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Complexity of Hsp90 in organelle targeting

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Abstract Heat shock protein 90 (Hsp90) is an abundant and highly conserved molecular chaperone. In *Arabidopsis*, the *Hsp90* gene family consists of seven members. Here, we report that the *AtHsp90-6* gene gives rise to two mRNA populations, termed *AtHsp90-6L* and *AtHsp90-6S* due to alternative initiation of transcription. The *AtHsp90-6L* and *AtHsp90-6S* transcription start sites are located 228 nucleotides upstream and 124 nucleotides downstream of the annotated translation start site, respectively. Both transcripts are detected under normal or heat-shock conditions. The inducibility of *AtHsp90-6* mRNAs by heat shock implies a potential role of both isoforms in stress management. Stable transformation experiments with fusion constructs between the N-terminal part of each *AtHsp90-6* isoform and green fluorescent protein indicated import of both fusion proteins into mitochondria. *In planta* investigation confirmed that fusion of the AtHsp90-5 N-terminus to green fluorescent protein (GFP) did result in specific chloroplastic localization. The mechanisms of regulation for mitochondria- and plastid-localized chaperone-encoding genes are not well understood. Future work is needed to address the possible roles of harsh environmental conditions and developmental processes on fine-tuning and compartmentalization of the AtHsp90-6L, AtHsp90-6S, and AtHsp90-5 proteins in *Arabidopsis*.

Keywords Alternative transcription · Chloroplasts · Heat shock · *Hsp90* · Mitochondria · Targeting

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Introduction

Heat shock proteins 90 (Hsp90s) have been assigned numerous and diverse functional roles in many pathways ranging from cellular homeostasis and signal transduction to development. Since they assist client protein functional competence at a proper time and place, multiple cellular compartments may require the presence of Hsp90.

The Hsp90s are evolutionarily conserved proteins found from *E. coli* to humans (Emelyanov 2002). Under normal conditions, Hsp90 function is essential for the biogenesis and support of numerous cellular proteins that control cell physiology. They have key roles in signal transduction, protein folding, and protein degradation (Rutherford and Lindquist 1998). Hsp90 proteins are molecular chaperones acting to prevent misfolding and aggregation of unfolded or partially folded proteins (Young et al. 2001). They assist protein transport across the endoplasmic reticulum (ER) and organellar membranes (Schatz and Dobberstein 1996). In higher eukaryotes, Hsp90s form complexes with various client proteins, including steroid hormone receptors (Joab et al. 1984), helix-loop-helix transcription factors

(Wilhelmsson et al. 1990), tyrosine and serine/threonine kinases, nitric oxide synthase and telomerase (Wegele et al. 2004), the tumor suppressor p53 (Sepehrnia et al. 1996), and the type 1 tumor necrosis factor receptor (TNFR-1) (Song et al. 1995). Recent studies in *S. cerevisiae* have revealed that Hsp90s interact to an extended network of cofactors and substrates involved in a wide range of cellular functions (Zhao et al. 2005).

Although different isoforms have been identified, a role has not yet been assigned to each Hsp90 homolog, while gene duplication may not result in functional redundancy. The *Arabidopsis* genome contains seven *Hsp90* protein-encoding genes. These specify four cytoplasmic Hsp90 (*AtHsp90-1*, At5g52640; *AtHsp90-2*, At5g56030; *AtHsp90-3*, At5g56010; *AtHsp90-4*, At5g56000), one ER Hsp90 (*AtHsp90-7*, At4g24190), and two organellar Hsp90 proteins (*AtHsp90-5*, At2g04030; *AtHsp90-6*, At3g07770) (Milioni and Hatzopoulos 1997; Krishna and Gloor 2001; Chen et al. 2006). Genetic and biochemical studies are only beginning to define their function and the nature of their client proteins in plants. Studies in the fruit fly and *Arabidopsis* strongly suggest that Hsp90 acts as a capacitor for morphological evolution by most likely neutralizing the conformational consequences of several mutations, therefore buffering their potential phenotypic changes and turning them phenotypically silent (Rutherford and Lindquist 1998; Queitsch et al. 2002). Recent results in *Arabidopsis* have shown that Hsp90 restricts stochastic phenomena by minimizing perturbations, thereby canalizing development (Samakovli et al. 2007). The *Arabidopsis shd* mutant phenotype could be the result of the accumulation of *cryptic mutations*, promoting morphological evolution in the *Arabidopsis* genome (Ishiguro et al. 2002). In addition two Hsp90 isoforms, Hsp90-1 and Hsp90-2, were identified as essential factors for the pathogen resistance response mediated by several R proteins of the NB-ARC-LRR class in plants (Takahashi et al. 2003; Zhang et al. 2004; Sangster and Queitsch 2005; Boter et al. 2007).

Mitochondria and chloroplasts are the targets of different stresses, often resulting in deleterious effects such as apoptosis and cell cycle arrest. It has been suggested that Hsp90 is engaged in chloroplast biogenesis during normal growth and development (Cao et al. 2003). The *CR88* gene encodes a chloroplast-targeted Hsp90 homolog but its crucial role remains to be elucidated (Cao et al. 2003). In animals, a mitochondrial Hsp90 homolog (TRAP-1) appears to have a crucial role in cell cycle progression, cellular differentiation, and apoptosis (Felts et al. 2000; Masuda et al. 2004).

Our results provide strong evidence for an exclusive plastidic localization of *AtHsp90-5* and demonstrate that alternative transcription of the *AtHsp90-6* gene generates two different proteins transported into mitochondria.

Materials and methods

Plant material and transformation

Arabidopsis thaliana (ecotype Columbia) plants were grown under standard conditions at 22°C under 70% humidity with a light/dark cycle of 16 h/8 h. After transformation, seeds from individual transgenic plants were imbibed at 4°C overnight, surface sterilized, and layered on MS medium containing 50 mg l^{-1} kanamycin and 200 mg l^{-1} cefotaxime, under the same growth conditions. Transgenic plants were transferred to soil for further analysis.

Arabidopsis thaliana protoplasts were isolated as previously described (Doelling and Pikaard 1993).

Primer extension analysis

Total RNA was extracted from 28-day-old *Arabidopsis* plants using the RNeasy Plant Kit (Qiagen). Primer extension analysis was carried out using the Primer Extension System—AMV Reverse Transcriptase kit (Promega), according to the manufacturer's instructions. The following sequence-specific oligonucleotides were used: PRET-1, 5′-ACGGAGCGCTTAGAGAGCCTGATC-3′ (*AtHsp90-6L*) and PRET-2, 5′-CTGCACTGGATTCGTTC CGGTATC-3′ (*AtHsp90-6S*). PRET-1 and PRET-2 are located 3 bp and 23 bp downstream of the translation start sites, respectively.

RT-PCR analysis

Total RNA was extracted as described above. To assess heat induction, 28-day-old plants were incubated at 37°C for 2 h. After treatment, intact plants were harvested and used for RNA preparation. First strand cDNA synthesis was carried out using Expand Reverse Transcriptase (Roche Diagnostics). The primers were as follows: for *AtHsp90-6L*, RC4 5′-TCGCTCGATACGTTTGATGGTAC-3′ (RC4 is located 265 bp upstream of *AtHsp90-6L* transcription start site), RC2 5′-ACAAGCCAATAAGGTTTTAATCAGG-3′ (RC2 starts 4 bp upstream of *AtHsp90-6L* transcription start site), and RC6 5′-CTACCGAAATCAAAATCCACCATTC-3 (RC6 is located 169 bp downstream of *AtHsp90-6L* transcription start site); for *AtHsp90-6S*, RC5 5′-TTCAT TTCAATTTCCTTCATC-3′ (RC5 starts 14 bp upstream of *AtHsp90-6S* transcription start site), RC3 5′-TGATTGGA TTACTTACAGTGTCACTA-3′ (RC3 is located 78 bp downstream of *AtHsp90-6S* transcription start site), rRC3 5′-TAGTGACACTGTAAGTAATCCAATCA-3′ (the same position as for RC3), and rRC5 -5′-GATGAAGGAAA TTGAAATGAA-3′ (the same position as for RC5). Two primers were designed in common for *AtHsp90-6L* and

AtHsp90-6S cDNAs: RC15, 5′-GATTCCATTCTCCTTG TCTGCATAG-3′; RC1, 5′-CCGATGGTGAAACAGGTG GCACATA-3′ (RC15 and RC1 are located 698/483 bp and 1434/1219 bp downstream of *AtHsp90-6L*/*AtHsp90-6S* transcription start sites, respectively). The following primers were used for the semiquantitative reverse transcriptase-mediated (RT) PCR experiments: *AtHsp90-1*, 5′-CGCAT GTTCAGATGGCTGATGC-3′ and 5′-AGCAGAGTAGA AACCAACACC-3′; for *AtHsp90-5*, 5′-CTAATGGCTCC TGCTTTGAGTAGAAG-3′ and 5′- ACCAAACTGTCCG ATCAAACCGT-3′; for *AtHsp90-6L*, RC15 5′-GATTCCA TTCTCCTTGTCTGCATAG-3′ and RC6 5′-CTACCGAA ATCAAAATCCACCATTC-3′; for *AtHsp90-6S*, RC15 5′-GATTCCATTCTCCTTGTCTGCATAG-3′ and RC7 5′-GGATCCGGTCATTTGCTCAATTGAACATG-3′; and for GFP, GFP-1 5′-GGAGATATAACAATGAGTAAAGGA GAA-3′ and GFP-2 5′-TTATTTGTATAGTTCATCCAT-3′. For the analysis of differential expression, three rounds of RT-PCR were conducted with two independently isolated total RNA samples. RT-PCR was performed for 15, 20, 25, 28, 30, and 35 cycles to determine the linearity of the PCR. The thermal cycling parameters used for the RT-PCR for all genes were as follows: 94°C for 15 s, 62°C for 30 s, and 72°C for 1 min; followed by 72°C for 10 min. As a positive control, a 540 bp *AtGAPDH* fragment was amplified under the same RT-PCR conditions for a total of 20 cycles using the primer pair 5′-GCAATGCATCTTGCACTACCAACTGTC-3′ and 5′-CTGTGAGTAACCCCATTCATTATCSTACCA-3′. The sequence identity of all RT-PCR products obtained was confirmed by sequencing. The amplified cDNAs were subcloned using the pGEM-T vector system (Promega) and sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with fluorescent sequencing (FS) AmpliTaq DNA polymerase (Perkin-Elmer). Database searches were performed on the NCBI web server by using the Basic Local Alignment Search Tool (BLAST) network service. Subcellular localization predictions were performed using Predotar (http://www.inra.fr/predotar/), TargetP (http://www.cbsdtu.dk/services/TargetP/), ChloroP (http://www.cbs.dtu.dk/services/ChloroP/), and MitoProt (http://ihg.gsf.de/ihg/mitoprot.html).

In vitro transcription-translation assays

In order to produce plasmids for in vitro transcription and translation reactions, an *AtHsp90*-6_{AUG1} open reading frame (ORF) (584 amino acid encoding sequence) and an *AtHsp90*-6_{AUG2} ORF (518 amino acids encoding sequence) were subcloned into pGEM vector (Promega). The *AtHsp90*-6_{AUG1} ORF and *AtHsp90*-6_{AUG2} ORFs were amplified with the common reverse primer TRANS-C 5′-TACTACTTCAAGTCCTTTCTCCAGC-3′ and the forward primer TRANS-1 5′-TCTCTTCCGAGATTTT AGAAGTTTGC-3′ and TRANS-2 5′-CAATCTAAGAA TAGTGGGTCAT-3′, respectively. The plasmids were linearized downstream of the T7 transcription terminator and the corresponding proteins were synthesized by using the TNT coupled transcription-translation reticulocyte lysate system (Promega) according to the manufacturer's instructions. ^{35}S methionine-labeled proteins were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis on 12.5% gels.

GFP reporter gene constructs

The plasmid constructs for *Arabidopsis* transformation were assembled as illustrated in Figs. 5 and 6. For targeting analysis of the protein encoded by *AtHsp90-5* transcripts, an N-terminal fragment of the *AtHsp90-5* gene (576 bp) was amplified by using the following primers: 121F 5′-CTAAT GGCTCCTGCTTTGAGTAGAAG-3′ and 121R 5′-GTTG TCAGCACCAAGGTCCTTGTT-3′. For the construct based on the long transcript of the *AtHsp90-6* gene, a 510 bp fragment was amplified with primers RC21 5′-TAGAA GTTTGCGACGATGAT-3′ and RC15, whereas for the corresponding construct of the short transcript, a 317 bp fragment of *AtHsp90-6* was amplified with primers RC20 5′-GGTCATTTGCTCAATTGAACATG-3′ and RC15. The amplified fragments were subsequently fused in-frame upstream of the smGFP sequence and cloned into the pBI101 binary vector under the control of the *AtHsp90-1* promoter (Haralampidis et al. 2002). The constructs obtained were named as follows: pK90-5GFP, N-terminal 190 amino acid residues of AtHsp90-5 fused to GFP; pK90-6LGFP, N-terminal 164 amino acid residues of AtHsp90-6L fused to GFP; and pK90-6SGFP, N-terminal 98 amino acid residues of AtHsp90-6S fused to GFP. All constructs were sequenced to check the accuracy of amplification and translational fusions and were used to transform the *Agrobacterium tumefaciens* strain GV3101::pGV2260 by the direct transfer method (An et al. 1988). Transgenic *Arabidopsis* plants were obtained by the floral dip method (Clough and Bent 1998).

Fluorescence microscopy

Localization of GFP fusions was analyzed by epifluorescence microscopy using an Olympus BX50 fluorescence microscope. Images were captured with an Olympus DP71 microscope digital camera. The *Arabidopsis* transgenic plants were heat shocked for 2 h at 37°C before GFP fluorescence was analyzed. Mitochondria were visualized with the fluorescent probe MitoTracker Orange CMTMRos (M7510, Molecular Probes, USA) as a counterstain. The plants were suspended in MS medium containing 25 nM MitoTracker and incubated for 30 min at 22°C before

analyzing the fluorescence. The following filter sets were used: for GFP, exciter HQ470/40, dichroic Q495LP, and emitter HQ525/50; for MitoTracker Orange CMTMRos, exciter BP 546/12, beam-splitter FT 580, emitter LP 590; and for chlorophyll autofluorescence, 633-nm excitation and 680 nm emission.

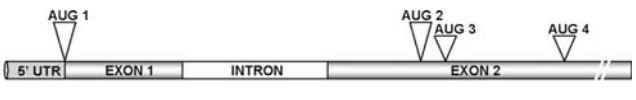
Results

AtHsp90-6 gene generates multiple transcripts

The full-length Hsp90-6 protein of the *Arabidopsis thaliana* is reported in the NCBI database to contain 803 amino acid residues. However, the open reading frame of the corresponding cDNA (*NM_111652*) contains four in-frame translation initiation codons at the N-terminal region (107 aa) (AUG1-AUG4; Fig. 1). Therefore the cDNA could potentially encode for proteins starting at any initiation codon. The consensus sequence for translational initiation is defined as (-6)GCCA/GCCAUGG/A(+4) with positions −3 and +4 being the most critical (Kozak 1986). Inspection of the genomic sequence of *AtHsp90-6* revealed that the first two AUGs (AUG-1 and AUG-2) are located within a sequence context matching the demand of Kozak's rule. In contrast, AUG-3 and AUG-4 are located within a poor Kozak configuration to be considered as initiation codons. These observations led us to investigate whether *AtHsp90-6* could be a multifunctional gene, coding for various forms of Hsp90 by alternative use of transcription/translation initiation codons.

Primer extension analysis was performed to determine the potential transcription initiation sites of *AtHsp90-6*. The results indicated that two major transcript ends can be detected in *A. thaliana*: one mapping 228 nucleotides upstream of the first translation start site (AUG-1) and one mapping 210 nucleotides upstream of the second translation start site (AUG-2) (Fig. 2). Consequently, it is very likely that the *AtHsp90-6* gene has multiple transcriptional start sites. The first exon of the longer transcript (designated *AtHsp90-6L*) is identical to that deposited in the *Arabidopsis* genome database. The ATG start codon, of the shorter transcript (designated *AtHsp90-6S*), is located within the second exon of the *AtHsp90-6L*. The 5′-region of the *AtHsp90-6L* sequence contains a predicted intron of 137 bp (Fig. 2a). The DNA sequence conservation rule near the exon–intron boundaries is fulfilled, with GT located at the initiation and AG at the termination sites of the intron, respectively. To corroborate the presence of two different mRNA populations corresponding to *AtHsp90-6L* and *AtHsp90-6S*, RT-PCR analysis was performed using forward and reverse oligonucleotide primers specific for the 5′-UTR (RC4, RC2 and RC6), intron 1 (RC5, RC3 and rRC3), exon 4 (RC15), and exon 12 (RC1), as shown in Fig. 3. DNA fragments of different sizes were amplified and the two transcript populations were confirmed by nucleotide sequencing of the RT-PCR products. The corresponding cDNAs did not contain any intron sequences. As anticipated, no amplification products were obtained using primer combinations RC4-RC1 and RC5-RC1 while the primer combination RC3-RC1 produced the predicted fragment. Since both RC3 and RC5 primers are located within the first intron and the latter covers the determined initiation of transcription (Fig. 2), the existence of the smaller mRNA is expected. To rule out the possibility that the PCR products originated from contaminating genomic DNA, additional RT-PCR reactions were performed. Reverse primer rRC3 (located in the first intron) together with the sense primers RC6 or RC2 did not produce any amplification fragments. Sequencing of both cDNAs deriving from the long and short transcripts showed that they were identical except for the 5′ ends. We therefore concluded that *AtHsp90-6* produces at least two different mRNAs encoding AtHsp90-6L (799 amino acids from 20 exons) and AtHsp90-6S (733 amino acids from 19 exons) proteins. It should be noted that the *AtHsp90-6* gene in the NCBI database possesses 21 exons. However a careful comparison of the genomic sequence to the cDNA sequences obtained in the course of the present study revealed that *AtHsp90-6* consists of 20 exons. This error is due to a misannotation of the intron/exon boundaries for exons 16 and 17.

In silico analysis of the 1 kb region upstream of the *AtHsp90-6* gene transcription start sites revealed the presence of a heat-shock element (HSE) consisting of three perfect and one imperfect core units (cTTCaaaTCcaGAAgcTTCg) (Fig. 2A). Two CCAAT sequences were identified near the putative HSE. It has been reported that CCAAT-box sequences (representing the binding sites for the C/EBP transcription factors) act cooperatively with



AUG	CONTEXT											ORF
AUG 1	C	G	A	C	G	**A**	**U**	**G**	A	T	C	799 aa
AUG 2	T	G	A	A	C	**A**	**U**	**G**	A	A	A	733 aa
AUG 3	G	T	T	T	A	**A**	**U**	**G**	G	G	A	728 aa
AUG 4	G	C	C	T	C	**A**	**U**	**G**	G	A	C	692 aa
	-5	-4	-3	-2	-1	+1	+2	+3	+4	+5	+6	

Fig. 1 Nucleotide context of AUGs contained within the first 107 amino acids of the *A. thaliana* Hsp90-6. Upper: positions of alternative translational initiation codons (AUG1-AUG4) are designated by arrowheads. Lower: table showing the nucleotide sequence flanking the four AUGs (indicated in bold) and the length of the resulting ORFs

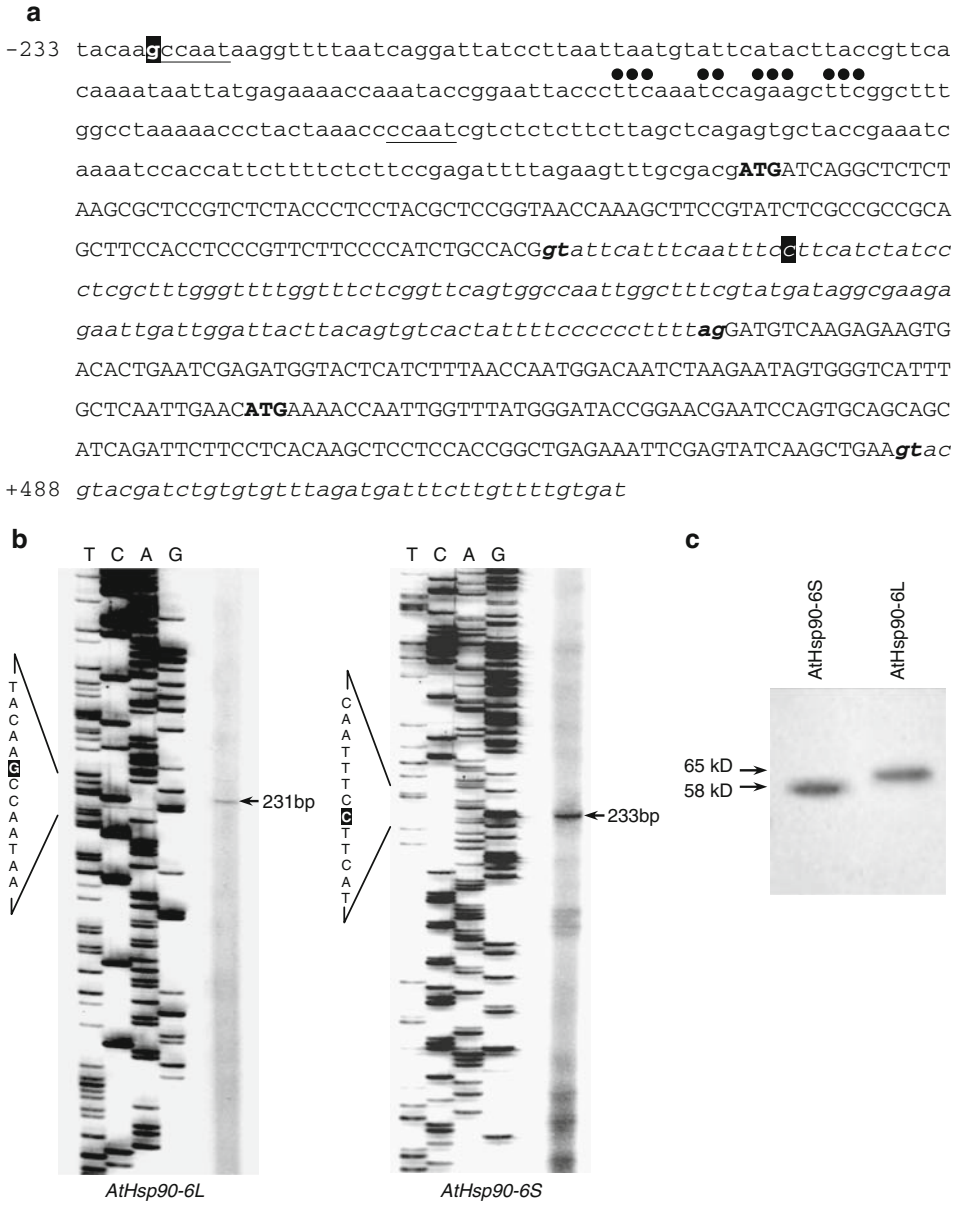
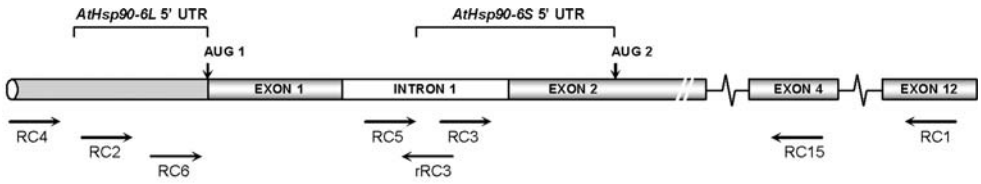


Fig. 2 Alternative transcription start sites of the *Arabidopsis AtHsp90-6* gene. (**a**) Partial sequence of the 5′ region *AtHsp90-6* gene showing the two in-frame ATG codons (indicated in boldface). The first intron of the *Hsp90-6L* transcript is represented in lowercase italics, and boundaries in bold letters. Potential start sites for *AtHsp90-6L* and *AtHsp90-6S* transcripts are highlighted black. Potential heat shock element (HSE) is indicated by black dots. CCAAT boxes are underlined. (**b**) The transcription initiation sites of the *AtHsp90-6L* and *AtHsp90-6S* mRNAs were determined by primer extension analysis. The same primers (PRET-1 and -2) were used for sequencing of an appropriate fragment. The products of extension and sequencing analysis were electrophoresed on the same gel. In the vicinity of the alternative transcription start sites, the nucleotide sequence is shown to the left of each gel. Arrows show the nucleotide positions of the 5′ ends of the transcripts relative to PRET-1 and PRET-2. (**c**) The *AtHsp90-6L* and *AtHsp*90-6S transcripts were translated in a rabbit reticulocyte lysate system using [^{35}S]Met. Samples were separated on a 12% SDS polyacrylamide gel. Bands corresponding to AtHsp90-6L and AtHsp90-6S are indicated by arrows. The estimated molecular masses are also indicated.

HSEs (Rieping and Schoffl 1992; Prasinos et al. 2004). Interestingly, no perfect TATA boxes were identified. Further inspection of the promoter region revealed the presence of potential binding sites for various transcription factors including activating-enhancer protein 1 (AP-1), mitochondrial stress response element (MSR-like), and stress response element (STRE) (data not shown). These *cis*-elements play important roles in regulating expression of genes in response to heat shock, heavy metal, dehydration, low temperature, light, and pathogen elicitors (Haralampidis et al. 2002; Zhao et al. 2002; Takahashi et al. 2003). However, the biological significance of the



Forward/Reverse primer pair	RC2	RC3	RC4	RC5	RC6
RC1	+	+	-	-	+
rRC3	-	-	-	-	-
RC15	+	+	-	-	+

Fig. 3 Schematic representation of the strategy used to confirm the existence of two different *AtHsp90-6* mRNAs. The relative position and the orientation of primers used to amplify the *AtHsp90-6L* and *AtHsp90-6S* transcripts are shown by arrows. A common reverse primer (RC15) was used in combination to different sense primers in order to obtain a consistent 3′ end and varying ends at the 5′. The amplification results were also validated by another common reverse primer (RC1). The primers RC3, RC5, and rRC3 were designed inside the first intron of *AtHsp90-6L*, hence fragments specific to *AtHsp90-6S* mRNA were amplified. For further details see the 'Materials and methods' section. The schematic representation has not been drawn to scale

putative elements within the promoter region of the *AtHsp90-6* remains to be verified experimentally.

Since our results suggested that the annotated Met codon (AUG-1) might not serve as the only site for the initiation of translation, we investigated whether this Met codon was dispensable for translation of the gene. Given that every ATG codon is not necessarily used for translation initiation, we attempted to translate in vitro the two *AtHsp90-6* coding sequences. Open reading frames encoding 584 or 518 amino acids of the *AtHsp90-6L* or *AtHsp90-6S* cDNA, respectively, were cloned into the pGEM vector and the resulting polypeptide products were ^{35}S methionine labeled in a TNT Coupled Reticulocyte Lysate System. As shown in Fig. 2c, two translation products of 65 or 58 kDa were produced from the long or the short *AtHsp90-6* template, respectively. These results suggested that both ATGs are suited for translation initiation, as predicted by the presence of typical Kozak sequences. We therefore propose that the Hsp90-6 isoforms were not generated by alternative splicing but resulted from the utilization of alternative transcription initiation sites.

Subcellular localization of AtHsp90-6L:GFP and AtHsp90-6S:GFP fusion proteins

The majority of nuclear-encoded organellar proteins are translated by cytosolic ribosomes and directed to the appropriate organelle by the N-terminal signal peptides. Sequence comparison between members of the AtHsp90 protein family revealed that AtHsp90-6L, AtHsp90-6S, and AtHsp90-5 contain a highly variable N-terminal region composed of 28–94 amino acids (Fig. 4). In the case of AtHsp90-6L, the targeting peptide is predicted to be 31 amino acids long as determined by in silico analysis

Fig. 4 Sequence alignment of the N-terminal regions of AtHsp90-5, AtHsp90-6L, and AtHsp90-6S. Identical or highly similar residues are highlighted black or grey, respectively. Dashes indicate gaps. Numbers indicate the amino acid position. Residues in white boxes show the predicted transient peptide of the organellar Hsps. Sequences used to generate the translational GFP fusion constructs are underlined. Accession numbers: AtHsp90-5, NP 849932; AtHsp90-6, NP187434

(MitoProt). The intracellular prediction program TargetP indicated that AtHsp90-6L could be localized to both mitochondria and chloroplasts, whereas Predotar predicted only mitochondrial localization. Interestingly, in the case of AtHsp90-6S, in silico protein targeting analysis indicated that the translation initiation at the AUG-2 start codon did not contain any organelle target sequence. To determine the intracellular targeting of AtHsp90-6L and AtHsp90-6S proteins *in planta*, pK90-6LGFP and pK90-6SGFP reporter constructs were used for *Arabidopsis* transformation (Fig. 5a). *Arabidopsis* transgenic lines expressing the fusion proteins were established and analyzed by epifluorescence microscopy. Both pK90-6LGFP and pK90-6SGFP fusion proteins were detected in numerous spherical bodies of root-hair cells (Fig. 5b). Staining with the mitochondria-specific dye MitoTracker Orange (Molecular Probes) revealed co-localization with the GFP fluorescent signals, confirming that pK90-6LGFP and pK90-6SGFP fusion proteins were delivered into mitochondria. In control plants, only red and no green fluorescence was detected (data not shown). To investigate whether any of the two fusion proteins had a dual targeting to both mitochondria and chloroplasts, isolated *Arabidopsis* leaf protoplasts were analyzed. Using the autofluorescence of chlorophyll as a marker, it was clear that the GFP signal did not co-localize with the red chlorophyll autofluorescence (Fig. 5b lower panel). The above results indicate that the N-terminal sequences present in pK90-6LGFP and pK90-6SGFP include a mitochondrial targeting sequence that is necessary and sufficient to transport the fusion proteins into mitochondria.

The predicted transit peptide targets AtHsp90-5 exclusively to chloroplasts

There are many and varied ways by which cross-compartment targeting is achieved by nuclear-encoded organellar proteins including N-terminal and internal signal

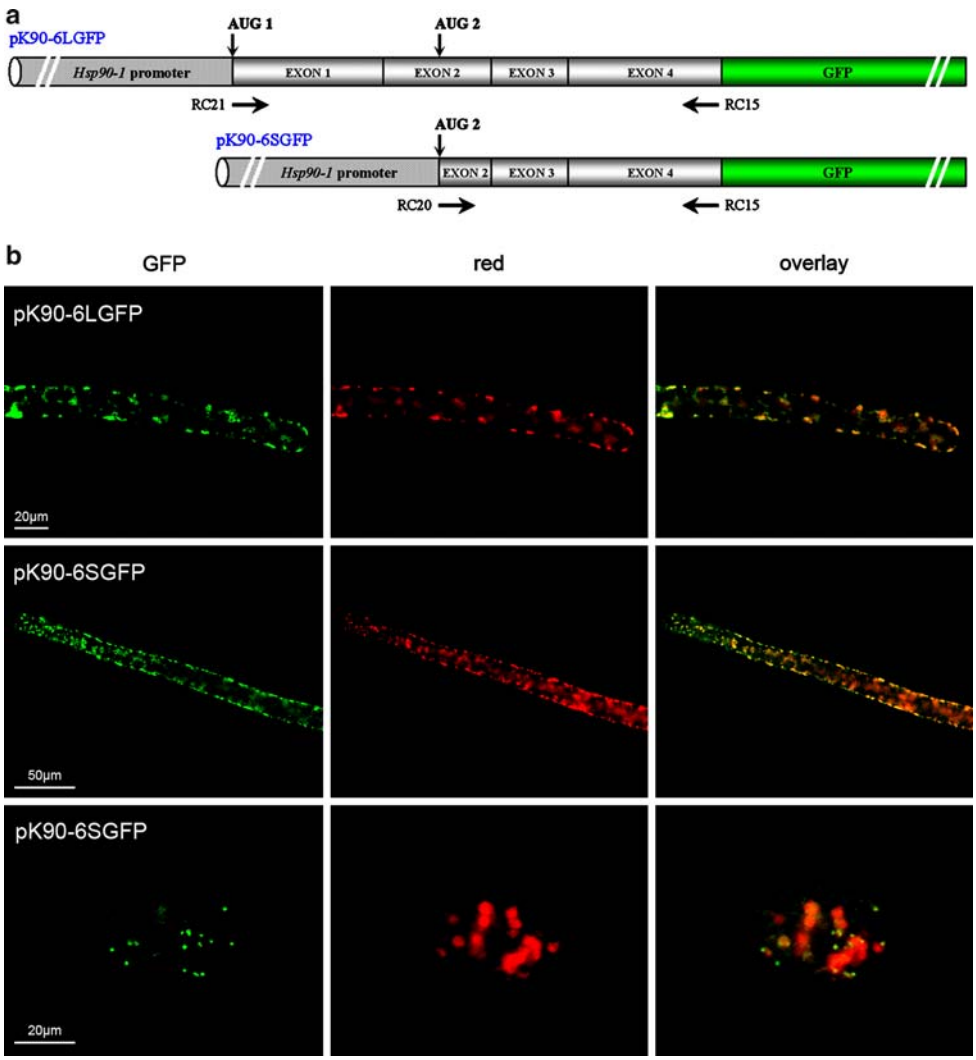


Fig. 5 In vivo targeting of AtHsp90-6L::GFP and AtHsp90-6S::GFP fusion proteins in *Arabidopsis*. (**a**) Depiction of the *AtHsp90-6* fusion constructs used for stable transformation of *Arabidopsis thaliana* plants. Expression was controlled by the *AtHsp90-1* promoter. (**b**) Root-hair cells (upper and middle panels) and isolated protoplasts (lower panel) of stable *A. thaliana* transformants expressing AtHsp90-6L::GFP or AtHsp90-6S::GFP fusion proteins. Mitotracker Orange (red channel) was used as a mitochondrial fluorescent marker in root-hair cells. The red channel in protoplasts shows the autofluorescence of chloroplasts. Overlay panels are merged images of the GFP and Mitotracker or GFP and chloroplast autofluorescence

sequences. Computer-assisted analysis for localization signals predicted that the AtHsp90-5 protein has a 60-amino-acid transit peptide (TP) (Fig. 4). The intracellular prediction program TargetP indicated that the AtHsp90-5 could be localized to both chloroplasts and mitochondria, while Predotar predicted only chloroplastic localization. To independently verify the plastidic localization of AtHsp90-5 *in planta*, a pK90-5GFP fusion construct was used to generate stably transgenic *Arabidopsis* plants. Targeting of the encoded fusion protein was monitored using epifluorescence microscopy presented in Fig. 6. The green fluorescence of GFP clearly co-localized with the red autofluorescence of chlorophyll (Fig. 6b, middle panels), demonstrating that the protein was efficiently targeted to chloroplasts. To investigate whether the fusion protein was also targeted to mitochondria, *Arabidopsis* root cells were analyzed. Using Mitotracker as a fluorescent marker for mitochondria, it was clear that the detected GFP signal in plastids of root cells did not overlap with the red Mitotracker fluorescence signal (Fig. 6b lower panel). In transgenic lines harboring the GFP control construct (pK90-1GFP), the fluorescence was observed in the cytoplasm, corroborating the principally cytosolic localization reported for wild-type GFP (Fig. 6b, upper panel).

Exogenously *Hsp90* mRNAs resemble the corresponding endogenous mRNAs

In order to investigate how the abundance of *AtHsp90-5*, *AtHsp90-6L*, and *AtHsp90-6S* was regulated under control or heat-stress conditions, we determined the levels of the corresponding transcripts using semiquantitative RT-PCR on RNA isolated from 28-day-old plants. *AtHsp90-1* mRNA levels were also monitored (Fig. 7a). Whereas a strong *GAPDH*-derived band was observed, neither *AtHsp90-5*- nor *AtHsp90-6*-derived transcript was detectable after 25 cycles of PCR (data not shown). After 30 cycles of PCR amplification, *AtHsp90-6S* mRNA was detected under heat-stress conditions whereas after 35 cycles, both *AtHsp90-6L* and *AtHsp90-6S* mRNAs were amplified under control or heat-shock conditions. The results indicate that the expression profile of both transcript forms is similar; however *AtHsp90-6S* was expressed at higher levels under the conditions tested. *AtHsp90-5*

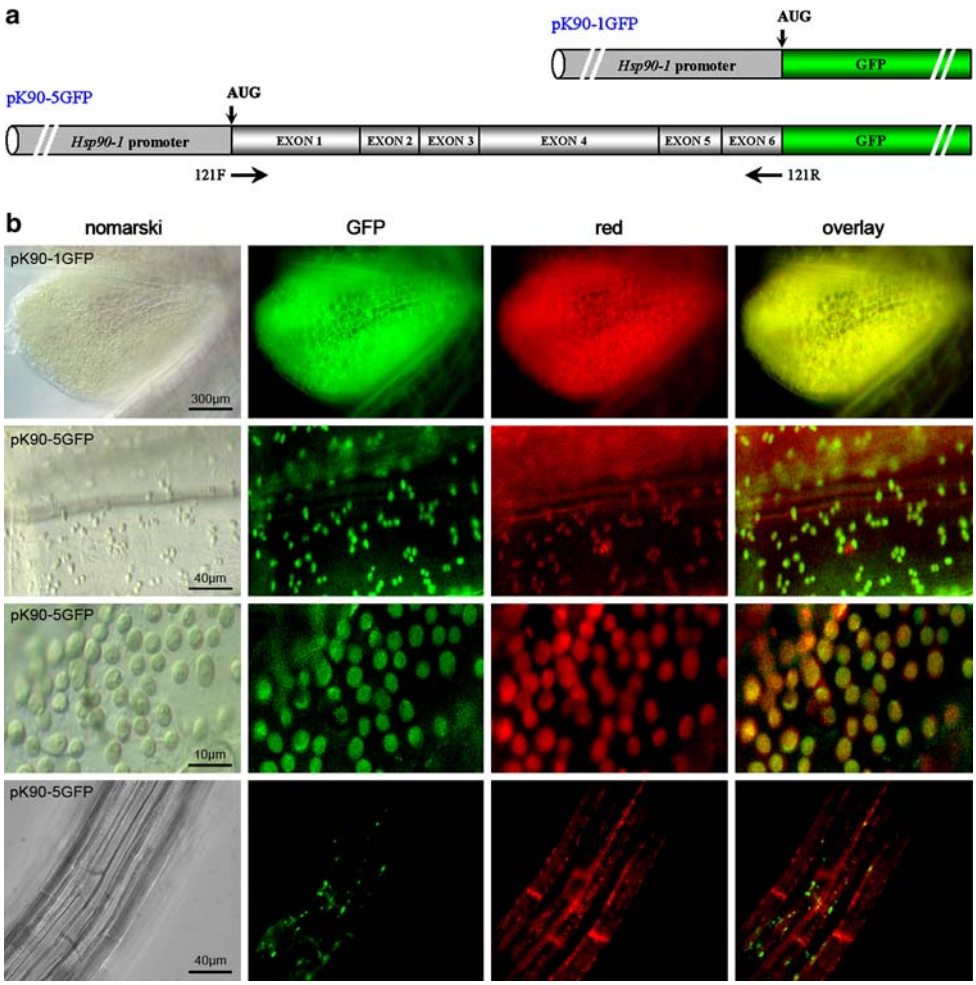


Fig. 6 In vivo targeting of the AtHsp90-5::GFP fusion protein into *Arabidopsis* chloroplasts. (**a**) Depiction of pK90-1GFP (control) and pK90-5GFP fusion constructs used for stable transformation of *Arabidopsis thaliana* plants. Expression was controlled by the *AtHsp90-1* promoter. (**b**) Plant tissues were inspected by differential interference contrast (DIC) microscopy and fluorescence signal was detected by epifluorescent microscopy. The red channel shows autofluorescence of chlorophyll (upper three panels) in photosynthetic tissues and Mitotracker fluorescence (lower panel) in roots. The merged images of GFP and chlorophyll autofluorescence confirm the plastidic targeting of the AtHsp90-5. In roots, GFP plastidial localization did not co-localize with Mitotracker stained mitochondria. GFP alone (pK90-1GFP) shows the characteristic fluorescence in the cytosol

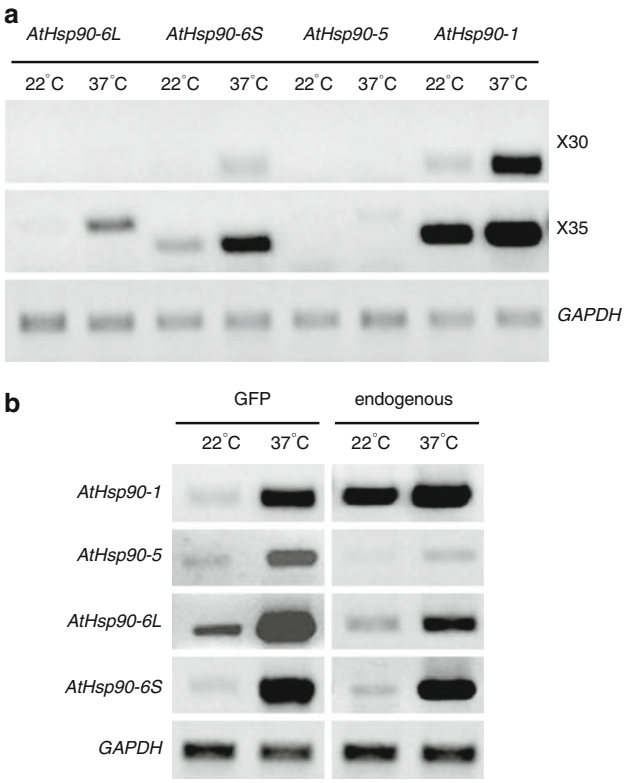


Fig. 7 Expression patterns of the organellar *AtHsp90* genes (**a**) Semiquantitative RT-PCR analysis of endogenous *AtHsp90-1*, *AtHsp90-5*, *AtHsp90-6L*, and *AtHsp90-6S* expression levels under normal or heat-stress conditions as detailed in the 'Materials and methods' section. PCR amplification was carried out for 30 or 35 cycles. (**b**) Semiquantitative RT-PCR analysis of *Hsp90* genes (endogenous) or GFP (GFP) driven by the AtHsp90-1 promoter alone or in combination with the respective transit peptide sequence. RNA was isolated from 4-week-old *Arabidopsis* transgenes harboring pK90-1GFP (control), pK90-5GFP, pK90-6LGFP, and pK90-6SGFP under normal or heat-stress conditions. In both (**a**) and (**b**) the *Arabidopsis GAPDH* gene was used as an internal control for normalization

expression levels were low under control or heat-shock conditions. As shown previously, *AtHsp90-5* mRNA levels were almost undetectable in mature plants, suggesting that the expression of *AtHsp90-5* is developmentally regulated (Cao et al. 2003).

To determine whether the expression pattern of the fused genes in the transgenes harboring the constructs pK90-1:GFP, pK90-5:GFP, pK90-6L:GFP or pK90-6S:GFP resembled that of the corresponding endogenous *AtHsp90* mRNA levels, RT-PCR analysis was performed. Twenty-eight-day-old transgenic *Arabidopsis* plants harboring a single transgene copy were exposed to 37°C for 0 or 2 h. RNA was reverse-transcribed from a pool of ten independent transgenic lines and the expression profiles of the *AtHsp90-1*, *AtHsp90-5*, *AtHsp90-6L*, and *AtHsp90-6S* were determined. Transcription patterns of the GFP gene constructs and the corresponding endogenous genes were similar for both GFP transgenes and wild-type plants (Fig. 7a, b). However, in transgenic plants, the GFP fusion transcript levels were higher than the endogenous under heat-shock conditions, suggesting a stronger interaction of the *AtHsp90*-1 promoter to Hsfs and/or a difference in mRNA stability (Salvador et al. 2004).

Discussion

In *Arabidopsis* the organellar Hsp90 subfamily of proteins consists of two members, designated AtHsp90-5 (At2g04030) and AtHsp90-6 (At3g07770). In this paper we show that *AtHsp90-5* encodes a protein localized specifically to the chloroplasts but not to the mitochondria, while *AtHsp90-6* encodes two different gene products, both localized into mitochondria. Chloroplasts and mitochondria are remnants of free-living prokaryotes that lost their autonomy during evolution by establishing an endosymbiotic relationship with their host cells. However, it has been suggested that none of the organellar Hsp90s were derived from endosymbiotic events during eukaryote evolution, in marked contrast to Hsp60 and Hsp70 (Stechmann and Cavalier-Smith 2004).

Alternative transcript initiation and alternative splicing are two notable mechanisms for subcellular localization (Silva-Filho 2003). A number of alternative transcription events have been reported in plants (Obara et al. 2002; Wachter et al. 2005; Parsley and Hibberd 2006). The results obtained by primer extension analysis are consistent with the notion that the *AtHsp90-6* gene generates at least two transcripts, allowing the production of a second, previously undetected mRNA that encodes a novel Hsp90 isoform (named AtHsp90-6S). The first exon of the long transcript is identical to that predicted for the *AtHsp90-6* gene deposited in the genome database. The alternative transcriptional initiation start site of the short transcript was identified within the first intron of the long form. The occurrence of the *AtHsp90-6L* and *AtHsp90-6S* mRNAs was demonstrated by cloning of the corresponding cDNAs. To evaluate whether the observed transcript heterogeneity is a unique feature of *A. thaliana*, computational search for expressed sequence tags (ESTs) from different plant species was performed. The presence of two *Hsp90-6*-like transcript populations derived from the *mtHsp90* (Os12g32986) gene was identified in the rice genome sequence database (http://www.gramene.org/Oryza_sativa/geneview). The overall genomic structure of the Os12g32986 rice gene resembles that of the *AtHsp90-6* gene from *Arabidopsis*. Alignment of the amino acid sequences of AtHsp90-6 and the Os12g32986 protein product revealed 84.4% homology and 91.5% similarity at the amino acid level. Some collinearity exists between the rice and

Arabidopsis genomes and initial comparative microarray analysis has revealed some basic similarities between the two transcriptomes (Ma et al. 2005). The above observation indicates that an alternative transcription/translational pattern may be conserved in organellar *Hsp90* genes of monocotyledonous and dicotyledonous plants. However, the biological significance of this phenomenon in plants has yet to be determined. This different transcriptional regulation may facilitate spatial and temporal regulation of *Hsp90* gene expression, fine-tune Hsp90-6 protein function at different stages of development, and/or provide adaptive responses to the environment. In animal systems, alternative transcription of the *Hsp70-1* gene might be primarily linked to the pathophysiology of human depression or secondary to specific pleiotropic effects (Shimizu et al. 1999). It has been postulated that multiple transcription initiation sites and alternative splicing events are frequently used to create diversity and flexibility in the regulation of gene expression. Global analysis of alternative splicing events has revealed that about 12–20% of *Arabidopsis* nuclear genes produce multiple RNAs (Wang and Brendel 2006). In the case of the *SHD* gene (At4g24190), encoding an ER-targeted Hsp90, genome-wide analysis of pre-mRNA splicing in *Arabidopsis* revealed the presence of different transcript populations due to alternative terminal (AT) exon splicing (Iida et al. 2004). Maize mitochondrial *Hsp22* has also been shown to be subject to alternative intron splicing (Lund et al. 2001).

In silico studies on promoter *cis*-elements could allow the functional dissection of the *Arabidopsis* mitochondrial *Hsp90* gene. In mammals when cells are exposed to stress conditions, CHOP (GADD153), which belongs to a bZIP transcription factor family, has been shown to activate nuclear genes encoding mitochondrial stress proteins (Zhao et al. 2002). The modification of nuclear gene expression in response to changes in mitochondrial status has been termed mitochondrial retrograde regulation (MRR). Although little is known about the mechanism of communication between mitochondria and the nucleus in plants, it has been reported that MRR can occur during heat stress (Yu et al. 2001; Rhoads et al. 2005). An MSR-like *cis*-acting regulatory element was found in the promoter region of the *Arabidopsis AtHsp90-6* gene, suggesting that *Hsp90-6* could potentially be regulated by an *Arabidopsis* CHOP-like transcription factor.

Our investigation has indicated differential accumulation of *AtHsp90-6L* and *AtHsp90-6S* transcripts in heat-stressed *Arabidopsis* plants, implying that AtHsp90-6 may fulfill multiple roles. The expression of *AtHsp90-6* or *AtHsp90-5* is strongly induced in response to a number of stress treatments, including heat, arsenite, and light treatment (Milioni and Hatzopoulos 1997; Cao et al. 2003). A range of defense strategies such as protection of existing mitochondria's matrix enzymes by the synthesis of soluble protein-folding molecular chaperones such as Hsp90 appears to exist in plants, which could potentially help the organism to minimize the damage generated by various stress conditions (Taylor et al. 2005). However, it is plausible that developmental processes could be involved in the regulation of *AtHsp90-5* (Cao et al. 2003) or *AtHsp90-6* expression.

The subcellular localization analysis provided evidence for an organellar role of AtHsp90-5, AtHsp90-6L, and AtHsp90-6S proteins. Previous work has shown that the in vitro translation product of *AtHsp90-5* was imported into the pea chloroplast stroma compartment (Cao et al. 2003). Herein, we demonstrated *in planta* the chloroplastic localization of the protein by using a partial *AtHsp90-5* cDNA fused to GFP. Furthermore, we showed that AtHsp90-6L and AtHsp90-6S were targeted into the same cellular compartment. Both proteins could import the fused GFP into mitochondria. Although AtHsp90-6L signal peptide has the characteristics of a mitochondrial presequence, AtHsp90-6S did not contain any mitochondrial localization signal. This indicates that the presence of AtHsp90-6S in mitochondria may be due to the existence of an unconventional mitochondrial-sorting signal. Independent support of Hsp90-6 localization into *Arabidopsis* mitochondria is provided by proteomic studies using analytical methods (Heazlewood et al. 2004; Millar et al. 2005). However, an in-depth inspection of the pK90-6SGFP micrographs showed that a number of green fluorescent spherical bodies did not stain with the MitoTracker Orange. Since the Hsp90 function is essential for basic cellular activities, it is anticipated that this chaperone could be present in most compartments (organelles) of the cell. In plants, mitochondria are known to contain the chaperones Hsp70, Cnp60, small Hsps, and a form of Hsp100 (Clp), which are involved in protein import, remodeling, folding, and assembly (Sigler et al. 1998; Merlin et al. 1999; Sun et al. 2002; Peltier et al. 2004). However, mitochondrial Hsp90 has been thus far characterized only in animals but not in yeast or plants. In mammals, the Hsp90-related protein tumor necrosis factor-associated protein 1 (TRAP1) is primarily a mitochondrial matrix protein. However, it has also been localized to various extramitochondrial sites such as secretory granules, nuclei, and at the cell surface (Cechetto et al. 2000). Furthermore, in differentiating *Dictyostelium* prespore cells TRAP1 is located in the prespore-specific vacuole (PSV), a unique cell-type-specific organelle (Yamaguchi et al. 2005). TRAP1 has been implicated in protecting mitochondria against damaging stimuli via a decrease of reactive oxygen species (ROS) production (Im et al. 2007). Hsp90 chaperone does not act to fold nonnative proteins but rather binds to substrate proteins at a late stage of folding (Pearl and Prodromou 2002) and accompanies proteins involved in signal

transduction (Zhao et al. 2005). Glucocorticoid receptors (GRs) have been shown to be located within the mitochondria, eliciting apoptosis in some animal cell types (Sionov et al. 2006). It is plausible that GR is transported to the mitochondria by a heat-shock protein, as its ligand binding (558–580) domain overlaps with one of its Hsp90 binding sites (Schaaf and Cidlowski 2002). Although it is alluring to speculate that the *A. thaliana* mtHsp90 may function in a mitochondrial stress-response mechanism that acts to prevent cellular damage and to re-establish cellular homeostasis or sustain the function of proteins participating in intracellular signal transduction networks, further studies are required to relate this molecular chaperone to specific targets.

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