Tight regulation of expression of two Arabidopsis cytosolic Hsp90 genes during embryo development

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Abstract
The spatial and temporal distribution of expression of two cytosolic members of the AtHsp90 gene family was assessed during early development. In stressed transgenic plants bearing the AtHsp90-3 promoter, β-glucuronidase (GUS) activity was strong in meristematic tissues. Expression was also detected in vascular tissues, leaf veins, siliques, and in pollen sacs. The promoter induced gene expression after heat shock in a time-course dependent manner. AtHsp90-1 promoter activity was low throughout the early stages of embryo development but high just before embryo maturation, with expression most prominent in cotyledons. AtHsp90-3 promoter activity was almost constant and restricted to the root and the cotyledon tips of the embryo. This highly specific spatial distribution of GUS activity changed when the tissues were heat-stressed. Both promoters were also active in unstressed mature pollen grains and during pollen germination. The results shown here indicate that different regulatory and developmental mechanisms control and differentiate the expression of the two cytosolic members of the Arabidopsis AtHsp90 gene family under normal conditions. The developmental and restricted pattern of expression of the AtHsp90-1 and −3 gene promoters in unstressed transgenic plants suggest prominent and distinctive roles of these two genes during different developmental processes.

Key words: Arabidopsis, Hsp90 genes, pollen and embryo expression, promoter analysis.

Introduction
Immobile organisms like plants respond to various acute environmental changes by modulating the expression pattern of a number of genes. This altered pattern results in altered biochemical and physiological activity of the cell and developmental pursuit of the organism. A new expression pattern is driven by the biosynthesis of heat shock proteins (Hsps) when plants are exposed to high temperatures (for review, see Lindquist and Craig, 1988; Vierling, 1991; Miernyk, 1999). The heat-shock response in plants is similar to that of other organisms. The induction of heat-shock genes is known to be regulated mainly by the trimerization and activation of heat shock factors (HSFs). HSFs act in the promoter region of the Hsp genes through a well-defined and highly conserved heat shock element (HSE). At least three core units are required for efficient HSF binding (Amin et al., 1988; Xiao and Lis, 1988; Schoeffl et al., 1998). The contribution of individual HSEs, and their recognition by distinct protein factors during heat shock or development has been observed and analysed (Yabe et al., 1994; Mars and Sinibaldi, 1997). Moreover, several well-characterized elements (i.e. CCAAT-box and STRE elements, scaffold attachment regions: see Rieping and Schoeffl, 1992; Chinn and Comai, 1996; Haralampidis et al., 2002) have been shown to contribute quantitatively to the expression of different classes of heat shock genes.

Hsps are not only present after stress stimulation. The same proteins have been shown to be essential components of cells and developmental processes under normal physiological conditions (Rutherford and Lindquist, 1998). Accumulating results reveal that most Hsps serve as molecular chaperones (Georgopoulos and Welch, 1993; Bukau and...
Horwich, 1998; Pratt et al., 2001). The Hsp90s are abundant cytosolic proteins in eukaryotes and are highly conserved. Unlike other heat shock proteins, the chaperone activity of the Hsp90 is exerted on a number of target substrates or client proteins including steroid hormone receptors, signal transduction pathway components, cell cycle kinases, proteolytic machinery, and microtubule dynamics (Czar et al., 1997; Nathan et al., 1997; Garcia-Gardena et al., 1998; Holt et al., 1999; Pratt et al., 2001). However, the high abundance of Hsp90 compared with that of its client proteins, the high levels of expression in response to stress, and the existence of endoplasmic reticulum (ER), chloroplasts, and mitochondria homologues suggests that this is a very narrow view of the Hsp90 cellular activity and further indicates that it may contribute to additional functions in the cytosol and organelles under physiological or stress conditions.

Several members of the plant Hsp90 gene family have been isolated. Studies on the expression of Hsp90 genes revealed that some members are bona fide heat shock genes while others are constitutively expressed. Chemical treatments such as cadmium or arsenite induce the expression of AtHsp90 genes (Takahashi et al., 1992; Milioni and Hatzopoulos, 1997). In addition, some members have been shown to be specifically expressed during embryogenesis (Marrs et al., 1993), seed germination (Reddy et al., 1998), in shoot and root meristematic apices (Koning et al., 1992), in flowers (Takahashi et al., 1992; Krishna et al., 1995), and during pollen grain development (Marrs et al., 1993; Haralampidis et al., 2002).

Recently, there has been growing evidence that Hsp90s participate in different developmentally, hormonally, and morphogenetically regulated processes (Dhaubhadel et al., 1999; Ludwig-Muller et al., 2000; Berardinini et al., 2001; Ma et al., 2002; Muessig et al., 2002). Notably, inactivation of Hsp90s reveals cryptic genetic variation indicating that Hsp90 machinery plays an important role in buffering capacity of genetic variation (Queltisch et al., 2002), consistent with the role for Hsp90s in maintaining the structure and function of key elements of signal transduction pathways. A similar buffering capacity has also been observed in Drosophila (Rutherford and Lindquist, 1998). The above data suggest that dedicated Hsp90s should be specifically synthesized as a result of differential regulation and expression in response to environmental and developmental processes. In concert with this, the Arabidopsis genome contains seven members of the AtHsp90 gene family. Four of these have a cytosolic localization while the other three have been predicted in silico to be translocated into ER, mitochondria, and plastids (Milioni and Hatzopoulos, 1997; Krishna and Gloor, 2001). The chloroplastic member of the Hsp90 gene family has been shown recently to possess a pronounced role in plastidial development (Cao et al., 2003). Since the expression of the heat shock genes is known to be regulated mainly at the transcriptional level, it is important to unravel the cis elements within the promoter region that regulate specific and developmental expression.

In the present study, by using transgenic plants and the β-glucuronidase (GUS) reporter gene system, the contribution of promoter dynamics to the expression of the Arabidopsis AtHsp90-3 gene was explored under normal conditions or heat stress. Furthermore, a quantitative and qualitative study of AtHsp90-1 and AtHsp90-3 expression during development has been assessed in Arabidopsis transgenic plants under normal conditions.

Materials and methods

Plant material and growth conditions

A. thaliana (Columbia) plants were used in all transformation experiments. Wild-type and transgenic plants were grown under standard conditions at 22 °C, 50% humidity in a light/dark cycle of 16/8. Seeds from individual transgenic plants were imbibed at 4 °C overnight, surface-sterilized for 2 min with 70% ethanol and 5 min with 15% sodium hypochlorite containing 0.1% Tween 20. After several washing steps with sterile deionized water, seeds were germinated 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 development. Mature pollen grains were germinated according to Hodgin (1983).

Plasmid construction and plant transformation

A 1.8 kb BglII genomic fragment, containing the promoter region, the first exon and part of the first intron (AJ010947) of the A. thaliana AtHsp90-3 gene (Milioni and Hatzopoulos, 1997) was cloned into the BamHI site of the pBluescript Stragenate(La Jolla, CA) vector. The promoter region was recovered by PCR reaction. The Kas −40 primer (from the pBluescript) was used as forward and the specific primer 5’-TGTGCTAGATCGGA-3’ (PRIM161) as reverse was used to remove the initiation of the translation start codon (ATG) from the native AtHsp90-3 gene. A fragment of approximately 700 bp was amplified, cloned into PGEM-T vector and its sequence was determined by dideoxy-nucleotide sequencing using the Sequenase 2.0 sequencing kit. Double digests with the restriction enzymes BamHI and HindIII of both the 700 bp cloned promoter region and the binary vector pBI101.1 (Clontech, Palo Alto, CA) were used for directional cloning into the binary vector upstream of the GUS reporter gene. The plasmid pBI161G was recovered and verified. Routine DNA manipulations were carried out as described by Sambrook et al. (1989).

The pBI161G was introduced into the Agrobacterium tumefaciens strain C58C1::pGV2260 by the direct transfer method (An et al., 1988). A. thaliana (Columbia) plants were transformed using the in planta Agrobacterium infiltration method as described (Bechtold et al., 1993). For AtHsp90-1 promoter gene analysis, transgenic plants bearing the pK1445 construct containing the 1445 bp upstream sequence of the AtHsp90-1 gene fused to GUS was used (Haralampidis et al., 2002).

Heat-stress treatment

Transgenic plants were germinated on MS medium plates containing 50 mg l⁻¹ kanamycin. Seedlings and flowering plants were heat-shocked for various times at 37 °C or for 1 h at various temperatures. Five-day-old seedlings were incubated at 22 °C for 6 h in liquid MS medium containing 10 mM of Na₂HAsO₃·7H₂O. After each treatment material was either frozen in liquid nitrogen and kept at −80 °C until further use or treated to histochemical GUS staining.

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I-PCR and RT-PCR analysis

Genomic DNA was isolated from independent T۳ A. thaliana plants using the CTAB method (Rogers and Bendich, 1994) and digested (6 μg) with the restriction enzyme HindIII. There is only one HindIII recognition site within the T-DNA region of the pBI161G binary vector at the distal end of the AtHsp90-3 promoter. Digested DNA was purified and self-ligated (1 μg) in 250 μl reaction volume using 40 units of T۴ DNA ligase. Circularized DNA was purified and used for I-PCR reactions (100 ng) in PTC-200 Peltier Thermal Cycler (MJ Research), for 30 cycles and 7 min extension time. Two sets of primers were used: (i) 5′-TCATGGGCAAGTTTTATGGAATG-3′ (BEGSTA) and 5′-GGCAAGGAGGCAATGATCAACACT-3′ (BEFGIN) containing the initiation and stop codons of the GUS gene, respectively. (ii) 5′-CTCGTGTGGCCCC-AGTCATAGCCGAAATAGGATGACGCTGCACG-3′ (BEPTII) and 5′-GTATGGTTGTTGTGGGAATGTGGACGCTGCACG-3′ (POL2466) containing the initiation codon of the NPTII gene and a sequence from T۴ OS 2466 bp from the right border of pBI161G and to the left of the HindIII recognition site, respectively. The products amplified from the first set of primers would be expected to contain, besides the NOS terminator and the 700 bp AtHsp90-3 promoter region, genomic DNA from the transformed plants and should be at least 1700 bp in size. The products amplified from the second set of primers should consist of the NOS promoter, the T-DNA right border, and genomic DNA from the transformed plants, and should have a size of at least 450 bp. To avoid any false positive amplified fragments, amplified DNA using the first set of primers was digested with HindIII which cuts only once in the T-DNA region. A 700 bp fragment consisting of the promoter region of AtHsp90-3 would result when the correct positive amplified fragments were digested with HindIII.

Total RNA was isolated from different plant tissues using a phenol–chloroform extraction procedure (Haralampidis et al., 2002). RNA concentration was determined spectrophotometrically (At5g52640.1) has an extra intron not found in the other three cytosolic members (Milioni and Hatzopoulos, 1997). AtHsp90-2 and AtHsp90-4 have a head-to-head orientation and are separated by a 1.2 kb intergenic region while AtHsp90-3 has the same orientation as AtHsp90-2 (Milioni and Hatzopoulos, 1997).

Approximately 700 bp of the AtHsp90-3 promoter (position −563 to +96) was analysed. This fragment (Fig. 1) contains a sequence of 563 bp upstream of the putative initiation transcription site and 96 bp of the 5′ untranslated region up to the first codon. In order to search the Arabidopsis AtHsp90-3 promoter region for potential binding sites of regulatory transcription factors, the transcription factor databases Genomatix and TRANSFAC were used and analysed in silico using specific motifs. In addition, a region of approximately 1 kb upstream of the −563 bp promoter region was also analysed. This region is located between the AtHsp90-2 and AtHsp90-3 genes and contains the coding region of the At5g56020 putative gene (TAIR database). A putative TATA box (TATAAT) was detected at position −32, upstream of the putative initiation transcription site. Proximal to the putative TATA box, two AT-rich regions (92%) were found. Another AT-rich region (93%) located at around −500 was also observed. About 1500 bp upstream sequences were analysed for putative transcription factor-binding sites, i.e. consensus motives for HSF (Wu, 1995). The promoter region up to −563 bp was also searched for C/EBP (Akira et al., 1990), STRE

Fluorometric and histochemical GUS assays

Independent transgenic plants bearing one or two copies of AtHsp90-3 were selected and used for quantitative or qualitative GUS assays. T۳, T۴ or T۵ transgenic Arabidopsis lines in which the insert segregated homozygously were used. Quantitative GUS assays were carried out essentially as described by Jefferson (1987) on the transgenic plants. Following heat shock, young seedlings were immediately homogenized in 50 μl ice-cold phosphate buffer (50 mM sodium phosphate pH 7, 40 mM 2-mercaptoethanol, 10 mM Na۴ EDTA). The samples were centrifuged for 5 min at 4 °C and GUS activity was measured under standard conditions using buffers containing 4-methylumbelliferon-β-d-glucuronide (MUG; Sigma, St Louis) with the L550B fluorometer (L550B; Perkin Elmer Instruments, Norwalk, CT). Standard curves were prepared with 4-methylumbelliferone (4-MU, Sigma). Specific GUS activity is expressed as nmol 4-methylumbelliferone (4-MU) produced mg⁻¹ protein min⁻¹. The experiments were repeated three times on five independently transformed plants. One-way ANOVA was used to determine whether differences between means were statistically significant at P ≤ 0.05. AtHsp90-1 promoter-driven GUS quantitative analysis was previously reported (Haralampidis et al., 2002).

Histochemical staining for GUS activity was performed on embryos, siliques, seedlings, mature parts of the plant, and pollen grains at different stages of development using 5-bromo-4-chloro-3-indolyl-β-d-glucuronide (X-glu) as a substrate (Jefferson, 1987). Tissues were stained at 37 °C in X-Gluc reaction buffer (50 mM sodium phosphate buffer pH 7.2, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 2 mM X-gluc), for the time periods indicated, dehydrated by series of ethanol washes and kept in 3.7% formaldehyde, 50% ethanol, and 5% acetic acid at 4 °C before being photographed.

Results

Analysis of the AtHsp90-3 promoter sequence

The Arabidopsis AtHsp90-1 to −4 genes are localized on chromosome V. The three highly similar genes AtHsp90-2 (At5g56010.1, TAIR database), −3 (At5g56030.1), and −4 (At5g56000.1) are localized within a chromosomal region of about 15 kb. AtHsp90-1 (At5g2640.1) has an extra intron not found in the other three cytosolic members (Milioni and Hatzopoulos, 1997). AtHsp90-2 and AtHsp90-4 have a head-to-head orientation and are separated by a 1.2 kb intergenic region while AtHsp90-3 has the same orientation as AtHsp90-2 (Milioni and Hatzopoulos, 1997).

The steady-state levels of AtHsp90-1 and AtHsp90-3 transcripts were estimated in various tissues. The AtHsp90-1 primers used for RT-PCR analysis were 5′-CGCATGTTTCCATGCGCTGTCG-3′ (forward) and 5′-AGCAAGTAAAGAAACCACC-3′ (reverse). The AtHsp90-3 primers used were 5′-GTATGGCTGAGCTGCAAACAT-3′ (forward) and 5′-GTTGGTTGTGTAAGTATGTCG-3′ (reverse). As a control, a part of the coding region of the Arabidopsis β-tubulin gene (At5g12250, TAIR database) was amplified with the specific forward primer 5′-GGAATCTTCTAGATCTGCG-3′ and the reverse 5′-GGCTCAACAGTCTGCA-3′. PCR products were performed using the specific primers and gene-specific or tubulin primers and carried out for different numbers of cycles in order to ensure that reactions remained in the log-linear range.

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The upstream region up to −563 bp of the AtHsp90-3 gene contains one HSE (Fig. 1A) that conforms to the canonical heat-shock consensus of three core units of the repeating pentanucleotide sequence 5′-uGAAu-3′, arranged in alternate orientation (Amin et al., 1988; Xiao and Lis, 1988). This element is located 100 nucleotides upstream of the putative initiation transcription site. The heat-shock element (aGAAcgaTCtaGAAg) consists of two perfect and one imperfect units. This HSE is very close to the putative TATA-box and in silico analysis showed that it is present only once within the 1500 bp upstream sequences.

Within the promoter region (up to −563 bp), three perfect CCAAT sequences representing the binding sites for the C/EBP transcription factors are also present at positions −207, −465, and −521 bp (Fig. 1A). It has been shown that CCAAT enhancer sequences act co-operatively with HSEs to increase promoter activation (Williams and Morimoto, 1990; Rieping and Schoeffl, 1992).

MREs have been identified in a number of heavy metal-induced promoters in animal and plant metallothionein genes (Karin et al., 1987; Culotta and Hamer, 1989; Whitelaw et al., 1997). Since the AtHsp90-3 gene responds to heavy metal stress (Milioni and Hatzopoulos, 1997), the promoter region was searched to identify possible MREs binding sites that would suggest the involvement of metals in its transcriptional regulation. One MRE-like box (TGCAGAC) matching six of the seven nucleotides of the consensus sequence TGCPuCNc (Culotta and Hamer, 1989) was identified at position −258 bp (Fig. 1A).

**Determination of transgene copy number**

The pBI161G construct was introduced into Arabidopsis via Agrobacterium tumefaciens-mediated in planta transformation. Thirty-six independent T2 transgenic lines were generated. Figure 2 shows the results of an analysis of six lines. In order to determine the transgene copy number, DNA was digested with HindIII, self-ligated, and further amplified using inverse specific primers. The transgenic DNA was also used to amplify fragments using a second set of specific primers. Three transgenic plants (A, B, and F, Fig. 2) contain one copy of the chimeric construct, two plants (D and E, Fig. 2) contain two copies and one plant (C, Fig. 2) most likely contains three copies. The digested fragment of 700 bp representing the promoter region of AtHsp90-3 gene was present in all six transformants, indicating the integrity of the promoter in the transgenic plants.

**Activity of AtHsp90-3 promoter under stress conditions**

AtHsp90-3 gene transcripts are present in Arabidopsis plants grown under normal conditions. Nevertheless,
substantially elevated AtHsp90-3 transcript levels were observed in heat-stressed plants (Milioni and Hatzopoulos, 1997). In order to investigate the temporal distribution of AtHsp90-3 promoter-driven gene expression, GUS activity was assayed in vitro and greenhouse-grown Arabidopsis transformants. The levels of GUS activity were assayed quantitatively in five independently transformed 14-d-old seedlings. Figure 3A summarizes the time-course of the fluorometric GUS activity assayed in young heat-shock treated seedlings. The results showed that plants displayed relative high levels of GUS activity under normal environmental conditions, when compared with that observed in AtHsp90-1 promoter-driven gene expression (Haralampidis et al., 2002). The former displayed 1341±846 units while the latter 341±67 units (Fig. 3A). The expression levels increased temporarily and reached the highest measured levels after 3 h at 37 °C. Extended incubation at 37 °C had very little effect on the levels of GUS activity. Previous results had also shown increasing levels of AtHsp90-3 gene transcripts when Arabidopsis seedlings were heat-shocked for 1–3 h (Milioni and Hatzopoulos, 1997). The expression levels in plants treated with heat shock, for 3 h at 37 °C increased 4.4-fold (5909 units). This increase in the level of induction of the reporter gene was substantially lower than the 300-fold increase of the AtHsp90-1 promoter-driven gene expression (Haralampidis et al., 2002). Nevertheless, the quantitatively measured GUS activity of the heat-shock treated transgenic plants bearing the AtHsp90-3 promoter was almost equivalent to that of AtHsp90-1 promoter carrying only one HSE (Haralampidis et al., 2002) (Fig. 3A).

Heavy metal toxicity is known to promote Hsp gene expression. In several species cadmium toxicity induces a number of stress proteins with molecular mass ranging from 10–70 kDa. Cadmium at 1 mM induces the expression of most Hsp90 genes in Arabidopsis plants. Arsenite has also been shown to have a profound effect on the induction of all the Hsp90 genes investigated (Milioni and Hatzopoulos, 1997). Analysis of the GUS reporter gene expression following exposure of AtHsp90-3 promoter-driven transgenic seedlings to 10 mM arsenite resulted in an increased gene expression compared with untreated control plants (data not shown). This increase was almost equivalent to that observed following heat shock.

Developmental expression of both AtHsp90-1 and AtHsp90-3 genes

Hsp90 genes have been shown to be specifically expressed during embryogenesis (Marrs et al., 1993) and during seed germination (Reddy et al., 1998). To determine the temporal and spatial distribution of both AtHsp90-1 and -3 promoter-driven gene expression, GUS activity was assessed in Arabidopsis transformants. Figure 3B summarizes the fluorometric GUS activity determined during seedling growth and embryo development. In order to investigate GUS activity during embryo development, siliques were grouped into four stages according to their sizes. Mature seeds were also examined. The results showed that at early stages of embryo development, the promoter activity of AtHsp90-1 was constant and almost negligible. However, when zygotic embryos were reaching maturation (siliques >1 cm) there was a rapid increase in promoter activity (Fig. 3B). This increase was almost 30-fold and occurred immediately prior to embryo maturation indicating tight regulation of AtHsp90-1 gene expression.

The promoter activity of AtHsp90-3 showed a different profile of expression. A slight but steady increase was detected in siliques of 2–3 mm up to 1 cm in length, and on the average, the activity was almost 7-fold higher than that measured for the promoter of the AtHsp90-1 gene (Fig. 3B). When embryos were approaching maturation (>1 cm), the activity of AtHsp90-3 promoter declined slightly and was almost 5-fold less than that observed for the AtHsp90-1 promoter (Fig. 3B). The above-mentioned results indicate that the two genes are differentially regulated during embryogenesis. To determine GUS activity in mature embryos, these were dissected out and assessed. The activities of both the AtHsp90-1 and -3 promoters were low, indicating a rapid decrease in the expression of both genes during the final stages of embryo maturation. Low activity for both promoters was also observed in the early stages of seed germination and in young seedlings. In 10-d-old seedlings the promoter activity of AtHsp90-3 measured was almost four times higher than that of AtHsp90-1 (Fig. 3B).
In order to determine tissue-specific expression, histochemical analysis was made. While GUS staining was prominent in bundles of siliques, funiculus, and embryos for the \textit{AtHsp90-3} promoter-driven gene, GUS activity from the \textit{AtHsp90-1} promoter–driven gene was detected in only a small proportion of embryos within siliques (Fig. 4). As silique development proceeded, a higher proportion of embryos showed GUS staining driven by the \textit{AtHsp90-1} promoter (Fig. 4). This result corroborates the data presented in Fig. 3B in which \textit{AtHsp90-1} promoter activity increased rapidly in embryos just before maturation.

Embryos from \textit{AtHsp90-1} and -3 transgenic lines were dissected from siliques (>1 cm) and stained. The results showed that in the \textit{AtHsp90-3} promoter, GUS staining was
restricted to two distinct regions, in the root and at the tips of the cotyledons. The promoter-driven GUS staining of \( \text{AtHsp90-1} \) was most prominent in the cotyledons and at a restricted area at the tip of the root (Fig. 4). The tissue specificity of both promoters was lost when plants were heat shocked. GUS staining was prominent in all silique tissues tested, indicating that the tight developmentally regulated expression of both \( \text{AtHsp90-1} \) and \( \text{AtHsp90-3} \) promoters under normal conditions changed radically when tissues were heat shocked (Fig. 4). Furthermore, the highly specific \( \text{AtHsp90-1} \) and \( \text{AtHsp90-3} \) promoter-driven gene expression in embryos changed quantitatively and qualitatively when embryo tissues were heat shocked (Fig. 4) indicating that both genes are regulated spatially and temporally in specific regions of the embryos.

\textbf{Tissue specificity of AtHsp90-3 promoter activity under heat-stress conditions}

Histochemical analysis of heat-shock-treated transgenic plants harbouring the construct of the pBI161G plasmid showed detectable levels of GUS activity. Reporter gene activity was prominent in the root meristematic region of up to 3-d-old heat-stressed germinated seeds (Fig. 5B). In 5-d-old heat-stressed seedlings, high levels of GUS activity were observed mainly in the root, meristems, and vascular system. However, barely detectable levels were also observed in the upper meristematic region (Fig. 5C). Furthermore high levels of GUS activity were particularly prominent at the border of the root and the hypocotyl, in the transition zone where vascular bundle reorganization occurs (Fig. 5C).

In 7-d-old heat-stressed seedlings high levels of reporter gene activity were also present in shoot meristem and cotyledon veins (Fig. 5E). However, the promoter activity was higher in the root meristem when compared with that of the upper meristem (Fig. 5F, G). In mature, stressed plants, significant levels of GUS activity were localized predominantly in flowers, in the stigma, the styles, and especially the anthers and the filaments (Fig. 5H, M). In the developing siliques of stressed plants, high levels of expression were observed in the receptacles and funiculus (Fig. 5K, M). In the leaves of 4-week-old stressed transgenic plants, reporter gene activity was significant in the leaf veins and in regions of active transpiration (Fig. 5I).

\textbf{Expression of AtHsp90-1 and AtHsp90-3 genes during pollen development and germination}

Although unstressed transgenic plants carrying the construct of the pBI161G plasmid cultivated under normal conditions generally showed little GUS activity, high levels of reporter gene activity was detected in developing and mature pollen grains (Fig. 6). Significant GUS activity was also observed for the \( \text{AtHsp90-1} \) promoter-driven reporter gene in pollen grains (Haralampidis et al., 2002) and in released pollen grains (Fig. 6). Pollen was the only part of the \textit{Arabidopsis} flower that showed prominent GUS activity under normal conditions for both the \( \text{AtHsp90-1} \) and \( \text{AtHsp90-3} \) promoter-driven reporter gene.

To examine the \( \text{AtHsp90-1} \) and \( \text{AtHsp90-3} \) expression profiles, reporter gene activity was further assessed during pollen tube development. Pollens were germinated \textit{in vitro} and
in vivo on the stigma of Arabidopsis flowers, and then collected and stained. Histochemical GUS staining was prominent in germinating pollen at almost all stages of pollen tube elongation of the isolated pollen grains (Fig. 6). It was also prominent in germinating pollen on the stigma (Fig. 6). Almost equivalent staining was observed for both the AtHsp90-1 and -3 promoters.

Semi-quantitative RT-PCR analysis showed that the AtHsp90-1 and -3 transcript levels were higher from flowers bearing anthers than from flowers where the anthers were removed, and even higher than those from 20-d-old plants (Fig. 6).

Discussion

The Arabidopsis genome contains a gene family of four cytosolic Hsp90 proteins, all located on chromosome V. Three of these genes AtHsp90-2, -3, and -4 are located very close to each other, share the same intron/exon structure and are highly similar. The cytosolic members of the Hsp90 gene family have been shown to have different patterns of expression (Milioni and Hatzopoulos, 1997). In order to understand the regulatory mechanisms controlling and differentiating the expression of the cytosolic members of the AtHsp90 gene family, a series of promoter fusions were constructed with the GUS reporter gene, and the temporal and spatial expression of the promoter-driven GUS during development or following heat treatment was examined. Since AtHsp90-2, -3, and -4 genes are highly similar in nucleotide and predicted amino acid sequence, the promoter activity of the AtHsp90-3 gene under normal and heat-shock conditions was analysed. Furthermore, the spatial and temporal distribution of promoter-driven GUS from both the AtHsp90-1 and -3 genes during embryo development was compared.

AtHsp90-3 gene transcripts were detected at low levels in untreated plants and were increased in response to heat shock. AtHsp90-1 gene transcripts were undetectable in the absence of stress conditions, but were highly heat-inducible in Arabidopsis plants (Milioni and Hatzopoulos, 1997). Consistent with these results the AtHsp90-3 promoter displayed relatively high GUS activity levels (Fig. 3A) under normal conditions. This activity was almost 5-fold greater than that attributed to the full-length promoter of the AtHsp90-1 gene, and was equivalent to the activity observed for GUS fusions containing approximately half of the full AtHsp90-1 promoter (Haralampidis et al., 2002). These results indicate that different regulatory mechanisms control the activation or repression of AtHsp90-1 and AtHsp90-3 gene expression under normal conditions.
Heat-shock resulted in a 4.4-fold increase in the \textit{AtHsp90-3} promoter-driven reporter gene expression when the transgenes were stressed at 37 °C for 3 h, and the same levels of GUS activity was detected when the plants were treated for up to 24 h. The transcriptional activation of heat-shock genes under heat stress depends on the interaction of HSFs with HSEs, the highly conserved \textit{cis}-acting DNA sequences. All HSEs contain multiple units of the repeating 5 bp consensus sequences 5'-nGAAu-3' arranged in alternate orientation (Amin \textit{et al.}, 1988; Xiao and Lis, 1988). The degree of homology of each of the three pentameric units required for heat-inducible expression to the consensus motif can vary. Barros \textit{et al.} (1992) showed that the G/C bp (G and complementary C) at position one of the unit is more important than the A/T base in the third position. In the \textit{AtHsp90-3} promoter, the HSE deviates from the consensus HSF-binding site only at the second core unit (nATC\textsubscript{n} instead of nTTC\textsubscript{n}). During heat shock, the \textit{AtHsp90-3} promoter activity was substantially lower than that of the \textit{AtHsp90-1} full promoter, but equivalent to that observed for the \textit{AtHsp90-1} promoter containing one HSE element (Haralampidis \textit{et al.}, 2002). In \textit{silico} analysis of the \textit{AtHsp90-3} promoter revealed, in addition to the HSE, CCAAT-boxes and MRE\textit{cis}-regulatory elements known to be involved in a number of stress responses in different organisms. These motifs were found to have quantitative effects on the induction of certain
heat-shock genes. In plants, there is accumulating evidence for the involvement of CCAAT-box elements and HSE in stress-induced transcription (Rieping and Schoeffl, 1992; Schoeffl et al., 1998; Haralampidis et al., 2002). CCAAT-boxes in the AtHsp90-3 promoter may contribute to the induction of the gene under heat stress.

Arsenate treatment showed increased transcript levels of the AtHsp90 genes (Miliioni and Hatzopoulos, 1997). Both heat-shock and arsenite stress share many features at the molecular level inducing a number of Hsps. Both phenomena lead to oxidative stress and to up-regulation of HSF phosphorylation, and hence Hsp induction. However, Elia et al. (1996) suggested that the HSF phosphorylation induced by heat stress is different from that induced by arsenite, implying distinct mechanisms of transcriptional regulation. In Arabidopsis, 21 potential HSF genes have been identified, indicating the complexity of the stress response and the tight regulation of expression (Schoeffl and Prandtl, 1999). MREs have been identified in the promoters of a number of heavy metal-induced genes such as those encoding for human and mouse metallothionein (Karina et al., 1987; Culotta and Hamer, 1989), the mouse and chicken haem oxygenase genes (Alam, 1994; Lu et al., 1998), tomato type II metallothionein gene (Whitelaw and chicken haem oxygenase genes (Alam, 1994; Lu et al., 1998), tomato type II metallothionein gene (Whitelaw et al., 1997), as well as the AtHsp90-1 gene (Haralampidis et al., 2002). The MRE in the AtHsp90-3 promoter, in combination with the HSE, could contribute to the expression of this gene after arsenite treatment.

Both the AtHsp90-1 and -3 genes have distinctive profiles of expression during embryo development. These results showed that AtHsp90-3 promoter activity remained almost constant as the silique growth proceeded or embryos developed. The activity of the AtHsp90-3 promoter was not detected throughout the tissues of the silique or embryo but was restricted to very defined areas, for example, the root and cotyledon tips of the embryos. The activity of the AtHsp90-1 promoter was very low as silique growth proceeded and increased abruptly just before embryo maturation, suggesting a possible coupling to the hormonal status at different embryo developmental stages. It is known that the maturation of the embryos in the silique does not follow a regular pattern. GUS staining revealed that the AtHsp90-1 promoter activity was high and prominent in the cotyledon tissues of embryos approaching maturation in the silique. This highly specific pattern of spatial and temporal expression changed radically when the tissues were heat shocked, indicating that the gene expression follows a developmental profile of tight regulation. Studying the expression profiles of both AtHsp90-1 and -3 promoter-driven reporter gene it might be postulated that the two genes together are expressed more or less in all cells of the embryo, suggesting overlapping functional roles for the products derived from these two genes. This assumption, however, might be simplistic as it suggests that the functional redundancy of the proteins is solely determined by the tissue specificity of cis-

elements present in the promoter regions of the two genes. However, the almost constant profile of AtHsp90-3 promoter activity and the rapid increase in the activity of the AtHsp90-1 promoter just before embryo maturation, in combination with the restricted spatial distribution of expression, suggests that the products of these two genes play distinctive roles during embryo development.

Recently, new roles have been assigned to the Arabidopsis Hsp90-1 and Hsp90-3 proteins. Hsp90-1 has been found to interact with TWD1, a 42 kDa FK506-binding protein with similarity to multidomain PPIases such as the mammalian FKBP51 or FKBP52 proteins known to be components of steroid hormone receptor complexes (Kamphausen et al., 2002). Furthermore, TWD1, a plasma-membrane protein crosslinks with ABC transporters, a family of genes which are involved in polar auxin transport and auxin-mediated development (Geisler et al., 2003; Perez-Perez et al., 2004). These results suggest the participation of Hsp90-1 in multiprotein complexes regulating steroid- and auxin-mediated signalling (Silverstein et al., 1999; Kamphausen et al., 2002; Perez-Perez et al., 2004). In another approach using micro-array screening, the AtHsp90-3 gene has been shown to respond to brassinosteroid treatment (Goda et al., 2002). The above data also show distinctive roles or physiological assignments for the two members of the AtHsp90 gene family.

GUS staining of heat-shock transgenes bearing the AtHsp90-3 promoter was most prominent in the meristematic regions and the vascular bundles of the vegetative tissues. This pattern of GUS staining was similar to that observed with the AtHsp90-1 promoter region containing one HSE element. Meristematic cells are known to have the highest rates of cell division. Since it is reasonable to assume that heat stress may be most detrimental to rapidly dividing cells, the stain in these most vital parts of the seedling may reflect the significant role of AtHsp90-3 as a chaperone.

GUS staining of unstressed mature transgenic tissues revealed high expression of both genes at a level equivalent to that observed in heat-stressed mature transgenic plants only in pollen grains. Both promoters were also active during pollen germination. This observation is consistent with previous results in maize and Arabidopsis (Marrs et al., 1993; Magnard et al., 1996; Haralampidis et al., 2002) indicating the significance of chaperones, and particularly the prominent roles of AtHsp90-1 and -3 in Arabidopsis pollen grain development and germination. However, GUS-stained anthers bearing a promoter region of 473 bp from the AtHsp90-1 gene showed no stain in pollen grains (Haralampidis et al., 2002). This pattern was different from that observed in the AtHsp90-3 promoter transgenes, indicating that distally or proximally located elements within the promoters of the AtHsp90-1 or AtHsp90-3 gene, respectively, are necessary for the induction of expression in pollen. In silico analysis of the AtHsp90-1 and -3 promoters has revealed a number of elements known to be involved in pollen-specific expression.
(Bate and Twell, 1998; Rogers et al., 2001; Hulzink et al., 2003). Whether all of these elements are responsible for the induction of expression in pollen is unknown. The functionality of these sequences regulating gene expression in a pollen-specific manner remains to be tested.

These results indicate that different regulatory mechanisms control the expression of the cytosolic members of the AtHsp90 gene family during embryo development under normal conditions. The developmentally and spatially restricted patterns of expression of the AtHsp90-1 and -3 genes in unstressed transgenic plants suggest prominent and distinctive roles for these two genes during growth and development. Further analysis of the AtHsp90 promoter elements and use of these transgenes as marker lines in crosses with mutants may lead to a better understanding of the regulatory mechanisms controlling the developmental and tissue-specific nature of AtHsp90 gene expression.

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References


