Plant Molecular Biology **28**: 73–82, 1995.

Developmental regulation and tissue-specific differences of heat shock gene expression in transgenic tobacco and *Arabidopsis* plants

Ralf Prändl, Eva Kloske and Fritz Schöffl*
Universität Tübingen, Biologisches Institut, Lehrstuhl für Allgemeine Genetik, Auf der Morgenstelle 28, D-72076 Tübingen, Germany (author for correspondence)*

Received 18 August 1994; accepted in revised form 7 February 1995

Key words: development, glucuronidase reporter, heat shock genes, *in situ* hybridization, tissue specificity, transgenic plants

Abstract

The heat shock (hs) response during plant growth and development was analyzed in tobacco and *Arabidopsis* using chimaeric β-glucuronidase reporter genes (hs-Gus) driven by a soybean hs promoter. Fluorimetric measurements and histochemical staining revealed high Gus activities in leaves, roots, and flowers exclusively after heat stress. The highest levels of heat-inducible expression were found in the vascular tissues. Without heat stress, a developmental induction of hs-Gus was indicated by the accumulation of high levels of Gus in transgenic tobacco seeds. There was no developmental induction of hs-Gus in *Arabidopsis* seeds. *In situ* hybridization to the RNA of the small heat shock protein gene *Athsp17.6* in tissue sections revealed an expression in heat-shocked leaves but no expression in control leaves of *Arabidopsis*. However, a high level of constitutive expression of hs genes was detected in meristematic and provascular tissues of the *Arabidopsis* embryo. The developmental and tissue-specific regulation of the hs response is discussed.

Abbreviations: hs, heat shock; Hsp, heat shock protein(s); hs-Gus: heat-inducible Gus gene(s); HSE, heat shock element(s); HSF, heat shock factor; X-gluc, 5-bromo-4-chloro-3-indolyl-β-*D*-glucuronide; Gus, β-glucuronidase; DAF, days after flowering; SAR, scaffold attachment region.

Introduction

The heat shock response is a stress phenomenon in many organisms including plants. This response, induced by an elevation of the ambient temperature, is characterized at the molecular level by *de novo* synthesis of heat shock proteins (Hsp). At the same time, cells and organisms acquire an increase in thermotolerance. The functional role of Hsp as molecular chaperones has been demonstrated for almost all Hsp belonging to different molecular weight groups [for review see 35] including the small Hsp (HSP20 group) of human [17] and plant cells [19]. The plant-specific aspects of the hs response, such as the abundant synthesis of small Hsp in soybean and other species, are not yet understood. There may be a higher demand for this group of heat-inducible chaperones in the cytoplasm, inside the chloroplast, and in the absence of hs during cer-

tain stages in embryogenesis. Developmental regulation of Hsp synthesis is indicated by the presence of mRNAs [3, 8, 10, 21, 37] and Hsp [1, 5, 7, 11, 14, 35] in seeds of several plant species. Interestingly, small Hsp seem also to play a role in experimentally induced embryogenesis as shown by their induction during microspore embryogenesis in *Brassica napus* [22].

A major question with regard to Hsp synthesis during plant growth and development concerns the temporal and spatial regulation of the hs response. In the present study, we have addressed this question by using a suitable hs promoter-driven Gus gene for qualitative and quantitative measurements of the hs response in different tissues of transgenic tobacco and *Arabidopsis* plants. It has been previously shown that the soybean hs promoter of *Gmhsp17.3-B* is faithfully regulated by hs in tobacco leaves. Promoter elements for hs induction (HSE) and for enhancement of transcription have been characterized [23, 27, 28, 30]. In this paper, we report on quantitative differences for heat-inducible expression between different tissues and for the developmental induction during embryogenesis between tobacco and *Arabidopsis*. The term 'developmental regulation' used in this paper refers to the induction of hs gene expression in the absence of an environmental stress during embryogenesis. High levels of developmental expression of hs genes in the *Arabidopsis* embryo, as shown by *in situ* hybridizations of the mRNA, were found in meristematic and provascular tissues.

Materials and methods

Plant material

Different transgenic lines of *Nicotiana tabacum* cv. Samsun containing the heat-inducible β-glucuronidase gene were used: (1) a −321/−12-Gus-transformed line containing the *Gmhsp17.3-B* promoter (position −321 to −12 relative to the start codon) fused to the Gus reporter [30]; (2) a −593/−12-Gus-transformed line containing the *Gmhsp17.3-B* promoter with an upstream enhancer region (position −593 to −12 relative to the start codon) fused to the Gus gene [29]; (3) a SHS3252-transformed line containing −321/−12-Gus flanked at both sides by fragments of a soybean scaffold attachment region (SAR) for enhanced and stable expression in transgenic plants [30]. Thus, all heat-inducible Gus genes contain the same *Gmhsp17.3-B* promoter with multiple HSE, the binding sites for the transcription factor HSF [16]. The transcriptional start site is localized at position −100 [26]. A constitutive CaMV 35S-Gus expression line [24] was used as a control. Transgenic *Arabidopsis thaliana* plants (Columbia) containing the −321/−12-Gus construct or the SAR-flanked construct SHS3252 [30] were generated by *Agrobacterium* Ti-plasmid-mediated root transformation as described by Severin *et al.* [32]. For each construct, the transgenic line with the highest hs induction factor for hs-Gus was selected and taken for the experiments described.

Plant growth

Seeds were surface-sterilized for 20 min in a saturated calcium hypochlorite solution and then washed several times in deionized water. The seeds of transgenic tobacco were germinated on solid MS plant medium or on moistened Whatman 3MM paper with 100 mg/l kanamycin. After two weeks, plants were transferred to soil culture and grown for 1–6 months. *Arabidopsis* seeds were imbibed at 4 °C overnight and the incubation temperature was then shifted to 25 °C for germination. The standard growth conditions were 25 °C at 60% humidity in a light/dark cycle of 16 h/8 h for all experiments.

Heat stress treatment

Heat stress conditions for tobacco were 2 h at 40 °C and for *Arabidopsis* 2 h at 37 °C. The tissue was submerged in 1% sucrose and 1 mM KH_2PO_4 buffer, pH 6.0, in a shaking water bath. Control tissue was treated in the same buffer at 25 °C. Plant material was collected in different

developmental stages. Twenty individual seeds or seedlings were used at early growth stages. At later stages, four different plants were analyzed. From each plant, two opposite leaves at same developmental levels were collected and subjected to heat stress.

Fluorometric Gus assay

Activity of β-glucuronidase (Gus) was determined in protein extracts from plant tissues using the fluorometric assay described by Jefferson [18]. Aliquots of 0.5 ml containing 10–20 μg total protein and 1 mM 4-methylumbelliferyl-β-D-glucuronide were incubated for 0, 30, 60 and 90 min at 37 °C. The concentration of 4-methylumbelliferone was determined from the linear slopes of the fluorescence at 445 nm in a Hitachi F-2000 spectrofluorometer. Gus activity was calculated as pmol/min per mg protein. Protein concentrations were determined according to Bradford [4].

Histochemical Gus assay

Plant material was embedded in acryl-methacrylate (Historesin; Reichert and Jung) and stained with X-gluc (5-bromo-4-chloro-3-indolyl-β-*D*-glucuronide), a substrate for β-glucuronidase, as described by De Block and Debrouwer [6]. The embedding protocol was modified in that the steps of dehydration with water diluted acetone and infiltration with Historesin were performed under vacuum and shortened to one hour. The entire embedding protocol was consequently shortened to one day. Sections of 10 μm were cut using a microtome (Reichert and Jung) and slices were incubated in X-gluc overnight. The stained sections were photographed with a Zeiss Axioskop photomicroscope.

In situ *hybridization*

Leaves were fixed by vacuum infiltration with 4% paraformaldehyde in a buffer containing 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, and 0.3 M sucrose for one hour at 4 °C. This was repeated in the same buffer but with 0.5 M sucrose. Fixed leaves or dry seeds were embedded in Tissue-Tek O.C.T. Compound (Miles) and 10 μm sections were cut using a cryotome (Reichert and Jung). *In situ* hybridizations were performed according to Hogan *et al.* [15] using a ^{35}S-labelled riboprobe (10^5 dpm/μl) derived from the class I *Arabidopsis* hs gene *Athsp17.6* [12] or from the class I tobacco hs gene *Nthsp18P* [36]. For autoradiography, sections were coated with K.2 emulsion (Ilford) and subsequently exposed in the dark for up to 3 weeks. Processing of the K.2 emulsion was according to the manufacturer's instructions. The tissues were visualized by staining with toluidine blue. Sections were photographed using a Zeiss Axioskop photomicroscope.

Results

Differences of hs-Gus activities in tobacco

Transgenic tobacco containing the SAR-enhanced hs-Gus construct (SHS3252) was used in this analysis. The Gus activity in different tissues and developmental stages was determined with and without prior hs. For the quantitative determination of Gus activity, young tobacco leaves with a maximum leaf length of 2 cm, middle-aged leaves (not fully expanded and with a leaf length of about 8–10 cm) and fully expanded but not yet senescent leaves were separately collected and cut into leaf tip, leaf marginal region, leaf base, and the central area containing the main vascular tissue. Fully expanded leaves showed the highest level of heat-inducible Gus activity (Fig. 1). Leaf tips and marginal regions showed higher activities than central areas or the leaf bases and the levels were generally lower in younger leaves. Constitutive expression in leaves was not detectable. During flower development, there was no heat-inducible Gus activity detectable in young buds but it was detected in petals and immature seeds. At the time of seed maturation (at about 30

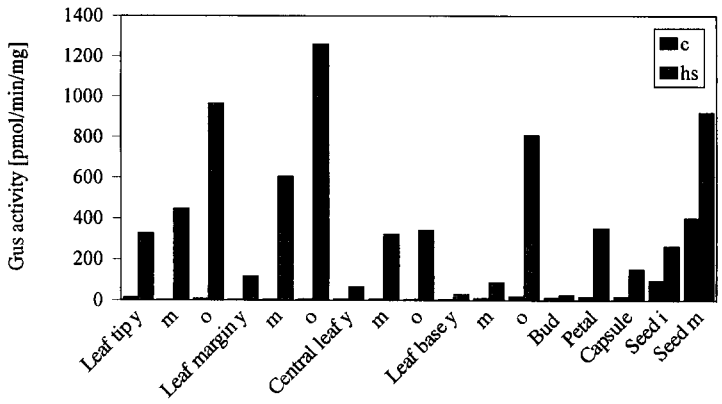


Fig. 1. Gus activities in different tissues of hs-Gus transgenic tobacco. Fluorometric measurements of Gus activity in untreated (c, control) and heat-shocked (hs) tobacco transformed with the SAR-enhanced hs-Gus construct. Leaf material was separated by age (y, young; m, middle-aged; o, old) and dissected into tip, leaf margins, central leaf, and leaf base. Reproductive organs (buds, petals, capsules, seeds (i, immature; m, mature)) were collected in the course of development.

DAF), constitutive activity of the hs-Gus construct increased to levels of up to 50% of the heat-inducible Gus activity.

Histochemical analysis of hs-Gus expression in tobacco and Arabidopsis

Histochemical staining of Gus activity was used for a higher resolution of tissue-specific differences in hs gene expression. Sections of transgenic tobacco and *Arabidopsis* tissues containing the same hs-Gus gene construct (SHS3252) were analyzed. Some of the original data for tobacco and *Arabidopsis* are exemplified in Fig. 2.

Gus staining in seeds was different between tobacco and *Arabidopsis*. No staining in seeds could be detected for transgenic *Arabidopsis*, neither with nor without prior heat stress (data not shown). In contrast, in transgenic tobacco Gus staining was visible in all tissues of the seed (Fig. 2A). The staining was independent of a prior heat stress. In the endosperm, the signal was weaker and showed some variation. The strongest signal was detected in the meristematic tissues (Fig. 2B). Wild type tobacco controls did not show any Gus staining (data not shown).

In the non-embryonic tissues under investigation, Gus staining was dependent on a prior hs. The leaf margin was in general more intensely stained than central parts of the tobacco leaf. In cross sections, staining is present in all cell types with the strongest signal in the xylem cells of the central vascular tissue and a weaker staining in cells of the epidermal layer and in parenchyma cells (Fig. 2C, E). In *Arabidopsis*, the staining decreased considerably from the base to the tip of the leaf as seen in serial cuts. Only a weak signal could be detected in the peripheral parts of the leaf (data not shown). The cells of the cambium in between xylem and phloem were preferentially stained (Fig. 2F). A much weaker staining was detected in the other cell types of the leaf. In both tobacco (Fig. 2D) and *Arabidopsis* (data not shown), the major staining areas of the roots are located above the root tip.

Developmental regulation of the soybean hs promoter in tobacco

The hs response of tobacco and *Arabidopsis*, as indicated by the induction of the chimaeric hs-Gus, was similar in the vegetative tissues but seemed to be different in the embryo. Due to the lower sensitivity of the histochemical analysis (compared to the fluorometric Gus assay), it could not be excluded that the differences between the two species were only quantitative and that lower levels of Gus activity escaped detection in *Ara-*

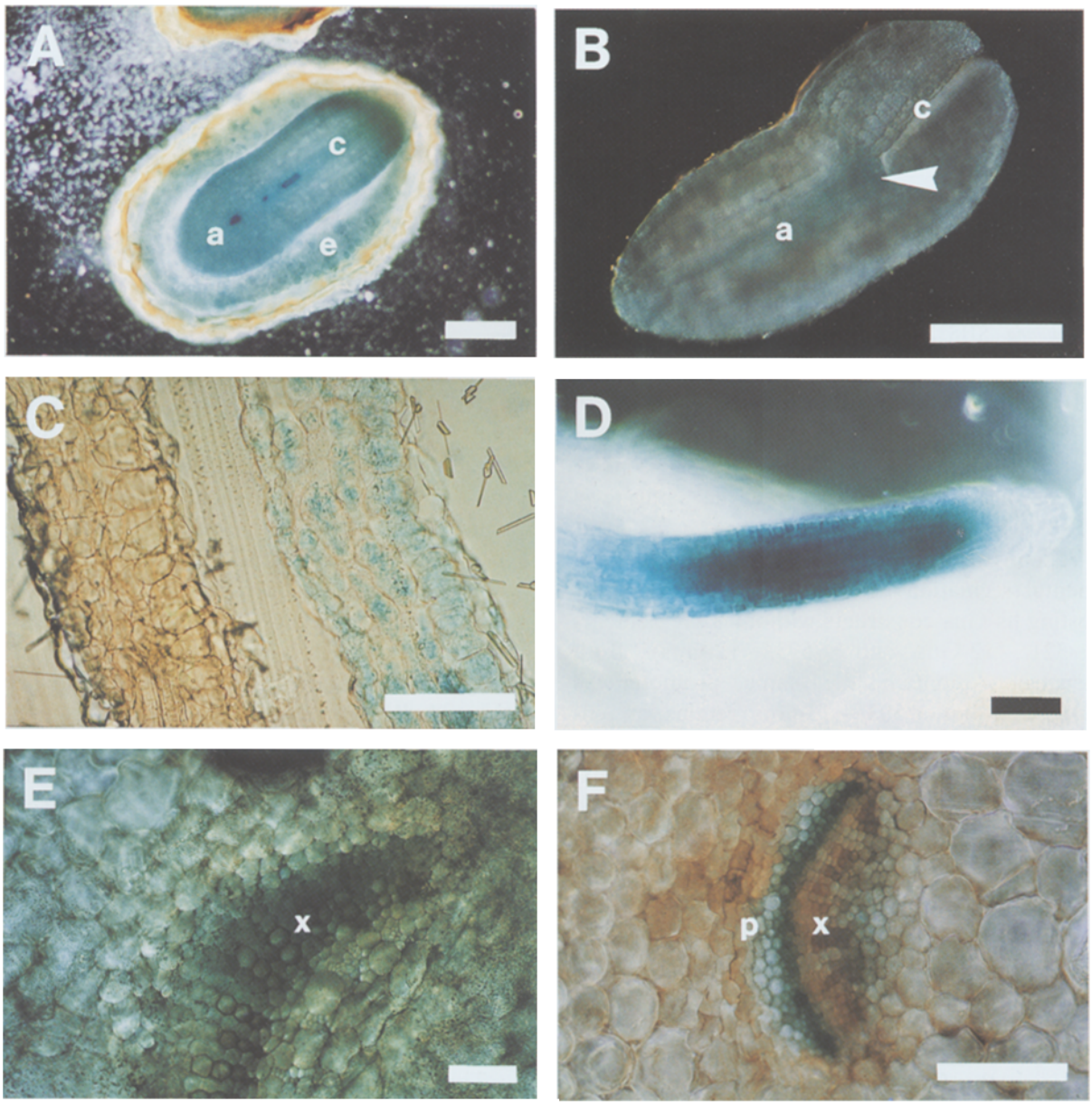


Fig. 2. Expression of hs-Gus in tobacco and *Arabidopsis* visualized by histochemical Gus staining. A longitudinal section of a tobacco seed (A) and an embryo from a mature seed (B) are shown (no prior heat shock). Tobacco leaves without (left) and after heat shock (right) were cross-sectioned through the marginal region (C). The longitudinal section of a tobacco root (D) and the cross sections of the central vascular system of a tobacco leaf (E) and of the central vascular system of the *Arabidopsis* leaf base (F) were obtained from heat-shocked tissues. Sections shown on the right of C and in E were from the same leaf. a, axis; c, cotyledons; e, endosperm; p, phloem; x, xylem; arrow, meristematic tissue of the shoot; bars: 200 μm.

bidopsis seeds. To answer this question, Gus activity was determined in seeds and leaves of the SHS3252-transformed lines of both species (Table 1). As expected, dry and imbibed seeds of tobacco but not of *Arabidopsis* showed constitutive levels of Gus activity. Heat shock had no significant effect on the Gus levels in seeds. Heat-inducible Gus activity was detectable in mature

Table 1. Gus activity (pmol/min per mg protein) in seeds and leaves of transgenic *Nicotiana tabacum* and *Arabidopsis thaliana*.

Species	Construct[a]	Seed		Leaf	
		c[b]	hs[c]	c[b]	hs[c]
N. tabacum	SHS3252	2741.3	2242.0	55.0	2343.9
	−593/−12-Gus	805.1	735.8	32.6	713.3
	−321/−12-Gus	59.1	88.0	12.3	183.2
	CaMV-35S-Gus	835.6	nd[d]	2246.0	nd[d]
A. thaliana	SHS3252	70.0	84.2	26.5	3210.0
	−321/−12-Gus	8.1	nd[d]	10.4	156.1

[a] See Materials and methods, [b] Control (without heat shock), [c] Heat shock, [d] Not determined.

leaves of both plants to about the same level (Table 1).

The involvement of SAR elements in developmental regulation in tobacco was excluded by testing hs-Gus constructs without SAR elements (−321/−12-Gus and −593/−12-Gus). Both constructs contain the same promoter as SHS3252 but −593/−12-Gus retains an enhancer upstream of the native hs promoter [2, 23]. With both constructs, constitutive Gus expression in tobacco seeds was detectable and with the high expression −593/−12-Gus construct reliable quantitative data were obtained. With the −593/−12-Gus construct, proportions of constitutive Gus activity in seeds and heat-inducible levels of Gus in leaves are very similar to those for SHS3252. No developmental induction of hs-Gus could be detected in *Arabidopsis* transformed with the construct −321/−12-Gus (Table 1).

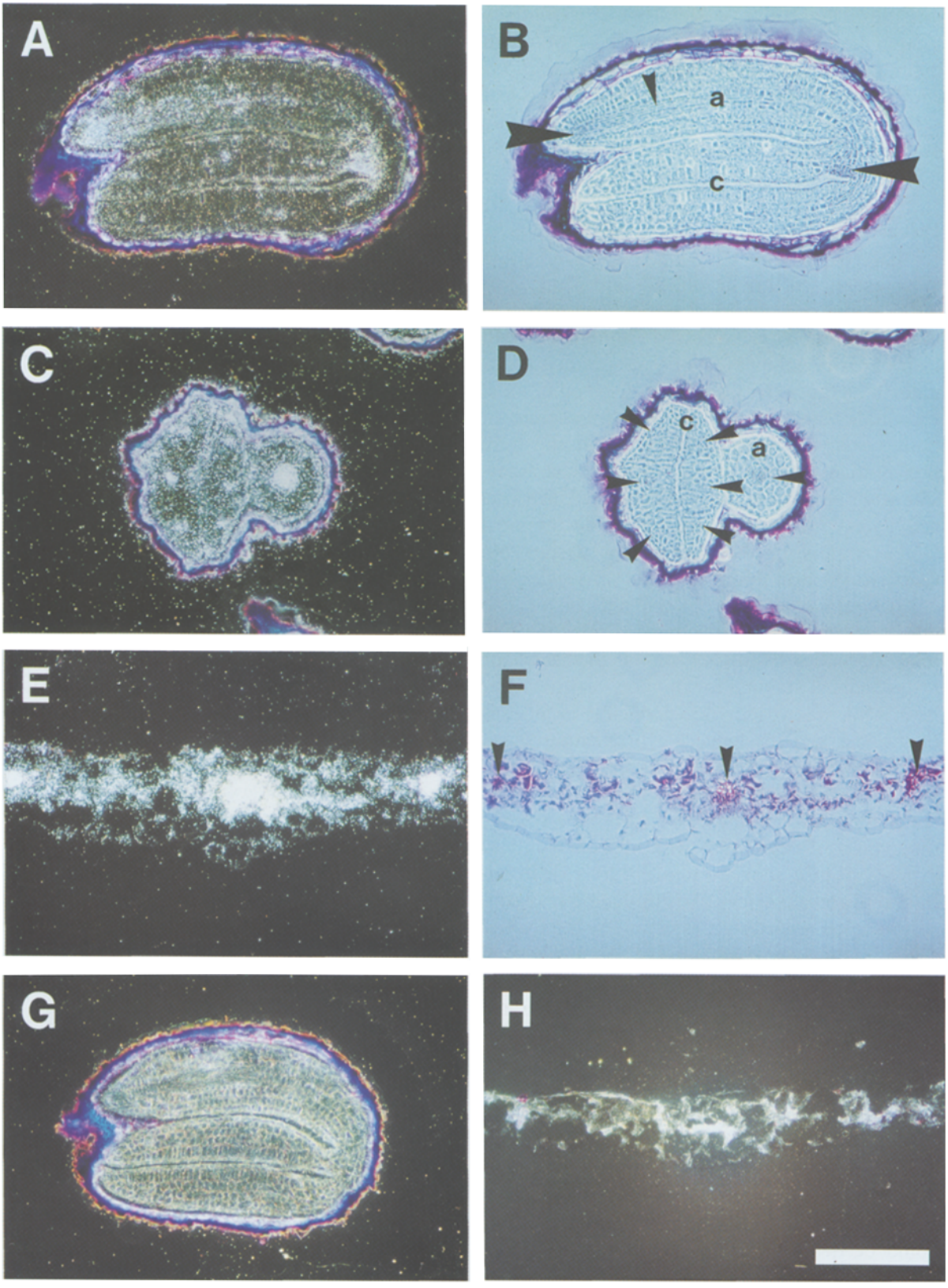
Using the CaMV 35S promoter-driven Gus gene as a control, constitutive levels of Gus activity were lower in the seeds than in the leaves of tobacco. This control excludes the possibility that developmental expression of hs-Gus is due to an unspecific stimulation of transcription in seeds compared to leaves. Hence, the elevated Gus activity in tobacco seeds must be a specific effect of developmental regulation of gene expression under control of the hs promoter.

Expression of Athsp17.6 *mRNA in the embryo and in leaves of* Arabidopsis

The endogeneous hs response in tobacco (data not shown) and *Arabidopsis* was investigated by *in situ* hybridization using radioactively labelled antisense RNA against *Nthsp18P* mRNA and *Athsp17.6* mRNA. Hybridization was detected in the seeds and the heat-shocked leaves of both plants. The results for *Arabidopsis* demonstrate a high density of silver grains in the embryo that is coincident with the meristematic tissues of the root tip, the shoot meristem at the base of cotyledons, and the provascular tissues (Fig. 3). In longitudinal sections, the root and shoot meristem (Fig. 3A, B) and partially the central procambium of the axis are stained with silver grains. Cross sections of the embryo clearly demonstrate a more intense staining of the vascular cords of the cotyledons (three in each one) and of the procambium of the axis than of the other surrounding tissues (Fig. 3C, D). The specificity of hybridization for the mRNA is confirmed by the lack of silver grains in control hybridizations using sense RNA probes that had been labelled to the same specific activity as the antisense probes. One example of a longitudinal section control is shown in Fig. 3G.

The *in situ* hybridizations to cross sections of mature *Arabidopsis* leaves show a high density of silver grains over the leaves that had been subjected to a prior heat shock. The grains accumu-

Fig. 3. Expression of small Hsp-mRNA in *Arabidopsis* visualized by *in situ* hybridization to tissue sections. Photomicrographs under dark-field illumination show the density of autoradiographic silver grains (A, C, E, G, H), whereas the histological structure was visualized under phase contrast conditions (B, D, F). The longitudinal section (A, B) and the cross section (C, D) of mature seeds and the cross section of a heat-shocked leaf (E, F) were hybridized with antisense RNA. For control, sections of seeds were incubated with sense RNA (G) and cross sections of leaves (without heat shock) were incubated with antisense RNA (H). a, axis; c, cotyledons; large arrows, meristematic tissues; small arrows, provascular tissues in axis and cotyledons; bar: 200 μm (for all photomicrographs).


A
B
a
c
C
D
c
a
E
F
G
H

late throughout the section but preferentially around the vascular tissues (Fig. 3E, F). Although hybridized to the same probe as the sections shown in Fig. 3A, B, C, D, E and F, the leaf sections from plants without heat stress show only a very low background signal (Fig. 3H).

Discussion

We have shown that in tobacco and *Arabidopsis* a Gus reporter gene under the control of a soybean hs promoter is expressed differentially with respect to development and tissue specifity. Heat-inducible Gus activity could be detected in most vegetative tissues of tobacco and *Arabidopsis*. In both systems, cells of the vascular tissue were preferentially stained in the histochemical assay. The seeds of transgenic tobacco showed high Gus activity even in the absence of hs with the most intense staining in the meristematic tissues. No Gus activity was found in seeds of *Arabidopsis*.

The *in situ* hybridizations to leaves of tobacco and *Arabidopsis* revealed a pattern of expression for endogenous hs-mRNA similar to that of the histochemical staining for the Gus reporter. The hs-mRNA was strictly heat-inducible in all cells of the leaf and the highest hybridization signal was localized in the vascular tissues. These findings are in accordance with previous experiments where the mRNA of small Hsp and tissue-specific differences of expression were detected only in extracts of heat-shocked tissues [20, 25]. In addition, the mechanism of heat inducibility is conserved when reporter genes driven by the respective promoters are examined [23, 27, 30, 31] and hs-induced mRNA levels of the *Gmhsp17.3-B* gene are almost identical in soybean and transgenic tobacco [2]. These results indicate that heterologous hs-Gus constructs are reliable reporters of the environmentally induced hs response in vegetative tissues.

On the other hand, the *in situ* hybridizations demonstrated the presence of hs-mRNA in all cells of tobacco and *Arabidopsis* seeds even without heat treatment. By far the strongest signal could be detected in the provascular and meristematic tissues. This pattern of expression is similar to the pattern of Gus expression in seeds of tobacco plants containing the hs-Gus construct. Moreover, hs-mRNA was detected in seeds of wheat [11], sunflower [1, 5] and pea [7] grown in the absence of heat stress. Beside the embryo-specific expression, developmental expression of small Hsp is indicated by the detection of mRNA during pollen development and starvation-induced microspore embryogenesis. The appearance of hs-mRNA is paralleled by elevated Gus activity during pollen development and microspore embryogenesis in tobacco transformed with the hs-Gus construct SHS3252 [36]. Thus, hs-Gus mirrors the developmental regulation of the hs response in this species.

In contrast to tobacco, no Gus activity was detectable in seeds of transgenic *Arabidopsis*, whereas *in situ* hybridizations proved the presence of the mRNA of small Hsp. In non-embryonic tissues, the Gus activity was faithfully regulated by hs. It is not known whether the lack of Gus activity in *Arabidopsis* seeds is due to an inefficient recognition of the promoter by the developmental signal or is a consequence of post-transcriptional events interfering with the Gus expression. Post-transcriptional difficulties in seeds seem possible since even an *Arabidopsis* hs promoter was unable to yield detectable levels of Gus activity in *Arabidopsis* seeds [33].

The first report on the histological distribution of small Hsp in seeds was published recently [5]. In contrast to the homogeneous staining of immunologically detected small Hsp in tissue prints of the sunflower embryo, our results with *in situ* hybridization reveal a high tissue specifity of the hs response. *In situ* hybridization specifically shows the abundance of the mRNA at the end of maturation and desiccation and identifies the tissues that rely on newly synthesized Hsp during early germination. In this context, it is important to note that plastid-localized HSP26 in barley is accumulated during early germination and may have probably been synthesized after imbibition [20].

What is the function of small developmentally induced Hsp in embryogenesis and germination?

In plants, small Hsp are strictly stress-induced in vegetative tissues and a protective role has been proposed because of the chaperone activity of small Hsp *in vitro* [17, 19]. During pea embryonic development, small Hsp appear when the embryo becomes tolerant to desiccation [7]. Therefore, it was been suggested that small Hsp function in the protection of the embryo from desiccation stress. Our results show that the mRNA levels in the seed are much higher in provascular and meristematic tissues which may be more important for growth and development of the plant than other tissues of the seed.

Acknowledgements

We are grateful to Drs Thomas Schmülling and Klaus Severin for the donations of transgenic tobacco containing CaMV-35S-Gus and transgenic *Arabidopsis* containing the SHS3252 construct. We appreciate the *Athsp17.6* cDNA provided by Dr Elizabeth Vierling (Tucson) and the *Nthsp18P* cDNA provided by Dr E. Heberle-Bors (Vienna). This research was supported by grants from the Deutsche Forschungsgemeinschaft and the EC (PTP programme).

References

1. Almoguera C, Jordano J: Developmental and environmental concurrent expression of sunflower dry-seed-stored low-molecular-weight heat-shock protein and Lea mRNAs. Plant Mol Biol 19: 781–792 (1992).
2. Baumann G, Raschke E, Bevan M, Schöffl F: Functional analysis of sequences required for transcriptional activation of a soybean heat shock gene in transgenic tobacco plants. EMBO J 8: 2195–2202 (1987).
3. Bouchard RA: Characterization of expressed meiotic prophase repeat transcript clones of *Lilium*: meiosis specific expression, relatedness and affinities to small heat shock protein genes. Genome 33: 68–79 (1990).
4. Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72: 248–254.
5. Coca MA, Almoguera C, Jordano J: Expression of sunflower low-molecular-weight heat-shock proteins during embryogenesis and persistence after germination: localization and possible functional implications. Plant Mol Biol 25: 479–792 (1994).
6. De Block M, Debrouwer D: *In situ* enzyme histochemistry on plastic embedded plant material. The development of an artefact-free β-glucuronidase assay. Plant J 2: 261–266 (1992).
7. DeRocher AE, Vierling E: Developmental control of small heat shock protein expression during pea seed maturation. Plant J 5: 93–102 (1994).
8. Dietrich PS, Bouchard RA, Casey ES, Sinibaldi RM: Isolation and characterization of a small heat shock protein gene from maize. Plant Physiol 96: 1268–1276 (1991).
9. Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM Isolation of the *Arabidopsis ABI3* gene by positional cloning. Plant Cell 4: 1251–1261 (1992).
10. Györgyey J, Gartner A, Nemeth K, Magyar Z, Hirt H, Heberle-Bors E, Dudits D: Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. Plant Mol Biol 16: 999–1007 (1991).
11. Helm KW, Abernethy RH: Heat shock proteins and their mRNAs in dry and early imbibing embryos of wheat. Plant Physiol 93: 1626–1633 (1990).
12. Helm KW, Vierling E: An Arabidopsis cDNA clone encoding a low molecular weight heat shock protein. Nucl Acids Res 17: 7995 (1989).
13. Helm KW, DeRocher A, Lauzon L, Vierling E: Expression of low molecular weight heat shock proteins during seed development. J Cell Biochem 14E: 301 (1990).
14. Hernandez LD, Vierling E: Expression of low molecular weight heat shock proteins under field conditions. Plant Physiol 101: 1209–1216 (1993).
15. Hogan BLM, Constantini F, Lacy E: Manipulating the Mouse Embryo: a Laboratory Manual, pp. 228–242. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY New York (1986).
16. Hübel A, Schöffl F: *Arabidopsis* heat shock factor: isolation and characterization of the gene and the recombinant protein. Plant Mol Biol, in press (1994).
17. Jakob U, Gaestel M, Engel K, Buchner J: Small heat shock proteins are molecular chaperones. J Biol Chem 268: 1517–1520 (1993).
18. Jefferson RA: Assaying chimeric genes in plants: the Gus gene fusion system. Plant Mol Biol Rep 5: 387–405 (1987).
19. Jinn TL, Wu SH, Yeh CH, Hsieh MH, Yeh YC, Chen YM, Lin CY: Immunological kinship of class I low molecular weight heat shock proteins and thermostabilization of soluble proteins *in vitro* among plants. Plant Cell Physiol 34: 1055–1062 (1993).
20. Kruse E, Liu Z, Kloppstech K: Expression of heat shock proteins during development of barley. Plant Mol Biol 23: 111–122 (1993).
21. Marrs KA, Casey ES, Capitant SA, Bouchard RA, Dietrich PS, Mettler IJ, Sinibaldi R: Characterization of two maize hsp90 heat shock protein genes and expression

during heat shock, embryogenesis, and pollen development. Devel Genet 14: 27–41 (1993).
22. Pechan PM, Bartels D, Brown DCW, Schell J: Messenger-RNA and protein changes associated with induction of Brassica microspore embryogenesis. Planta 184: 161–165 (1991).
23. Rieping M, Schöffl F: Synergistic effects of upstream sequences, CCAAT box elements, and HSE sequences for enhanced expression of chimaeric heat shock genes in transgenic tobacco. Mol Gen Genet 321: 226–232 (1992).
24. Schmülling T, Schell J, Spena A: Promoters of the A, B and C genes of *Agrobacterium rhizogenes* are differentially regulated in transgenic plants. Plant Cell 1: 665–670 (1989).
25. Schöffl F: Aspekte der Temperaturstress-regulierten Genexpression bei höheren Pflanzen. In: Dechema-Monographien, vol. 95, pp. 323–338. Verlag Chemie, Weinheim (1984).
26. Schöffl F, Raschke E, Nagao RT: The DNA sequence analysis of soybean heat-shock genes and identification of possible regulatory promoter elements. EMBO J 3: 2491–2497 (1984).
27. Schöffl F, Rieping M, Baumann G, Bevan M, Angermüller S: The function of plant heat shock promoter elements in the regulated expression of chimaeric genes in transgenic tobacco. Mol Gen Genet 217: 246–253 (1989).
28. Schöffl F, Rieping M, Raschke E: Functional analysis of sequences regulating the expression of heat shock genes in transgenic plants. In: Lycett GW, Grierson D (eds) Genetic Engineering of Crop Plants, pp. 79–94. Butterworth, London (1990).
29. Schöffl F, Rieping M, Severin K: The induction of the heat shock response: activation and expression of chimaeric heat shock genes in transgenic tobacco. In: Herrmann RG, Larkins B (eds) Plant Molecular Biology, vol. 2, pp. 685–694. Plenum Press, New York (1991).
30. Schöffl F, Schröder G, Kliem M, Rieping M: An SAR sequence containing 395-bp DNA fragment mediates enhanced gene-dosage-correlated expression of a chimaeric heat shock gene in transgenic tobacco plants. Transgen Res 2: 93–100 (1993).
31. Severin K, Schöffl F: Heat-inducible hygromycin resistance in transgenic tobacco. Plant Mol Biol 15: 827–833 (1990).
32. Severin K, Wagner A, Schöffl F: A heat-inducible *Adh* gene in *Arabidopsis* as a reporter of negative selection in transgenic *Arabidopsis*. Transgen Res, in press (1994).
33. Takahashi T, Naito S, Komeda Y: The *Arabidopsis hsp18.2* promoter/Gus gene fusion in transgenic *Arabidopsis* plants: a powerful tool for the isolation of regulatory mutants of the heat shock response. Plant J 2: 751–761 (1992).
34. Vierling E: The roles of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 42: 579–620 (1991).
35. Vierling E, Sun A: Developmental expression of heat shock proteins in higher plants. In: Cherry J (ed) Environmental stress in plants. Biochemical and physiological mechanism associated with environmental stress tolerance in plants, pp. 343–354. Springer-Verlag, New York (1987).
36. Zarsky V, Garrido D, Eller N, Tupy J, Vicente O, Schöffl F, Heberle-Bors E: The expression of a small heat shock gene is activated during induction of tobacco pollen embryogenesis by starvation. Plant Cell Environ (in press).
37. Zimmermann JL, Apuya N, Darwish K, O'Carroll C: Novel regulation of heat shock genes during carrot somatic embryo development. Plant Cell 1: 1137–1146 (1989).