious factors affecting growth, plants synthesize various heat shock proteins (Hsps) under sub-lethal heat stress conditions. These Hsps are grouped into five major families, according to their molecular masses: Hsp100, Hsp90, Hsp70, Hsp60, and small Hsps (Narberhaus, 2002). Additional discoveries have included single-chain chaperonins, in which chaperone and protease activities are combined on a single polypeptide (Spiess et al., 1999), as well as Hsp33, a novel redox-regulated chaperone class (Jakob et al., 1999).

The small heat shock proteins (sHsps) are abundant and ubiquitous in nature. They range in size from 12 to 43 kDa (the majority between 14 and 27 kDa), and can be found in complexes as large as 200 to 800 kDa (Narberhaus, 2002). sHsps share a sequence of about 90 residues, which is homologous to α-crystallin from the vertebrate eye lens (Caspers et al., 1995). Under heat stress, sHsp synthesis is highly induced (several hundred-fold) (Richmond et al., 1999), and may comprise up to 1% of the total soluble proteins in a plant (Hsieh et al., 1992). sHsps are also synthesized under oxidative stress (Banzet et al., 1998; Harndahl et al., 1999), γ-irradiation (Harndahl et al., 1999), and low temperatures (Sabehat et al., 1996; Ukaji et al., 1999). In plants, some stress-independent sHsps are also detected during zygotic embryogenesis (Carranco et al., 1997).

Plant sHsps have been organized into six classes, according to their structural similarity and cellular localization (de Jong et al., 1998). Three classes reside in the cytosol (I, II, III); the others are found in the chloroplasts, mitochondria, and endoplasmic reticulum (Scharf et al., 2001). In contrast to plant sHsps, mammalian sHsps localize only to the cytosol and nucleus (Narberhaus, 2002).

As important cellular organelles, the mitochondria perform many essential roles in plants. Their abnormal functioning can result in severely deleterious effects. Chou et al. (1989) have described a correlation between the amount of mitochondrial sHsps accumulated under high-temperature stress and the enhancement of heat tolerance in plant mitochondria. Protection of NADH: ubiquinone oxidoreductase in Complex I and citrate synthase by mitochondrial sHsps has also been demonstrated (Downs and Heckathorn, 1998; Shono et al., 2002). These studies have provided evidence that mitochondrial sHsps are related to the protection of that organelle from various stresses. Nevertheless, the understanding of their structure and functional mode

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is still in the early stage.

cDNA and genomic clones of mitochondrial sHsps have been characterized from various plant species, including *Lycopersicon esculentum* (Liu and Shono, 1999), *Arabidopsis thaliana* (Willett et al., 1996; Visioli et al., 1997; *Arabidopsis* Genome Initiative, www.arabidopsis.org/agi.html), and *Pisum sativum* (Lenne et al., 1995). *Nicotiana tabacum* has served as a popular model system for investigating various physiological, developmental, and genetic aspects in plants. Therefore, the objective of this study was to use that model species to examine the structure and expression patterns of mitochondrial sHsp genes under high-temperature stress.

MATERIALS AND METHODS

Plant Material and Heat Shock Conditions

Tobacco (*N. tabacum* L. cv. Wisconsin 38) plants were reared in a growth chamber at 25°C, with a 16-h photoperiod and approximately 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance. The heat-shock test conditions included temperatures of 30°C, 35°C, 40°C, or 45°C, over a period of 15 min, 30 min, 1 h, 2 h, or 4 h. Relative humidity during these treatments was maintained between 70 and 80%, as monitored with a hygrometer (Model DTH-50C, UEI, Korea).

Isolation of Genomic Clones

A tobacco genomic library in EMBL3 was plated on an LB agar plate containing an *Escherichia coli* strain KW251 bacterial lawn. The newly formed plaques were blotted to a nylon membrane (Amersham, UK), and hybridized with mitochondrial sHsp cDNA clone, *NtHSP24.6*, labeled with ^{32}P-dCTP. This clone had previously been named *TMLHSP1* (Choi and Hong, 2000; Youn, 2002). Phage clones with positive signals were amplified, and the phage DNA was extracted. To locate the coding sequence of NtHSP24.6 in the genomic clones, we performed Southern blot hybridization of the phage DNA. The smallest restriction fragment showing a positive signal with the ^{32}P-labeled *NtHSP24.6* was then subcloned into pBluescript II-SK(+). All the above procedures were conducted according to the methods of Sambrook et al. (1989).

Genomic DNA Blot Hybridization

Tobacco leaf tissue (0.2 g) was ground in a mortar with liquid nitrogen, then transferred to a 2 mL e-tube, to which 1.2 mL of genomic DNA lysis buffer [0.05 M Tris·Cl (pH 7.6), 0.1 M NaCl, 0.05 M EDTA, and 0.5% SDS], 0.84 μL of β-mercaptoethanol, and 50 $\mu g\ mL^{-1}$ RNaseA were added. After incubating for 1 to 2 h at room temperature, the mixture was centrifuged at 12,000 rpm for 15 min. The supernatant was extracted once with phenol, once with 1:1 phenol:chloroform, and once with chloroform. Genomic DNA was pooled down from the supernatant via isopropanol precipitation, followed by a wash with 70% ethanol. It was then digested overnight with restriction enzymes (EcoRI, HindIII, EcoRV, and BamHI), separated on a 0.8% agarose gel at 30V, and transferred to a nylon membrane (Amersham, UK). The blotted membrane was treated first with denaturation buffer (0.5 M NaOH and 1.5 M NaCl), then with neutralization buffer (1.5 M NaCl and 0.5 M Tris·Cl). Hybridization with the *NtHSP24.6* cDNA probe was carried out at 65°C in hybridization buffer [0.5% sodium phosphate buffer (pH 7.2) and 7% SDS]. The membrane was then washed with 2X SSC, 0.1% SDS for 15 min, followed by 1X SSC, 0.1% SDS for 15 min, and 0.1X SSC, 0.1% SDS at 65°C for 2 min. Detailed procedures were as described by Sambrook et al. (1989).

RNA Blot Hybridization

Tobacco leaf tissue (1.5 g) was ground in a mortar with liquid nitrogen, then transferred to a 15-mL polyethylene tube, to which 6 mL of RNA lysis buffer [50 mM LiCl, 25 mM Tris·Cl (pH 8.0), 35 mM EDTA, 35 mM EGTA, 0.5% SDS, and 0.2% PVP] was added. This mixture was extracted with 3 mL of phenol, then with 3 mL of chloroform. The supernatant was combined with an equal volume of 4 M LiCl, and stored overnight at –20°C. Afterward, the mixture was centrifuged at 12,000*g* for 15 min at 4°C. Following a 70% ethanol wash, the pellet was dissolved in DEPC-treated distilled water. RNA (20 μg) was separated on a 1% agarose gel containing 1X gel running buffer and 15% formaldehyde at 60V. After being transferred to a nylon membrane, it was hybridized with the ^{32}P-dCTP-labeled *NtHSP24.6* cDNA probe at 65°C in hybridization buffer [0.5% sodium phosphate buffer (pH 7.2) and 7% SDS]. The membrane was then washed at 65°C for 15 min each with 2X SSC, 0.1% SDS and 1X SSC, 0.1% SDS. Detailed methods were as described by Sambrook et al. (1989).

Nucleotide Sequencing and Analysis

We used a DNA sequencer 3730 (Applied Biosys-

tems, USA) to determine the nucleotide sequence. The NCBI database was searched for homologs to our identified nucleotide sequences and deduced amino acid sequences. Multiple alignment and homology percent calculations were carried via the GeneBee-Molecular Biology Server, which is managed by the A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University. The hydropathy plot was drawn according to the procedure of Kyte and Doolittle (1982).

RESULTS AND DISCUSSION

Genomic Cloning for Mitochondrial Small Heat Shock Protein from *N. tabacum*

Screening of a genomic library with the *NtHSP24.6* probe resulted in the isolation of seven putative genomic clones. Restriction maps for these clones revealed three that had partial coding sequences (data not shown), plus four with presumptive full coding sequences. Among the latter four clones, two showed identical restriction patterns. Thus, three genomic clones -- *TM2*, *TM4*, and *TM5* (Fig. 1) -- were chosen for further analyses. Subcloning of the smallest restriction fragments, including the putative coding sequences of *TM2*, *TM4*, and *TM5*, and nucleotide sequencing of the fragments revealed identical sequences from all three clones. The sequence extended for 2031 nucleotides, which fully covered the ORF for *NtHSP24.6*, and contained an intron sequence, promoter, and a 3' untranslated region (probably including a terminator). In the promoter region, heat-shock element sequences (i.e., GAA-- TTC) were found in a dozen locations. However, most of the putative heat-shock elements were either GAA or TTC, with a significant gap between the sequences. Although the typical TATA box was not present in the vicinity of the ORF starting point, a putative CAAT box was located at 85 nucleotides from the 5' region of the translation initiation codon. The intron sequence was flanked by the consensus sequences GT and AG. In the 3' region of the ORF, 298 nucleotides were determined. However, the most frequently denoted poly(A) additional signal, AATAAA, was not present in that region (Fig. 2). The hydropathicity calculated for the protein coded by *NtHSP24.6* indicated that it was strongly hydrophilic. However, the amino-terminus region was characterized as hydrophobic (Fig. 3).

Genomic DNA blot hybridization showed that *NtHSP24.6* is encoded by a small family gene. Restriction digests with EcoRI, HindIII, EcoRV, and BamHI resulted in four to six Southern bands, with one major band commonly appeared fromall the digests (Fig. 4).

Under heat-shock conditions, the expression pattern for *NtHSP24.6* transcript (Fig. 5) was similar to those of the cytosolic sHsp genes from tobacco (Park and Hong, 1998). RNA blot hybridization clearly showed the heat-inducibility of its transcription. Among the combinations of stress conditions that were tested, heat shock of 35°C induced the earliest transcript accumulation; 15 min after the treatment was initiated, a sig-

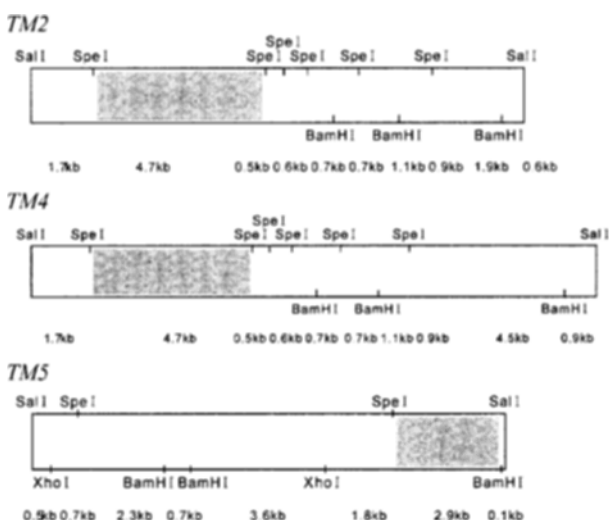

Figure 1. Restriction maps of genomic clones, *TM2*, *TM4*, and *TM5*, in an EMBL3 bacteriophage at the SalI site. Shadowed box represents portion of the genomic clones that probably contains the NtHSP24.6 coding region.

Figure 2. Nucleotide sequence of genomic clones and the amino acid sequence deduced for the open reading frame (ORF). Underlined sequences of GAA and TTC are putative heat-shock elements. Putative CAAT box is drawn in bold and underlined. Intron is 551 nucleotides long.

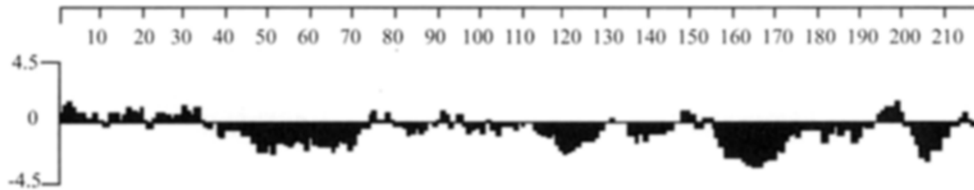

Figure 3. Hydropathy plot of NtHSP24.6. Positive value on Y-axis indicates hydrophobicity; negative value, hydrophilicity. X-axis indicates amino acid position.

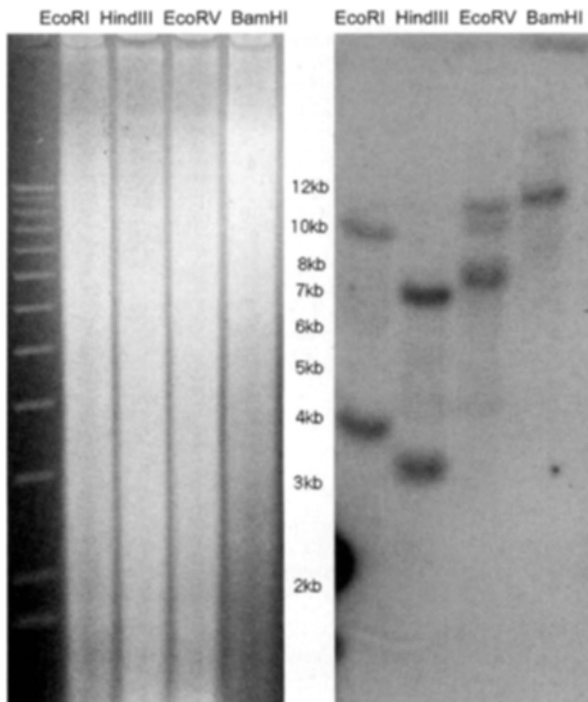

Figure 4. Genomic DNA blot hybridization with *NtHSP24.6* probe for *N. tabacum*. Genomic DNA was digested with EcoRI, HindIII, EcoRV, and BamHI.

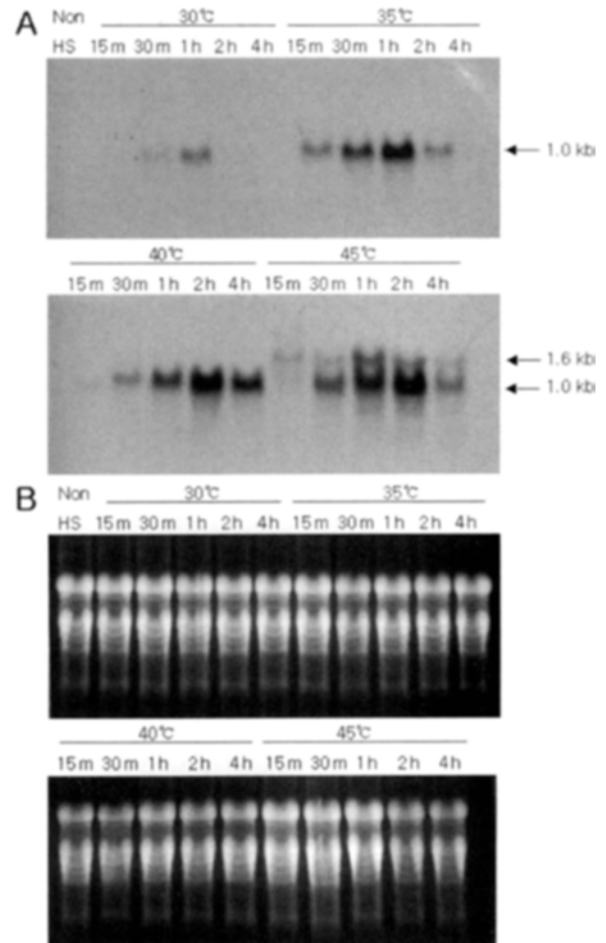

Figure 5. A, Expression pattern of *NtHSP24.6* gene under heat shock, based on RNA blot hybridization with *NtHSP24.6* probe. **B**, Ethidium bromide staining of a duplicate gel from (**A**).

nificant amount of *NtHSP24.6* transcript was already detectable. However, the overall level of transcript was greater at higher temperatures, i.e., 40°C and 45°C. In fact, the maximum was reached after 1 to 2 h, depending on the temperature treatment. Moreover, after this maximum was achieved, the accumulation of transcript declined when treatments were prolonged. We also observed that, at 45°C, one additional northern band appeared, which was approximately 600 nucleotides larger than those that were commonly found (Fig. 5).

Comparison of Plant Mitochondrial Small Heat Shock Protein Genes

When NtHSP24.6 was compared with other reported mitochondrial sHsps, we usually located a common, conserved α-crystallin domain, which is often represented as consensus region I, and consensus region II (Chen and Vierling, 1991). Most notably in consensus region I, from the 168th to the 210th amino acid of NtHSP24.6, --R--~-D-I-A-MKNGVL-V---K- in the shaded columns were completely conserved. The same was

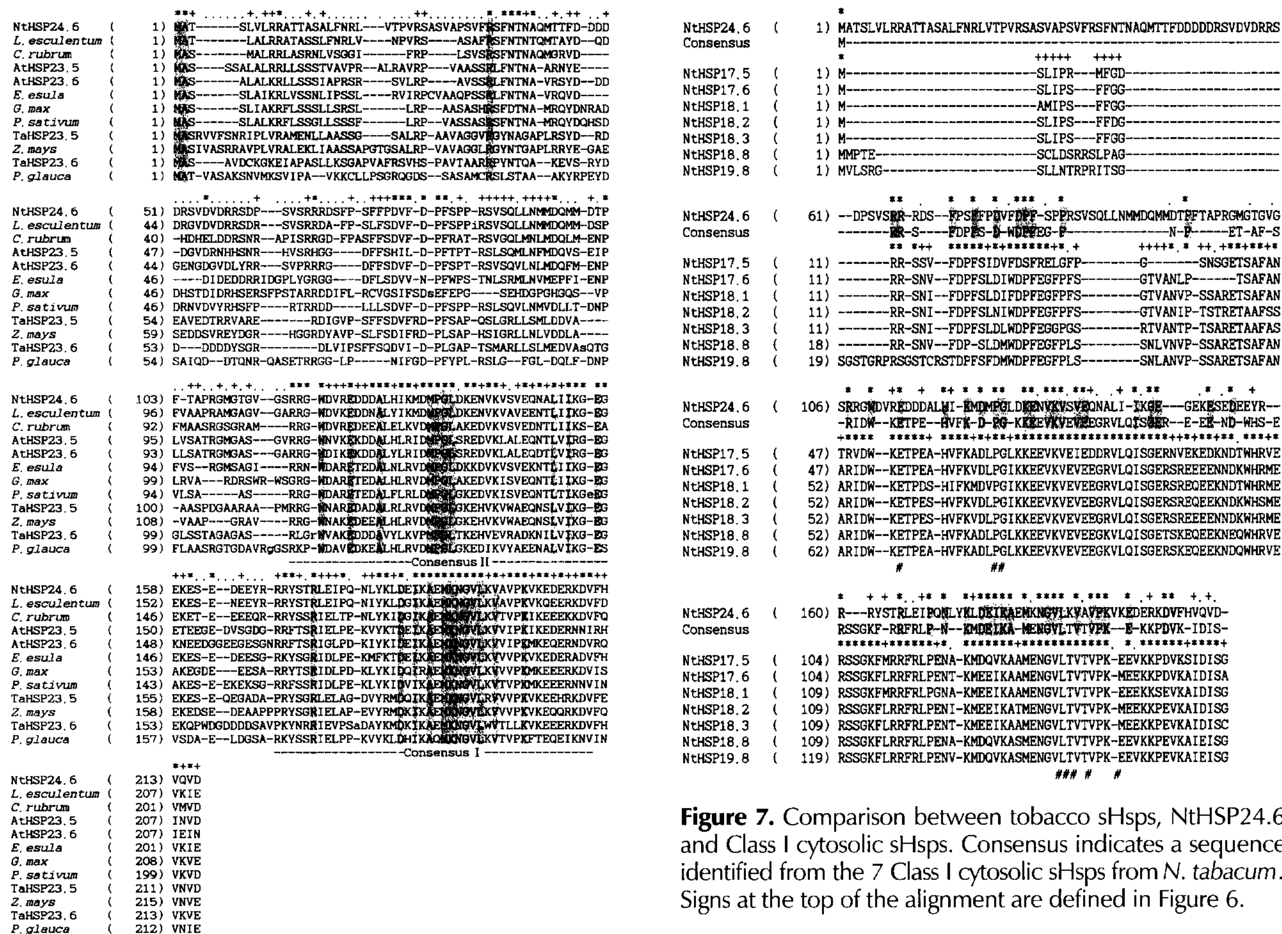

Figure 6. Comparison of deduced amino acid sequences for NtHSP24.6 and mitochondrial sHsps from *L. esculentum* (accession No. AB017134), *Chenopodium rubrum* (accession No. X15333), *Euphorbia esula* (accession No. AF237957), *A. thaliana* (*AtHSP23.6* accession No. X98375, U72958; *AtHSP23.5* accession No. BT008645), *P. sativum* (accession No. X86222), *Glycine max* (accession No. U21722), *Zea mays* (accession No. AF035460), *Picea glauca* (accession No. L47741), and *Triticum aestivum* (*TaHSP23.6* accession No. AF104108; *TaHSP23.5* accession No. AF104107).
Signs at the top of the alignment are defined as:
' ' - the average weight of column pair exchanges is less than weight matrix mean value
'.' - is less than mean value plus one SD
'+' - is less than mean value plus two SD
'*' - is more than mean value plus two SD

Figure 7. Comparison between tobacco sHsps, NtHSP24.6 and Class I cytosolic sHsps. Consensus indicates a sequence identified from the 7 Class I cytosolic sHsps from *N. tabacum*. Signs at the top of the alignment are defined in Figure 6.

true in consensus region II, from the 116th amino acid to the 157th amino acid, with -W---E---A------MPGL-------------L-I---E- in the shaded columns. In addition, alanine at the 2nd amino acid and arginine at the 35th amino acid were also completely conserved in a pool of many homologous amino acids throughout the proteins (Fig. 6).

Comparing NtHSP24.6 with the Class I cytosolic sHsps in *N. tabacum* revealed high homology, especially in consensus regions I and II, although this level of similarity was less than that represented in Figure 6. Nevertheless, NtHSP24.6 differed from the other proteins in having a long extension of about 60 amino acids to the amino terminus, a feature that was mostly lacking in the cytosolic proteins (Fig. 7).

Characteristics of Mitochondrial sHsp Genes in *N. tabacum*

By screening the genomic library for *NtHSP24.6*, we were able to isolate more than five different clones, which probably reflected family members of the mitochondrial sHsp genes in *N. tabacum*. This had also been the assumption based on the results of our genomic DNA blot hybridization. The identical nucleotide sequences from three genomic clones suggests that mitochondrial sHsp genes may have been duplicated during the genomic evolution of *N. tabacum*. Highly homologous restriction maps among those

clones also support this proposition. Likewise, if the amino terminus portion were not considered, the homology would be very strong between mitochondrial and cytoplasmic sHsps. This strong similarity suggests identical origin for the mitochondrial sHsp genes and cytoplasmic sHsp genes.

It is likely that the mitochondrial sHsp gene was developed from cytoplasmic sHsp genes by acquiring, for mitochondrial targeting, the first 26 amino-acid sequence (MATSLVLRRATTASALFNRLVTPVRS), as predicted by the MITOPROT program (Claros and Vincens, 1996), plus some additional sequences with unknown function. Further evidence for how little these mitochondrial sHsps have diverged among plant species is the low number of gene copies seen for mitochondrial sHsps in *A. thaliana* (i.e., possibly just one copy on Chromosome 4; *Arabidopsis* Genome Initiative Web Line: www.arabidopsis.org/agi.html), as well as the probably identical ORFs from all the mitochondrial sHsp genomic clones presented here.

The level of nucleotide sequence homology between *NtHSP24.6* and the genes for cytoplasmic sHsps (Park and Hong, 1998) was between 31.8% and 38.4%, and even in the consensus regions the rate was below 50%. Therefore, cross-hybridization of the cytoplasmic sHsp transcripts in the RNA blot hybridization was not likely. Because of this, the temporal expression pattern of the mitochondrial sHsp transcript that was highly homologous to that of the cytoplasmic sHsp transcripts suggests that the heat-shock response of mitochondrial sHsp gene transcription parallels that of the cytoplasmic. We detected an additional RNA band under severe heat-shock conditions, i.e., 45°C. Considering the size of the intron in *NtHSP24.6*, the larger-sized RNA that appeared in that environment was likely to be the mis-spliced mRNA of *NtHSP24.6*, namely the pre-mRNA still carrying the intron sequence. It is a good possibility that splicing malfunctioned at this high temperature, resulting in nonfunctional transcripts. In addition, Osteryoung et al. (1993) have found that intron processing can be disrupted by severe and abrupt heat shock, and that releasing the organism from such a stress can induce normal splicing of the intron, thereby promoting thermotolerance in the cells.

Molecular chaperone functioning has been well documented for cytoplasmic sHsps (Beissinger and Buchner, 1998; van Montfort et al., 2002). Considering the strong homology between our mitochondrial sHsp and the cytoplasmic sHsps, it is highly likely that the former possesses comparable chaperone activity, as has also been reported previously (Downs and Heckathorn, 1998; Shono et al., 2002). The presence of highly homologous consensus regions I and II, as peptides, suggests a similar, complex formation of mitochondrial sHsps in situ, i.e., a dodecameric double-ring structure. However, mitochondria still maintain many prokaryotic characters, e.g., circular genomes and their transcription and translation systems, and chemical environment is very different from other parts of the cell as they function in energy production. Therefore, we believe that the functional mode of mitochondrial sHsps deviates significantly from that of cytoplasmic sHsps as molecular chaperones. The major variation in their amino terminus regions, excluding the mitochondrial targeting sequence, may serve as the structural basis for those undefined differences.

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