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ORIGINAL RESEARCH

Combinatorial Interactions of Two *cis*-Acting Elements, AT-Rich Regions and HSEs, in the Expression of Tomato *Lehsp23.8* upon Heat and Non-Heat Stresses

Shu-ying Yi · Jian Liu

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Abstract We previously cloned and analyzed the 1,893-bp promoter region (−1,915 to −23) of the tomato (*Lycopersicon esculentum*) *Lehsp23.8* gene, whose expression is induced by treatment with high or low temperatures, heavy metal, or abscisic acid (ABA). In our present work, we examined how this expression is regulated. A comprehensive quantitative promoter deletion and base-substitution analysis was conducted under various environmental conditions. The proximal region (−565 to −23 bp) of the *Lehsp23.8* promoter harbors *cis*-regulatory elements that conferred high levels of heat-induced expression in transgenic tobacco. Mutation of the five proximal HSEs (HSE1 to 5) of that promoter led to an absence of heat inducibility. The AT-rich regions between −255 bp and −565 bp (AT-rich1 to 4) in the promoter might serve as enhancers for such heat-induced expression. Deletion and HSE mutation analysis indicated that other *cis*-acting elements also function in response to low temperature, heavy metal, and ABA and that HSE1 to 5 act at least as *cis*-acting elements in multiple-stress responses of *Lehsp23.8*. These results reveal that those five proximal HSEs and AT-rich regions function interdependently in the expression of *Lehsp23.8* in response to non-heat stresses. Furthermore, the putative elements CRT/DRE, AP-1, and ABRE in that promoter are not required for multiple-stress induction.

Keywords Deletion analysis · GUS activity · *Lehsp23.8* · Promoter · Stress

S.-y. Yi · J. Liu (✉)
College of Life Science, Shandong Normal University,
Jinan 250014, China
e-mail: ljlsd2002@gmail.com

S.-y. Yi
Department of Biochemistry, Taishan Medical College,
Tai'an 271000, China

Abbreviations

ABA	abscisic acid
GUS	β-glucuronidase
HSE	heat shock element
HSF	heat shock factor
HSP	heat shock protein

The generation of agricultural varieties tolerant to a range of growing conditions is a primary goal in biotechnological applications because, in the field environment, plants may encounter different types of stress in combination (Mittler 2006). Gene expression patterns are often similar when plants are exposed to various stresses, which indicate that crosstalk is extensive between signaling pathways (Seki et al. 2002; Sung et al. 2003). Therefore, focusing on those elements that overlap among such response pathways that underlie diverse forms of stress may advance our knowledge of cross-tolerance in plant species (Bowler and Fluhr 2000).

Plants react to elevated temperatures by expressing several families of evolutionarily conserved heat-shock proteins (HSPs) (Baniwal et al. 2004). Induction of heat shock genes is regulated mainly by the trimerization and activation of heat shock factors (HSFs) (Schöffl et al. 1998; Pockley 2001). An HSF acts through a highly conserved heat shock element (HSE), which is defined as the adjacent and inverse repeats of the motif 5′-nGAAn-3′ (Schöffl et al. 1998). The roles of individual HSEs and their recognition by distinct protein factors during heat shock have been analyzed (Marrs and Sinibaldi 1997). Moreover, several well-characterized elements, such as CCAAT-box, STRE elements, and scaffold attachment regions (SARs), have been shown to contribute quantitatively to the expression of different classes of heat shock genes (Haralampidis et al. 2002).

HSPs are involved in the cellular response to various forms of stress, e.g., heat, cold, heavy metals, and oxidation (Swindell

et al. 2007). Although the molecular pathways leading to HSP expression are not entirely understood (Sung et al. 2003), they do involve temperature-perception mechanisms coupled with multiple signal transduction pathways (Larkindale et al. 2005). Under chilling stress, specific *hsp* members are upregulated, such as those in the *hsp70* family in *Arabidopsis* (Sung et al. 2001), *hsp90* family in *Brassica napus* (Krishna et al. 1995), a cytosolic class I *Cshsp17.5* in chestnut (Lopez-Matas et al. 2004), and *hsp110* in rice (Singla et al. 1997). Expression of two sHSP genes, *tom66* and *tom111*, is induced at low temperatures only if first induced by high temperature (Sabehat et al. 1998). At least one CRT/DRE has been found in the promoters for strongly cold-inducible *hsp70* members, including *hsc70-3*, *hsp70*, and *mthsc70-2*. In contrast, CRT/DREs have not been identified within the promoter of the strongly cold-inducible *hsc70-2* (Sung et al. 2001). This implies that the HSF family can act in a gene-specific manner under low-temperature stress. Alternatively, some heat shock genes might be activated by a cold-responsive regulatory system aside from the HSF family. These reports all demonstrate the complexity and divergence of the mechanism for cold response of heat shock proteins.

Lehsp23.8 in tomato (*Lycopersicon esculentum*) expresses under a wide range of stimuli (Banzet et al. 1998; Sabehat et al. 1998; Liu and Shono 2001). This makes it a natural model for developing our understanding of the integration between regulatory networks associated with different kinds of stress. We previous isolated the 1,915-bp promoter region of *Lehsp23.8*, which contains HSEs, AT-rich regions, AP-1, ABRE, and CRT/DRE, as well as a putative TATA box (Yi et al. 2006). This promoter shows obvious activity when plants are treated with heat, low temperature, heavy metal, or exogenous abscisic acid (ABA) (Yi et al. 2006). Because expression of heat shock genes is regulated mainly at the transcriptional level (Wu 1995), it is important to unravel the *cis* elements within the promoter region that regulate specific expression.

Our objective in the current study was to further define the functional *cis* elements in the *Lehsp23.8* promoter. Here, we constructed chimeric genes composed of a series of mutant promoters and the β-glucuronidase (*gus*) gene. Quantitative GUS assays were performed under multiple-stress conditions in transgenic tobacco plants.

Materials and Methods

Plant Material and Growing Conditions

Tobacco (*Nicotiana tabacum* L. c.v. K_{326}) was used in all transformation experiments. Wild-type and transgenic plants were reared in growth chambers at 26/20°C (day/night), under natural illumination.

Construction of Chimerical Promoters

We obtained the DNA clone previously (Yi et al. 2006). Five forward primers (5′-ATTGTCGACAATTAACCCTCACTAAAGGG-3′, 5′-CTAGTCGACATCATCTAGTTACTCTGG-3′, 5′-ATCGTCGACCCTTTTTGTAGGAACTTG-3′, 5′-TAAGTCGACGTTTCTCGTGTTGGATCG-3′, and 5′-TAAGTCGACAAACCCAGAAGCGTTATG-3′; *Sal*I site underlined) and a reverse primer (5′-AGAGGATCCAATAACTTGCCGATTGAG-3′; *Bam*HI site underlined) were used to amplify DNA fragments harboring the *Lehsp23.8* promoter deletions (−1,915∼−23, −1,327∼−23, −871∼−23, −565∼−23, and −255∼−23 bp; numbers indicate the position from the *Lehsp23.8* start codon ATG). Amplified fragments were subcloned into the pBI101 vector (Clontech, Palo Alto, CA), thereby generating five 5′ end-deletion constructs (pK1915, pK1327, pK871, pK565, and pK255). The *gus* gene in recombinant plasmids was controlled by those inserted *Lehsp23.8* promoter deletions.

Three other internal deletions (342, 84, and 105 bp) were obtained by using combinations of appropriate primers and restriction enzymes. Constructs generated in the pBluescript KS background were cloned upstream of the *gus* reporter gene of the pBI101 binary vector. In this way, constructs pK△AT15, pK△AT34, and pK△AT12 were generated.

To obtain the 871-bp *Lehsp23.8* promoter with five mutated proximal HSEs, the sequences between −151 and −219 bp were turned to 5′-CTAGcgGCGTCTTCACaCATCCTCACGCCACAAgTCTGGAgCtcTCTACATtATGCTTCTAAAAGTgTC-3′ (underlining indicates the position of the consensus HSEs; wild-type sequences are in uppercase and introduced mutations are in lowercase). Finally, the HSE-mutated pK*HSE vector was generated.

Tobacco Transformation

The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 by the freeze–thaw method (Holsters et al. 1978). Following the leaf disk transformation protocol (Jin et al. 2001), we introduced the *gus* gene cassettes into tobacco. Plasmids pBI121 and pBI101 were also used to transform tobacco as positive and negative controls, respectively. Tobacco genomic DNA was isolated by the CTAB method (Doyle and Doyle 1990). Transgenic plants were identified via PCR, using a *gus*-specific primer combination (5′-TCGATAACGTGCTGATGGTGC-3′ and 5′-ACCGAAGTTCATGCCAGTCCAG-3′). The expected amplified product of 864 bp was analyzed on 1.0% agarose gels.

Treatments of Transgenic Plants

Independently transformed plants (30 or 42) from each construct were subjected to heat shock treatments for 3 h at

39°C. For each mutant construct, 10 transgenic lines with stronger GUS activities in response to heat were selected for further experiments. Cold stress was applied by exposing those plants to 2°C in a growth chamber for 48 h. For other analyses, transgenic plants were treated with either 1.0 mmol L^{-1} $PbCl_2$ or 0.1 mmol L^{-1} ABA for 6 h at 26°C.

Histochemical and Fluorometric GUS Assays

Histochemical staining for GUS activity was performed in transgenic plants, using 5-bromo-4-chloro-3-indolyl-β-*d*-glucuronide (X-gluc) as a substrate (Jefferson et al. 1987). Tissues were stained for 16 h 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), then dehydrated by a series of ethanol washes, and kept in 70% (*w*/*v*) ethanol at 4°C before being photographed.

Quantitative GUS assays were carried out as described by Jefferson et al. (1987). Fluorometric GUS activity was measured with 4-methylumbelliferyl-β-D-glucuronic acid (MUG; Sigma). Standard curves were prepared with 4-methylumbelliferone (4-MU; Sigma). Protein concentrations in samples were determined with Bradford reagent (Bio-Rad) and BSA as a standard. Specific GUS activities were defined as the number of units of pmol 4-MU produced per milligram of protein per minute. For all stress trials, measurements were repeated three times.

Results

Lehsp23.8 Promoter Mutant Constructs

To define the position and function of *cis* sequences that regulate *Lehsp23.8* expression, we constructed a series of 5′ end and internal deletions of the upstream promoter region and fused them to the *gus* reporter gene. Eight constructs (pK1915, pK1327, pK871, pK565, pK255, pKΔAT15, pKΔAT34, and pKΔAT12) were generated (Fig. 1). In addition, a fragment of promoter region (−871 ~ −23 bp) with five mutated proximal HSEs was fused to the GUS reporter gene (pK*HSE).

Generation of Transgenic Tobacco Plants

The constructed vectors were introduced into tobacco, and plasmids pBI121 and pBI101were used to transform tobacco as positive and negative controls, respectively. More than 30 independent transgenic plants were generated from each construct. These were confirmed by PCR, using a *gus*-specific primer combination. An 864-bp specific fragment was obtained from most transgenics (Fig. 2).

Response of Mutant Promoters to Stress Treatments

To investigate the contribution of specific regulatory sequences in gene expression under stress conditions, we measured GUS enzyme activities in transgenic lines of every construct after heat shock, low-temperature, heavy-metal, or ABA treatments.

Heat Shock

Histochemical staining of transgenic plants harboring construct pK1915, cultivated under control conditions, showed no detectable GUS activity (Fig. 3a, c). Following heat shock treatment, however, strong blue GUS staining was detected in their leaves, shoots, and roots (Fig. 3b). Examination of GUS expression revealed that the *Lehsp23.8* promoter was active in developing flowers after

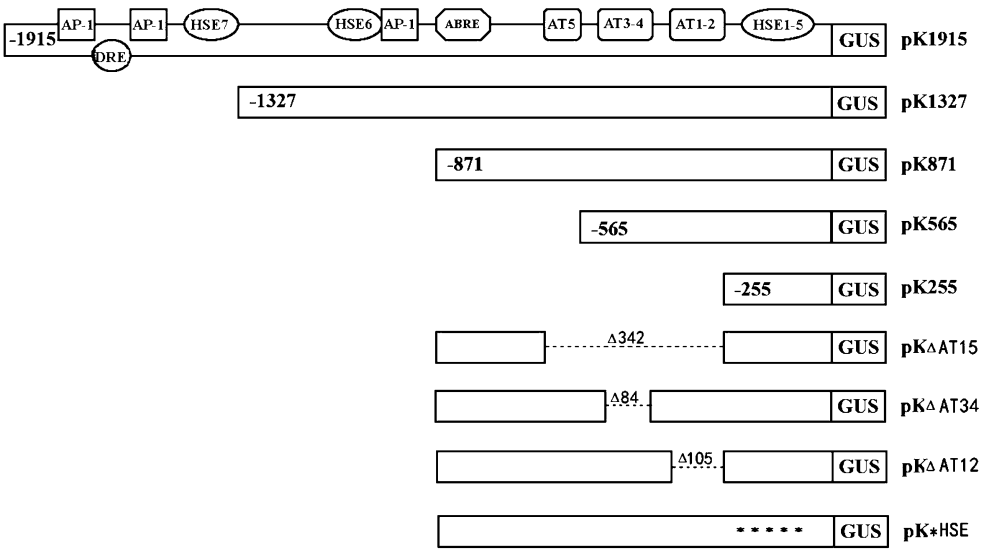

Fig. 1 Schematic structure of *gus* chimeric genes under control of various portions of *Lehsp23.8* promoter. *Numbers* represent positions from *Lehsp23.8* start codon ATG. Locations of putative regulatory elements are indicated. *Horizontal dashed lines* show internal deletions. Five mutant proximal HSEs are marked with *asterisks*. Construct names are on *right side*

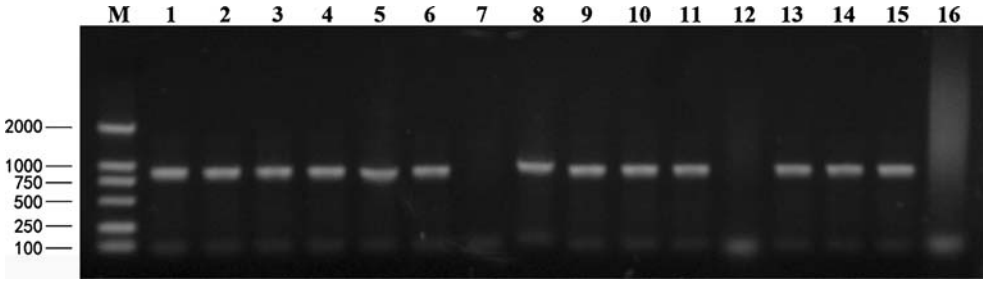

Fig. 2 PCR analysis of partial transgenic tobacco lines. *Lanes 1 to 15*, independent transformants; *Lane 16*, untransformed plants; *Lane M*, DNA marker. *Numbers on left side* are molecular mass standards (bp)

heat shock (Fig. 3d). Staining was most uniform in the earlier stages. However, in fully opened flowers, strong staining was observed in the ovaries, stigmas, sepals, and petals, while none was found in the styles, filaments, and anthers.

After heat shock, the GUS fluorometric activity for 5′ deletion constructs was studied in the leaves of 30 T1 transgenic tobacco lines. Plants harboring each construct had negligible activity under normal conditions, but this activity increased dramatically after heat shock (Fig. 4). Plants carrying the full-length promoter, construct pK1915, had an expression level of 8,328 units after 3 h at 39°C. Deletion construct pK1327 or construct pK871 had only a minor effect on GUS activities when compared with construct pK1915, although the deleted region contained two consensus HSEs (HSE6 and HSE7). A further deletion of the promoter to point −565 (construct pK565) abolished

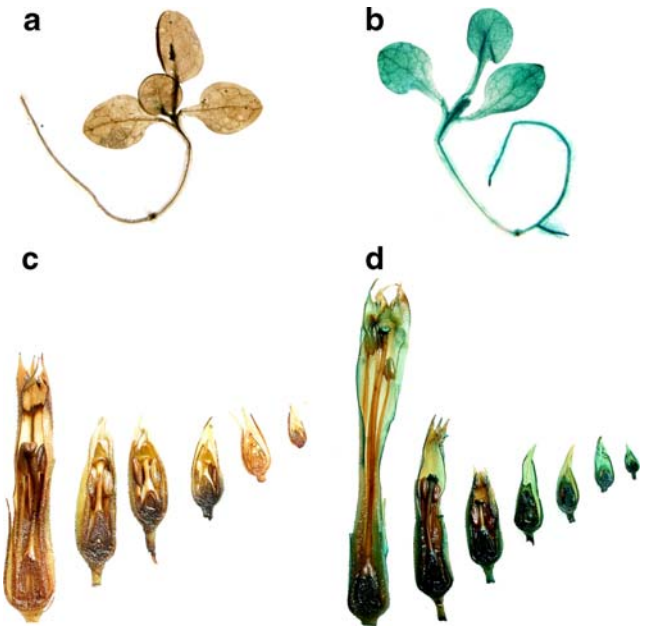

Fig. 3 Histochemical localization for pK1915 transgenic tobacco lines. GUS activity in untreated (**a**) and heat-shocked (**b**) seedlings; GUS activity in developing flowers from unstressed (**c**) and heat-shocked (**d**) plants

one AT-rich region (AT-rich5), resulting in a dramatic increase (approx. 38%) in gene expression (from 8,621 to 11,894 units) compared with pK871, i.e., being 43% higher relative to the full-length promoter. A further reduction in promoter size to −255 (construct pK255) resulted in the abolition of four AT-rich regions (AT-rich1 to 4), leading to a dramatic decrease (50%) in expression (from 11,894 to 5,972 units) compared with pK565. Tobacco plants transformed with the promoterless plasmid pBI101 showed negligible activity. These data indicated that the smallest promoter fragment (−255 ~ −23 bp) had the essential *cis*-acting elements and was sufficient for heat induction. Sequences that included AT-rich1 to 4 between −255 and −565 bp in the *Lehsp23.8* promoter enhanced heat-induced expression of the chimeric gene, while sequences with AT-rich5 between −871 and −565 bp repressed such heat-induced activity of that promoter. The two HSEs between −871 and −1915 bp (HSE6 and HSE7) were not necessary for increasing this activity.

To analyze in detail the role of proximal HSEs and AT-rich regions in the *Lehsp23.8* promoter under heat stress, we generated the internal deletion constructs pK△AT15, pK△AT34, and pK△AT12, as well as the HSE-mutated construct pK*HSE. GUS activity was then characterized in transgenic tobacco plants (Fig. 4). At 26°C, no activity was detected in the leaves from any construct. After heat treatment, however, the deletion of internal region AT-rich1–2 (construct pK△AT12) or AT-rich3–4 (construct pK△AT34) significantly affected heat-induced GUS expression. This suggested that AT-rich1–2 and AT-rich3–4 had important enhancer activities. However, GUS activities in the pKΔAT15 lines, which were similar to those of pK255, were unexpectedly a little higher than in either the pKΔAT34 or pKΔAT12 lines. The promoter harboring the mutant proximal HSEs (construct pK*HSE) lacked any heat-inducible activity.

Quantitative differences in transgene expression between independent transformants are generally ascribed to different integration sites of the transgene (van Leeuwen et al. 2001). Transgenes inserted into the plant genome can become inactive (gene-silencing). Our results also sug-

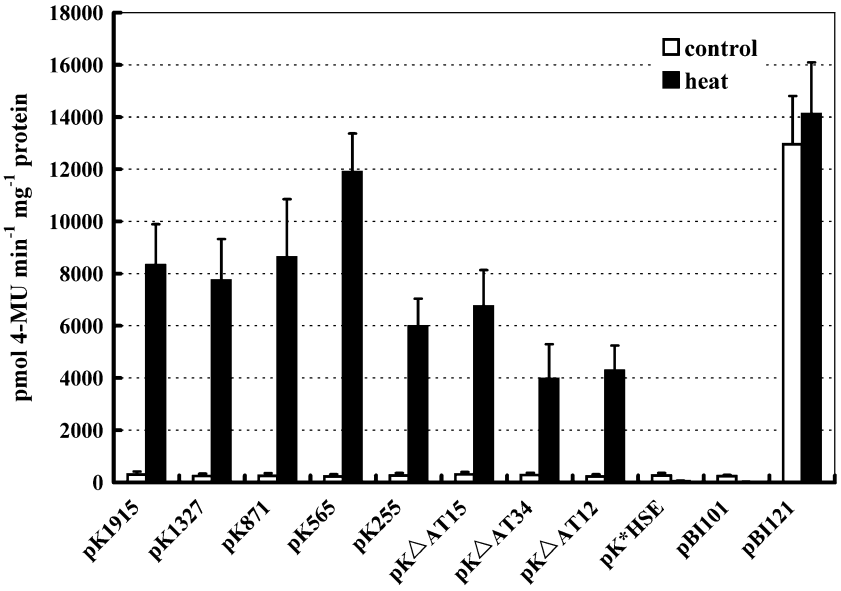

Fig. 4 GUS activity in tobacco transformed with *Lehsp23.8* promoter mutant constructs under heat shock. Transgenic plants (pK1915, pK1327, pK871, pK565, pK255, pK△AT15, pK△AT34, pK△AT12, and pK*HSE) were subjected to 39°C for 3 h. Fluorometric GUS assays of leaves were performed in triplicate; mean values were calculated for each treatment. PBI101 and pBI121 plants were assayed as negative and positive controls, respectively. *Errors bars* represent standard deviations of 30 independent transgenic lines

gested that the AT-rich regions could influence the rate of transgenic silencing. Here, we compared the degree of such *gus*-silencing among constructs pK△AT15, pK△AT34, pK△AT12, and pK871 (Table 1). Deletion of the internal AT-rich1–2 (construct pK△AT12), AT-rich3–4 (construct pK△AT34), or AT-rich1–5 (construct pK△AT15) resulted in an increased percentage of lines with transgenic silencing.

Low Temperature

We selected 10 transgenic lines that had stronger GUS activities in response to heat. These were then exposed to chilling at 2°C for 48 h before fluorometric assays were performed. The highest level of activity was detected in pK565 lines, being significantly increased, by approximately 32% (from 1,806 to 2,377 units), compared with pK1915 (Fig. 5). However, a deletion up to position −255 (construct pK255) resulted in a significant loss of cold-induced GUS expression. This suggested that the 565-bp region upstream of *Lehsp23.8* was sufficient for cold induction. The putative element CRT/DRE (−1,550 bp) was not involved in the stress response to low temperature. Nevertheless, the proximal HSEs (HSE1 to 5) and the AT-rich regions (AT-rich1 to 4) may, to some extent, have contributed to the multiple stress-responsive character of that promoter.

Transgenic plants harboring the internal deletion construct pK△AT12 or pK△AT15 showed negligible activity, while the chimeric gene with construct pK△AT34 was still induced by cold stress. This indicated that AT-rich1–2 of the *Lehsp23.8* promoter played an important role but AT-rich3–4 did not. The chimeric gene with the construct pK*HSE could not be induced by low temperature, suggesting that HSE1 to 5 were also important for the cold-induced expression of *Lehsp23.8* (Fig. 5). Together, both AT-rich1–2 and the five proximal HSEs proved indispensable to this cold response of *Lehsp23.8*.

Table 1 Effects of AT-rich regions in the *Lehsp23.8* promoter on gene-silencing in transgenic tobacco

Lines/construct	pK871	pK△AT15	pK△AT34	pK△AT12
Total	30	42	42	42
PCR-positive	29	41	39	40
Number silenced[a]	3	8	10	9
Percent (%)[b]	10.3	19.5	25.6	22.5

[a] Transgenic tobacco lines with GUS activity of ≤100 pmol 4-MU min^{-1} mg^{-1} protein

[b] Percentage of lines with silenced expression compared with lines that were PCR-positive

Heavy Metal

After exposing our transgenic plants to 1.0 mmol L^{-1} $PbCl_2$ for 6 h at 26°C, we performed fluorometric assays of GUS activity in their leaves. Among all transgenic lines, pK871 lines showed the highest activity (Fig. 6), while that of pK565 dropped by 30% compared with pK871. A further reduction in promoter size to −255 (construct pK255) that abolished four AT-rich regions resulted in negligible activities. This suggested that the 565-bp region upstream of *Lehsp23.8* was sufficient for heavy-metal induction. Likewise, the sequences from −871 to −255 bp enhanced lead-induced activity of the promoter. The putative element AP-1 (−884, −1,540, and −1,655 bp) in that promoter had only a small role in this stress response.

Transgenic plants harboring the internal deletion construct pK△AT34 and pK△AT12 were still induced by this heavy

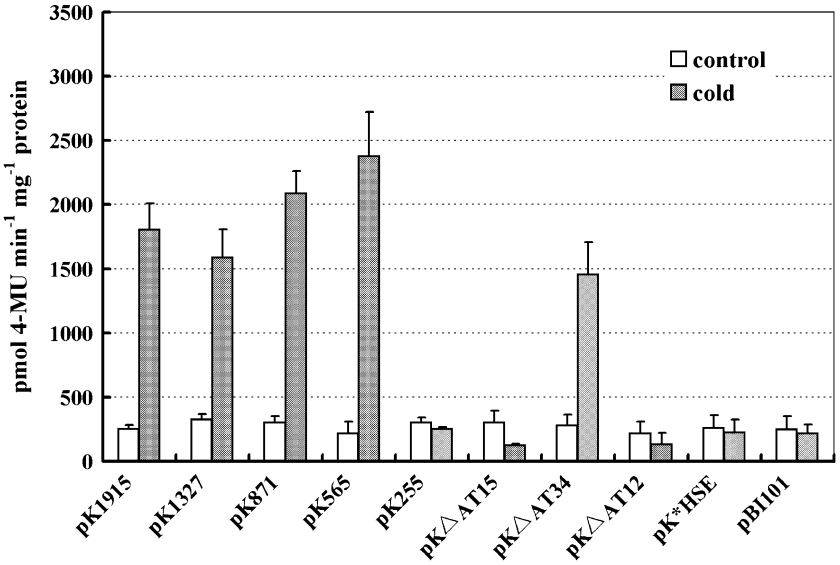

Fig. 5 GUS activity in tobacco transformed with *Lehsp23.8* promoter mutant constructs under low temperature. Transgenic plants (pK1915, pK1327, pK871, pK565, pK255, pK△AT15, pK△AT34, pK△AT12, and pK*HSE) were chilled at 2°C. Fluorometric GUS assays of leaves were performed in triplicate; mean values were calculated for each treatment. As negative control, pBI101 transgenic plants were assayed. *Errors bars* represent standard deviations of 10 independent transgenic lines

metal, while line pK△AT15 showed negligible GUS activity. This indicated that at least one of the AT-rich1–2 and AT-rich3–4 was essential for such induced expression of *Lehsp23.8*. Another possible mechanism for this phenomenon could have been other unidentified regulatory elements between AT-rich1–2 and AT-rich3–4 that affected promoter activity during this stress period. The chimeric gene with construct pK*HSE was not induced by lead exposure, suggesting that the proximal HSEs also played an important role in this induced expression (Fig. 6). All of these results indicated that both proximal HSEs and AT-rich1–4 were indispensable to chimeric gene expression under heavy-metal stress.

Abscisic Acid

Fluorometric GUS assays showed that expression patterns for all constructs after ABA treatment were similar to those found with lead, except that transcript levels of the report gene were relatively lower (Fig. 7). Likewise, tobacco plants transformed with pK255, pK△AT15, or pK*HSE displayed negligible activities. These similar GUS expression patterns between the ABA and Pb responses suggested a similar pathway existed.

Discussion

AT-rich Regions in the *Lehsp23.8* Promoter Enhance Heat-Induced Expression

Various heat shock genes contain conserved HSEs in their promoters, which are essential for heat-induced transcription (Wu 1995). Regulation of such expression is mediated by the conserved HSFs. Heat shock transcription factors are

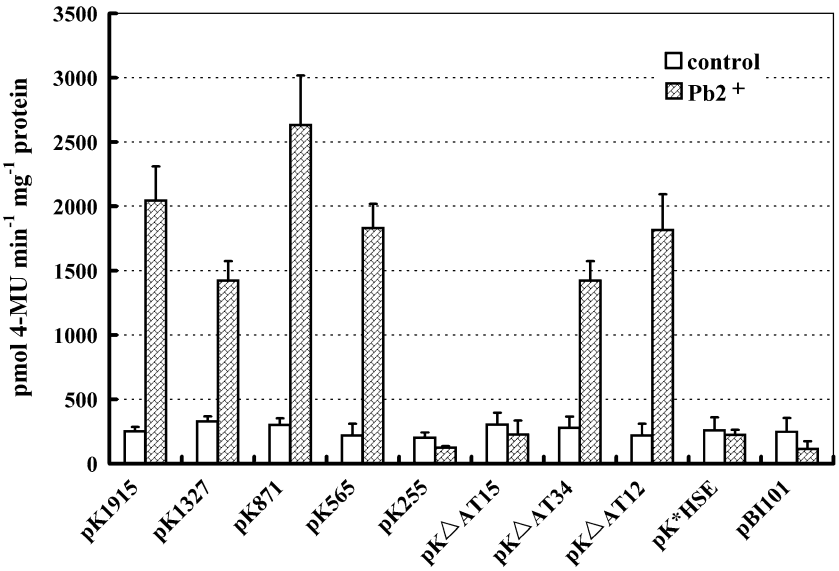

Fig. 6 GUS activity in tobacco transformed with *Lehsp23.8* promoter mutant constructs under heavy-metal stress. Transgenic plants (pK1915, pK1327, pK871, pK565, pK255, pK△AT15, pK△AT34, pK△AT12, and pK*HSE) were exposed to 1.0 mmol L^{-1} $PbCl_2$. Fluorometric GUS assays of leaves were performed in triplicate; mean values were calculated for each treatment. As negative control, pBI101 transgenic plants were assayed. *Errors bars* represent standard deviations of 10 independent transgenic lines

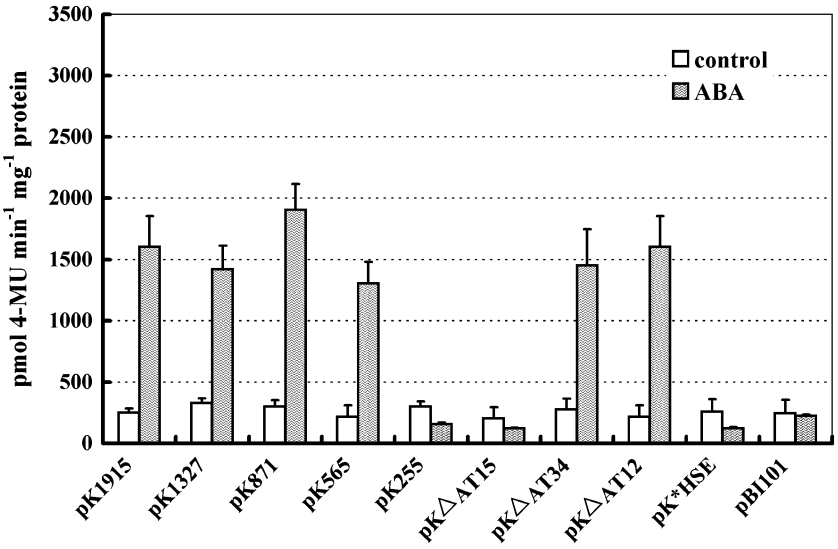

Fig. 7 GUS activity in tobacco transformed with *Lehsp23.8* promoter mutant constructs under ABA treatment. Transgenic plants (pK1915, pK1327, pK871, pK565, pK255, pK△AT15, pK△AT34, pK△AT12, and pK*HSE) were exposed to 0.1 mmol L^{-1} ABA. Fluorometric GUS assays of leaves were performed in triplicate; mean values were calculated for each treatment. As negative control, pBI101 transgenic plants were assayed. *Errors bars* represent standard deviations of 10 independent transgenic lines

present in a latent state under normal conditions but are activated upon heat stress by inducing trimerization and high-affinity binding to conserved HSEs (Baniwal et al. 2004). Here, our fluorometric assays showed that heat-induced GUS activity of the 565-bp promoter was strongest while that of the 255-bp promoter was lowest. Activities mediated by the 1,915-, 1,327-, or 871-bp promoters were similar. The 871-bp *Lehsp23.8* promoter within the five proximal HSEs mutation (pK*HSE) lost this activity. These results demonstrate that HSE1 to 5 within the 255-bp region upstream of *Lehsp23.8* are vital to the heat-inducible activity of that promoter. Distal HSEs may play less important roles for gene expression (Li et al. 2002). Our results confirmed that two distal HSEs (HSE6 and 7), between −871 and −1915 bp, were not necessary for enhancing *Lehsp23.8* promoter activity.

Heat-induced GUS activity of the 565-bp promoter was strongest, being significantly higher than that of the 255-bp promoter. Except for several AT-rich regions (AT-rich1 to 4), none of the HSEs were found between −255 and −565 bp in the *Lehsp23.8* promoter. Those regions may serve as enhancers for heat-induced expression of the chimeric gene. Similar AT-rich regions have been observed in several small heat shock protein promoters from soybean (Raschke et al. 1988). For example, in *GmHsp17.3-B*, an AT-rich upstream sequence (−81 to −306 bp) possesses enhancer-like properties (Baumann et al. 1987). The AT-rich 268-bp positive regulatory region of the pea *PetE* promoter also increases expression from both homologous and heterologous promoters in several transgenic tissues (Sandhu et al. 1998). Hoffmann and Binder (2002) have found that a 3-bp change in the AT-rich region causes *apt9* promoter activity to decline 70%. Therefore, we can speculate that the AT-rich regions between −255 and −565 bp contribute to the high heat-induced GUS activity of the 565-bp *Lehsp23.8* promoter. Although this activity from transgenic plants with pKΔAT34 or pKΔAT12 was significantly decreased, that level was only a little lower than those from plants with either pKΔAT15 or pK255. These results suggest that AT-rich1–2 and AT-rich3–4 have important enhancer activities and that both may interact to improve heat-induced expression of *Lehsp23.8*.

High mobility group (HMG) protein binding has been observed with AT-rich elements in several plant genes. Czarnecka et al. (1992) have demonstrated that nuclear proteins bind to scattered AT-rich sequences of the soybean *GmHsp17.5-E* promoter. These sequences are bound by HMGs and AT binding factors, which are believed to increase transcription through interaction with nuclear scaffold proteins (Czarnecka-Verner et al. 1994). However, the mechanism of the enhancing activity of AT-rich1–4 in the *Lehsp23.8* promoter remains to be tested.

Both Proximal HSEs and AT-rich Regions Play Important Roles for Multiple Inducibilities of the *Lehsp23.8* Promoter

The 871-bp *Lehsp23.8* promoter within the five proximal HSEs mutation (pK*HSE) lost its cold-inducible activity (Fig. 5). This demonstrated that HSE1 to 5 within the 255-bp region upstream of *Lehsp23.8* are vital to this activity. HSPs and HSFs also are involved in cellular responses to stresses other than heat (Swindell et al. 2007). Plant HSFs show great diversity, e.g., 21 in *Arabidopsis* and at least 34 in soybean (Miller and Mittler 2006). Moreover, there is a high degree of specialization in the response of certain *hsfs* to particular stress conditions. For example, *AthsfA9*, *AthsfA6a*, and *AthsfA6b* appear to be specific to cold stress (Zimmermann et al. 2004). Here, we speculated that some HSFs in tomato can act only under low temperatures, having a specific bond to the DNA-

binding domains of the *Lehsp23.8* promoter. Therefore, if HSFs are the activators for this cold response, further exploration is needed of those members that are involved.

How can HSF activate select *hsp* genes under chilling? We observed here that deletion of the promoter to point −255 bp, which contained the proximal HSE1 to 5, resulted in negligible cold-induced GUS activity, whereas deletion to point −565 bp led to obvious expression (Fig. 5). This indicates that the AT-rich region between −565 and −255 bp plays an important role in chimeric gene expression under cold stress. Further experiments revealed that AT-rich1–2 is indeed an important element for such activity. As discussed above, those AT-rich regions from −565 to −255 bp in the *Lehsp23.8* promoter serve as enhancers for heat-induced expression of the chimeric gene. In addition, a deletion of the internal AT-rich1–2, AT-rich3–4, or AT-rich1–5 resulted in increased transgenic silencing. In animals, HMG-I/Y competes with histone H1 to interact with the AT-rich sequence of DNA SARs (Zhao et al. 1993). However, it is unknown whether the existence of AT-rich1–2 in the *Lehsp23.8* promoter can change the native chromatin structure to make cold-induced HSF trimers bind the HSEs at low physiological concentrations.

Heavy-metal toxicity and abscisic acid are triggers of various HSP inductions (Coca et al. 1996; Heckathorn et al. 2004). Our results showed that a deletion of the *Lehsp23.8* promoter to −565 also led to gene expression under those types of stresses. Therefore, the putative element AP-1 (−884, −1,540, and −1,655 bp) and the abscisic acid-response element ABRE (−850 bp) of that promoter are not involved in plant responses to lead or ABA treatments. Furthermore, AT-rich1 to 4 and HSE1 to 5 proved vital for promoter activity under those stresses. In yeast, the CUP1 copper metallothionein gene is activated by excess copper and heat shock through the HSE sequences in the promoter (Liu and Thiele 1996; Peña et al. 1998). The *hsp70* promoter also is hyperactivated in an HSF1-dependent manner by combined exposure to heat and cadmium (Saydam et al. 2003). Finally, ABA appears to induce chimeric genes with the *HaHsp17.7G4* promoter, working synergistically with HSF3 (Rojas et al. 1999). Therefore, it is possible that *Lehsp23.8* expressions under heavy-metal and ABA stresses have similar pathways that are coupled with multiple signal transduction pathways leading to the binding of HSFs to HSEs.

Abscisic acid also accumulates during chilling periods, and many cold-induced genes respond to ABA stress, such as *kin1*, *cor6.6/kin2*, *cor15A*, *rd17/cor47*, and *rd29A/cor78* (Thomashow 1999; Seki et al. 2001). Our results showed that AT-rich1–2 has important roles in the activity of the *Lehsp23.8* promoter under cold stress but not ABA stress. This suggests that the pathways of *Lehsp23.8* expression differ between the two stimuli.

Regulation elements of *Lehsp23.8* represent an interaction between multiple stress-response pathways. Therefore, we propose that plant HSFs function as a network of transcription factors that controls *Lehsp23.8* expression upon different stresses. Our results have implications regarding the molecular basis of cross-tolerance in plant species and raise new questions for future experimental studies of this heat shock-response network.

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References

Baniwal SK, Bharti K, Chan KY, Fauth M, Ganguli A, Kotak S, Mishra SK, Nover L, Port M, Scharf KD, Tripp J, Weber C, Zielinski D, von Koskull-Döring P (2004) Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. J Biosci 29:471–487

Banzet N, Richaud C, Deveaux Y, Kazmaier M, Gagnon J, Triantaphylides C (1998) Accumulation of small heat shock proteins, including mitochondrial HSP22, induced by oxidative stress and adaptive response in tomato cells. Plant J 13:519–527

Baumann G, Raschke E, Bevan M, Schoffl F (1987) Functional analysis of sequences required for transcriptional activation of a soybean heat shock gene in transgenic tobacco plants. EMBO J 6:1161–1166

Bowler C, Fluhr R (2000) The role of calcium and activated oxygens as signals for controlling cross-tolerance. Trends Plant Sci 5:241–245

Coca MA, Almoguera C, Thomas TL, Jordano J (1996) Differential regulation of small heat-shock genes in plants: analysis of a water-stress-inducible and developmentally activated sunflower promoter. Plant Mol Biol 31(4):863–876

Czarnecka E, Ingersoll JC, Gurley WB (1992) AT-rich promoter elements of soybean heat shock gene Gmhsp17.5E bind two distinct sets of nuclear proteins in vitro. Plant Mol Biol 19 (6):985–1000

Czarnecka-Verner E, Dulce-Barros M, Gurley WB (1994) Regulation of the heat shock gene expression. In: Basra AS (ed) Stress induced gene expression in plants. Harwood, Newark, pp 131–161

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Haralampidis K, Milioni D, Rigas S, Hatzopoulos P (2002) Combinatorial interaction of cis elements specifies the expression of the Arabidopsis AtHsp90–1 gene. Plant Physiol 129:1138–1149

Heckathorn SA, Mueller JK, LaGuidice S, Zhu B, Barrett T, Blair B, Dong Y (2004) Chloroplast small heat-shock proteins protect photosynthesis during heavy metal stress. Am J Bot 91:1312–1318

Hoffmann M, Binder S (2002) Functional importance of nucleotide identities within the pea *atp9* promoter sequence. J Mol Biol 320 (5):943–950

Holsters M, de Waele D, Depicker A, Messens E, van Montagu M, Schell J (1978) Transfection and transformation of *Agrobacterium tumefaciens*. Mol Gen Genet 163:181–187

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jin TE, Kim IG, Kim WS, Suh SC, Kim BD, Rhim SL (2001) Expression of chromium (VI) reductase gene of heavy metal reducing bacteria in tobacco plants. J Plant Biotechnol 3:13–17

Krishna P, Sacco M, Cherutti JF, Hill S (1995) Cold-induced accumulation of *hsp90* transcripts in *Brassica napus*. Plant Physiol 107(3):915–923

Larkindale J, Hall JD, Knight MR, Vierling E (2005) Heat stress phenotypes of *Arabidopsis* mutants implicate multiple signaling pathways in the acquisition of thermotolerance. Plant Physiol 138:882–897

Li B, Liu HT, Sun DY, Zhou RG (2002) Signal transduction mechanism of plant heat shock response. J Plant Physiol Mol Biol 28(1):1–10

Liu J, Shono M (2001) Molecular cloning the gene of small heat shock protein in the mitochondria and endoplasmic reticulum of tomato. Acta Bot Sin 43(2):138–145

Liu XD, Thiele DJ (1996) Oxidative stress induced heat shock factor phosphorylation and HSF-dependent activation of yeast metallothionein gene transcription. Genes Dev 10:592–603

Lopez-Matas MA, Nunez P, Soto A, Allona I, Casado R, Collada C, Guevara MA, Aragoncillo C, Gomez L (2004) Protein cryoprotective activity of a cytosolic small heat shock protein that accumulates constitutively in chestnut stems and is up-regulated by low and high temperatures. Plant Physiol 134:1708–1717

Marrs KA, Sinibaldi RM (1997) Deletion analysis of the maize *hsp82, hsp81*, and *hsp17.9* promoters in maize and transgenic tobacco: Contributions of individual heat shock elements and recognition by distinct protein factors during both heat shock and development. Maydica 42:211–226

Miller G, Mittler R (2006) Could heat shock transcription factors function as hydrogen peroxide sensors in plants? Ann Bot (Lond) 98:279–288

Mittler R (2006) Abiotic stress, the field environment and stress combination. Trends Plant Sci 11:15–19

Peña MM, Koch KA, Thiele DJ (1998) Dynamic regulation of copper uptake and detoxification genes in *Saccharomyces cerevisiae*. Mol Cell Biol 18(5):2514–2523

Pockley AG (2001) Heat shock proteins in health and disease: therapeutic targets or therapeutic agents? Exp Rev Mol Med 3 (23):1–21

Raschke E, Baumann G, Schoffl F (1988) Nucleotide sequence analysis of soybean small heat shock protein genes belonging to two different multigene families. J Mol Biol 199:549–557

Rojas A, Almojuera C, Jordano J (1999) Transcriptional activation of a heat shock gene promoter in sunflower embryos: synergism between ABI3 and heat shock factors. Plant J 20(5):601–610

Sabehat A, Lurie S, Weiss D (1998) Expression of small heat-shock proteins at low temperatures. A possible role in protecting against chilling injuries. Plant Physiol 117:651–658

Sandhu JS, Webster CI, Gray JC (1998) A/T-rich sequences act as quantitative enhancers of gene expression in transgenic tobacco and potato plants. Plant Mol Biol 37(5):885–896

Saydam N, Steiner F, Georgiev O, Schaffner W (2003) Heat and heavy metal stress synergize to mediate transcriptional hyper-activation by metal-responsive transcription factor MTF-1. J Biol Chem 278(34):31879–31883

Schöffl F, Prandl R, Reindl A (1998) Regulation of the heat-shock response. Plant Physiol 117:1135–1141

Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y, Shinozaki K (2001) Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell 13:61–72

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J 31(3):279–292

Singla SL, Pareek A, Grover A (1997) Yeast HSP104 homologue rice HSP110 is developmentally- and stress- regulated. Plant Sci 125 (2):211–219

Sung DY, Vierling E, Guy C (2001) Comprehensive expression profile analysis of the *Arabidopsis* HSP70 gene family. Plant Physiol 123:789–800

Sung DY, Kaplan F, Lee KJ, Guy CL (2003) Acquired tolerance to temperature extremes. Trends Plant Sci 8:179–187

Swindell WR, Huebner M, Weber AP (2007) Transcriptional profiling of *Arabidopsis* heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways. BMC Genomics 8:125. doi:10.1186/1471-2164-8-125

Thomashow MF (1999) Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50:571–599

van Leeuwen W, Ruttink T, Borst-Vrenssen AW, van der Plas LH, van der Krol AW (2001) Characterization of position-induced spatial and temporal regulation of transgene promoter activity in plants. J Exp Bot 52:949–959

Wu C (1995) Heat shock transcription factors: structure and regulation. Ann Cell Dev Biol 11:441–469

Yi SH, Sun AQ, Sun Y, Yang JY, Zhao CM, Liu J (2006) Differential regulation of Lehsp23.8 in tomato plants: analysis of a multiple stress-inducible promoter. Plant Sci 171(3):398–407

Zhao K, Kas E, Gonzalez E, Laemmli UK (1993) SAR-dependent mobilization of histone H1 by HMG-I/Y *in vitro*. HMG-I/Y is enriched in H1-depleted chromatin. EMBO J 12:3237–3247

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol 136:2621–2632