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A Novel Transcriptional Cascade Regulating Expression of Heat Stress Proteins during Seed Development of *Arabidopsis* [W]

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Within the *Arabidopsis thaliana* family of 21 heat stress transcription factors (Hsfs), HsfA9 is exclusively expressed in late stages of seed development. Here, we present evidence that developmental expression of HsfA9 is regulated by the seed-specific transcription factor ABSCISIC ACID–INSENSITIVE3 (ABI3). Intriguingly, *ABI3* knockout lines lack detectable levels of HsfA9 transcript and protein, and further ectopic expression of *ABI3* conferred the ability to accumulate HsfA9 in response to abscisic acid in transgenic plantlets. Consequently, the most abundant heat stress proteins (Hsps) in seeds (Hsp17.4-CI, Hsp17.7-CII, and Hsp101) were not detectable in the *ABI3* knockout lines, but their expression could be detected in plants ectopically expressing HsfA9 in vegetative tissues. Furthermore, this seed-specific transcription factor cascade was reconstructed in transient β-glucuronidase reporter assays in mesophyll protoplasts by showing that ABI3 could activate the *HsfA9* promoter, whereas HsfA9 in turn was shown to be a potent activator on the promoters of *Hsp* genes. Thus, our study establishes a genetic framework in which HsfA9 operates as a specialized Hsf for the developmental expression of *Hsp* genes during seed maturation.

INTRODUCTION

As sessile organisms, plants have evolved a variety of mechanisms to respond to abiotic and biotic stresses. Central to the heat stress response in eukaryotes are the heat stress transcription factors (Hsfs), which belong to a family of proteins conserved throughout the eukaryotic kingdom (Wu, 1995; Nover and Scharf, 1997; Morimoto, 1998; Scharf et al., 1998b; Schöffl et al., 1998; Nakai, 1999; Baniwal et al., 2004). Hsfs have a modular structure with an N-terminal DNA binding domain characterized by a helix-turn-helix motif, an adjacent domain with a heptad hydrophobic repeat (HR A/B) required for oligomerization, a cluster of basic amino acid residues necessary for nuclear localization, and a C-terminal activation domain (AHA motifs) (Döring et al., 2000; Baniwal et al., 2004; Kotak et al., 2004). The *Arabidopsis thaliana* family of Hsfs comprises 21 members, which are grouped into three classes: A, B, and C (Nover et al., 2001). Meanwhile, the complexity of the Hsf family has been confirmed for other plants as well (Baniwal et al., 2004; Xiong et al., 2005). Interestingly, compared with plants, this multiplicity is much smaller in other organisms (i.e., *Drosophila melanogaster* and yeast with a single Hsf and vertebrates with three Hsf encoding genes, respectively; Sorger and Pelham, 1988; Wiederrecht et al., 1988; Clos et al., 1990; Rabindran et al., 1991; Sarge et al., 1991; Schuetz et al., 1991).

In the past few years, the role of selected *Arabidopsis* Hsfs has been characterized in the regulation of genes encoding molecular chaperones and other proteins providing cellular protection (Panchuk et al., 2002; Panikulangara et al., 2004; Busch et al., 2005; Davletova et al., 2005; Li et al., 2005; Schramm et al., 2006). Besides their role in the heat stress response in leaves, not much is known about other functions of Hsfs (e.g., during plant development). It has only been reported that Ha-*HsfA9* from sunflower (*Helianthus annuus*) is expressed during embryogenesis (Almoguera et al., 2002). However, so far, none of the genes have been identified that are involved in either developmental or stress-regulated expression of Hsfs.

Similar to other organisms, heat stress proteins (Hsps) in plants are expressed not only in response to stress, but also during various developmental programs, including pollen maturation, zygotic embryogenesis, and seed maturation (zur Nieden et al., 1995; Waters et al., 1996; Wehmeyer and Vierling, 2000). However, this phenomenon has been best characterized during seed development (Coca et al., 1994; De Rocher and Vierling, 1994; Wehmeyer et al., 1996). The putative role of Ha-HsfA9 as a transcriptional activator of the Ha-*Hsp17.7G4* gene has been demonstrated by transient reporter assays in sunflower embryos (Almoguera et al., 1998, 2002). The importance of heat stress elements as Hsf binding sites in the promoters of developmentally regulated *Hsp* genes of sunflower and tobacco (*Nicotiana tabacum*) has also been reported (Prändl et al., 1995; Coca et al., 1996; Prändl and Schöffl, 1996; Rojas et al., 1999). However, despite the occurrence of heat stress elements in the promoters of heat stress–inducible genes, only a subset of *Hsp* genes are expressed during seed development (Wehmeyer et al., 1996; Wehmeyer and Vierling, 2000; Hong and Vierling, 2001). These developmentally regulated Hsps accumulate late during the

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maturation phase, and then during germination the protein levels remain high for a few days and then decline rapidly (zur Nieden et al., 1995; Wehmeyer et al., 1996). The expression of particular isoforms of *Hsp* genes during seed development suggests that these Hsps might have a distinct function during seed maturation and that they are regulated by a defined developmental program.

The mechanisms that regulate *Hsp* expression during seed maturation remain largely unknown. In the *Arabidopsis* genome, several loci encoding transcriptional activators have been identified that specifically affect seed maturation, including *ABSCISIC ACID–INSENSITIVE3* (*ABI3*), *ABI4*, *ABI5*, *FUSCA3* (*FUS3*), *LEAFY COTYLEDON1* (*LEC1*), and *LEC2* (Koornneef et al., 1984; Giraudat et al., 1992; Bäumlein et al., 1994; Finkelstein et al., 1998; Lotan et al., 1998; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Stone et al., 2001). *Arabidopsis* plants with a desiccation-intolerant mutant allele of *ABI3* (*abi3-6*; Nambara et al., 1994) were shown to have no detectable Hsp17.4-CI in mature dry green seeds (Wehmeyer and Vierling, 2000). The absence of small Hsps (sHsps) correlates with a desiccation-intolerant phenotype, suggesting that sHsps might be required for desiccation tolerance in *Arabidopsis*.

In this study, we demonstrate that the seed-specific expression of *HsfA9* is regulated by ABI3. Additionally, we discuss the potential role of abscisic acid (ABA) in the context of our current model of the regulatory network involving *HsfA9* and *Hsp*. Furthermore, we have investigated the unique role of HsfA9 as a master regulator for expression of *Hsp* genes during seed development. Our data provide new insights into the mechanisms whereby an Hsf is regulated by another transcription factor and how it further plays a key role in the expression of *Hsp* genes during seed maturation.

RESULTS

HsfA9 Is a Seed-Specific Hsf

Using publicly available microarray data from different stages of *Arabidopsis* development (AtGenExpress), we identified HsfA9 as the only Hsf among the 21 members of the Hsf family that is exclusively expressed in the late stage of seed development and not during other stages of plant growth or during heat or other stresses (Figure 1A; see Supplemental Figures 1 and 2 online). *HsfA9* transcripts were detected at the onset of seed maturation, and the transcript level increased until seeds acquired desiccation tolerance and entered into dormancy (Figures 1A and 2A). Since Hsfs are known as transcriptional activators essential for the expression of *Hsp* genes (Wu, 1995; Nover and Scharf, 1997; Morimoto, 1998; Scharf et al., 1998b; Schöffl et al., 1998; Nakai, 1999), we further analyzed the AtGenExpress microarray data for expression of *Hsp* transcripts during silique development (Figure 1B; see Supplemental Figure 1 online). Among the 19 members of the sHsp family (Scharf et al., 2001), transcripts of only two genes were very abundant during seed maturation, *Hsp17.4-CI* and *Hsp17.7-CII* (Figures 1B; see Supplemental Figure 1 online). From the other Hsp families, the most abundant transcripts at this developmental stage encode Hsp70 and Hsp101 (see Supplemental Figure 1 online).

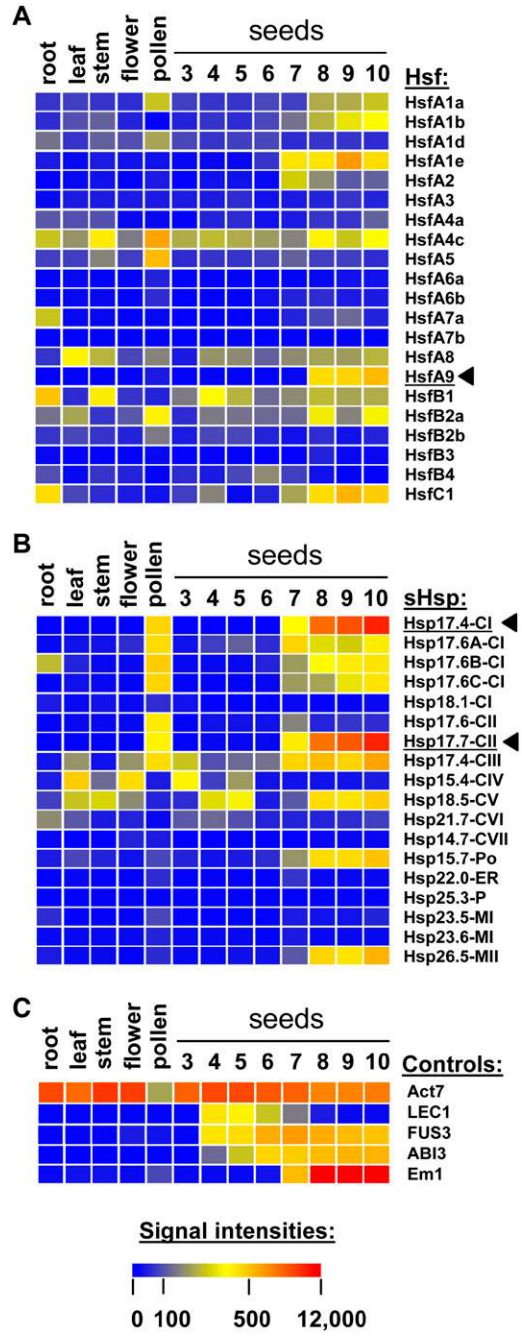

Figure 1. Expression Profiles of *Hsfs*, *sHsps*, and Genes Encoding Seed-Specific Transcriptional Activators in Different Developmental Stages of *Arabidopsis*.

Shown are normalized and averaged signal intensities visualized as heat maps with retransformed linear signal intensities from the AtGenExpress developmental series for the *Hsf* family **(A)**, and the *sHsp* family **(B)**, and the seed-specific transcription factors *LEC1*, *FUS3*, and *ABI3* **(C)**, with the late embryogenesis abundant protein encoding *Em1* transcripts and *Actin7* (*Act7*) as control. The corresponding color bar for the signal intensities of the transcript levels is shown below. For description of the selected samples (ATGE 93, 91, 27, 33, 73, 76, 77, 78, 79, 81, 82, 83, and 84) and further details, see Methods and Supplemental Figure 1 online.

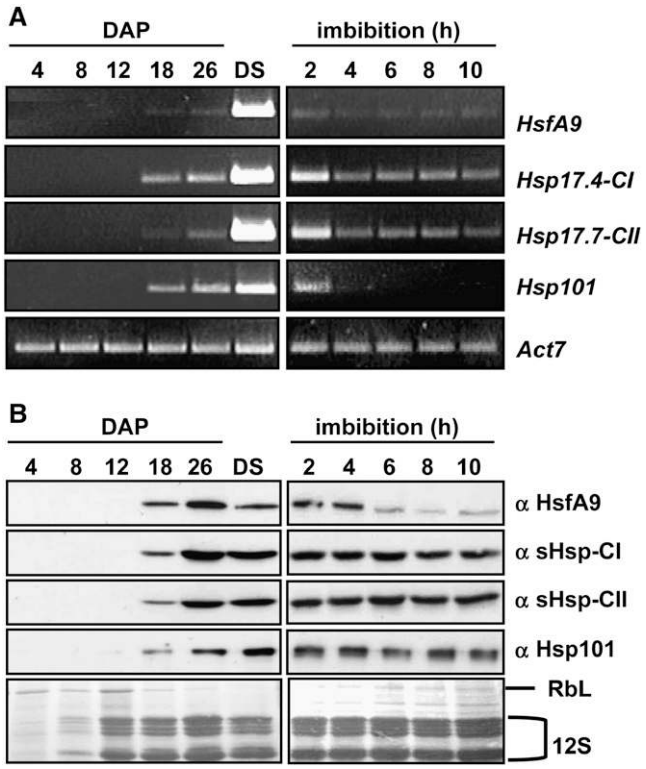

Figure 2. Expression of *HsfA9* and Selected *Hsp* Genes during Seed Development and Germination.

(A) Transcript levels of *HsfA9* and *Hsps* analyzed by RT-PCR from mRNA extracted from siliques from 4 to 26 d after pollination (DAP; see Supplemental Figure 3A online), dry seeds (DS), or 2 to 10 h of imbibition as indicated. As a control, the transcript levels of *Actin7* (*Act7*) were analyzed.

(B) The corresponding immunoblot analysis to **(A)** with specific antisera against HsfA9 and selected Hsps. As a control, the Ponceau-stained membrane is shown. Marked are the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RbL) and the 12S storage proteins (12S).

To compare the observed transcript levels with protein data, we raised polyclonal antibodies against the C-terminal domain (Kotak et al., 2004) of recombinantly expressed HsfA9. Immunoblot analyses demonstrated that the expression of HsfA9 is strictly developmentally regulated. Correlating with its transcript pattern, the HsfA9 protein is found only in the late stages of seed development, starting from 18 d after pollination (Figure 2B). The corresponding immunoblot analysis of class CI and class CII sHsp and Hsp101 correlates with the expression pattern of HsfA9 (Figure 2B). The protein levels detected in siliques in fact represent the amount in the seeds, since the proteins were not detectable in siliques from which the seeds were removed (data not shown).

HsfA9 Level Declines Rapidly during Seed Imbibition

To gain further insight into the relationship between *HsfA9* and *Hsp* expression during seed development, we monitored transcript and protein levels of *HsfA9* and the selected *Hsps* in seeds after different time periods of imbibition by RT-PCR and immunoblot analysis, respectively. As shown in Figure 2A, *HsfA9* transcripts were strongly reduced after only 2 h of seed imbibition, whereas the HsfA9 protein level declined drastically after 6 h but could be detected at a low level even after 10 h of seed imbibition (Figure 2B). Similarly, the levels of Hsp encoding transcripts were highly affected during seed imbibition (Figure 2A). While Hsp17.4-CI and Hsp17.7-CII transcript levels decreased after 4 h of imbibition to a level that does not change further up to 10 h of imbibition, the Hsp101 transcript level showed a rapid reduction, being almost undetectable after 4 h of seed imbibition. However, we could not detect any changes in the protein levels of any of the Hsp genes during the entire imbibition time course examined (Figure 2B).

Seed-Specific Elements Are Present in the Promoter Sequence of *HsfA9*

Next, we wanted to address the question that a transcription factor(s) may be involved in the regulation of *HsfA9* expression during seed development. By analyzing 1 kb of the sequence upstream of the open reading frame of *HsfA9* in the *Arabidopsis* genome sequence (http://www.arabidopsis.org), we found an RY/Sph and a RAV motif as putative seed-related regulatory elements (Figure 3A). The RY/Sph motif is an 8-bp sequence (CATGCATG) present in many seed-specific promoters, and it represents an essential binding site for ABI3 and FUS3 transcriptional activators found in seeds (Bobb et al., 1997; Ezcurra et al., 2000; Reidt et al., 2000; Mönke et al., 2004). The role of the RAV motif has also been documented as a potential binding site for RAV (related to ABI3/VP1) transcription factors (Kagaya et al., 1999; Yamasaki et al., 2004). However, we could not locate any abscisic acid–responsive elements, which are usually found in promoters of seed-specific genes (Nakabayashi et al., 2005). Further analysis of the available microarray data indicates that *ABI3* and *FUS3* transcripts accumulate before the onset of *HsfA9* and *Hsp* transcripts during silique development (Figure 1C); a similar observation for the expression pattern of *ABI3* and *FUS3* has been described previously (Parcy et al., 1994; Kagaya et al., 2005b). In contrast with this, expression of the *LEC1* gene, which encodes a CBF transcription factor shown to act in concert with *ABI3* and *FUS3* (Parcy et al., 1997; Lotan et al., 1998; Kagaya et al., 2005b), dramatically declined as seeds entered into the desiccation period (Figure 1C).

ABI3 Activates the *HsfA9* Promoter in Transient Reporter Assays

To test directly the potential of ABI3, FUS3, and LEC1 to activate *HsfA9* expression, we used a reporter construct containing 1 kb of upstream sequence from the *HsfA9* gene fused to β-glucuronidase (P_{HsfA9}*:GUS*) in transient assays using protoplasts from *Arabidopsis* suspension-cultured cells (Figure 3B). A *GUS* construct driven by the seed-specific promoter of the *USP* gene of *Vicia faba* (Bäumlein et al., 1991a) was used as a positive control for the seed-specific transcriptional activators ABI3 and FUS3, respectively (Reidt et al., 2000). As shown in Figure 3B,

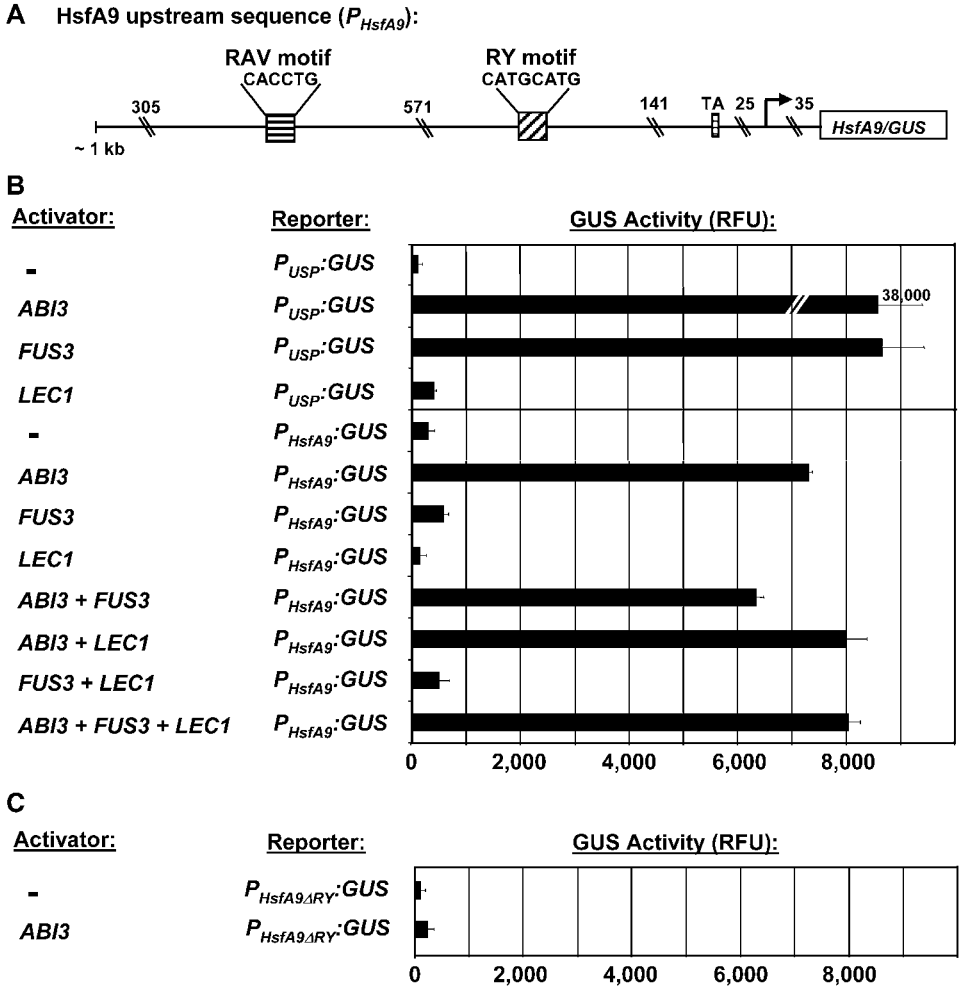

Figure 3. Activity of Seed-Specific Transcription Factors on the Promoter of *HsfA9* in Transient *GUS* Reporter Assays.

(A) Schematic diagram of 1-kb sequence upstream of the *HsfA9* open reading frame. Shown are the predicted RY and RAV motifs (see text for details), the putative TATA box (TA), and the transcriptional start site (arrow); the numbers above indicate the distance in base pairs between the marked points.
(B) The activator potential of ABI3, FUS3, and LEC1 was tested in *Arabidopsis* suspension culture protoplasts by transient cotransformation with a reporter construct containing a *USP* promoter fragment fused to *GUS* or containing a 1-kb promoter fragment of the *HsfA9* gene fused to GUS as indicated. GUS activities (relative fluorescence units [RFU]) are presented with error bars for the standard deviation of three independent replicates. For further details, see Methods.
(C) The activator potential of ABI3 analyzed as in **(B)** but using GUS fused to a 1-kb promoter fragment of the *HsfA9* gene from which the RY motif (see **[A]**) was deleted. For further details, see Methods.

ABI3 led to an activation of the P_{HsfA9}*:GUS* reporter construct, showing up to a 15- to 20-fold increase in GUS activity in comparison to the reporter alone. In contrast with ABI3, we did not observe any activation with FUS3 or LEC1 either alone or in combination with ABI3 (Figure 3B). Similar results were obtained using tobacco mesophyll protoplasts (data not shown).

Furthermore, to confirm that the predicted RY/Sph motif is a functional binding site for ABI3, we tested a mutated form of P_{HsfA9}*:GUS* having a deletion of the RY motif (Figures 3A and 3C). We could not measure any ABI3-driven GUS activity, indicating that the RY/Sph motif is essential for the transcriptional induction of *HsfA9* by ABI3 (Figure 3C). This is in agreement with the functional analysis of the RY/Sph motif in the promoters of other seed-specific genes (Bäumlein et al., 1992; Ellerström et al., 1996; Reidt et al., 2000; Mönke et al., 2004). Taken together, we conclude that ABI3 is a potent activator of the *HsfA9* promoter, and its RY/Sph motif is essential for it in homologous and heterologous plant cell systems.

HsfA9 Is Absent in ABI3 Mutant Lines

To investigate the biological relevance of the *ABI3*-encoded protein in the regulation of *HsfA9* expression in planta, we studied mutant lines of the *ABI3* gene (Figures 4A and 4B). In

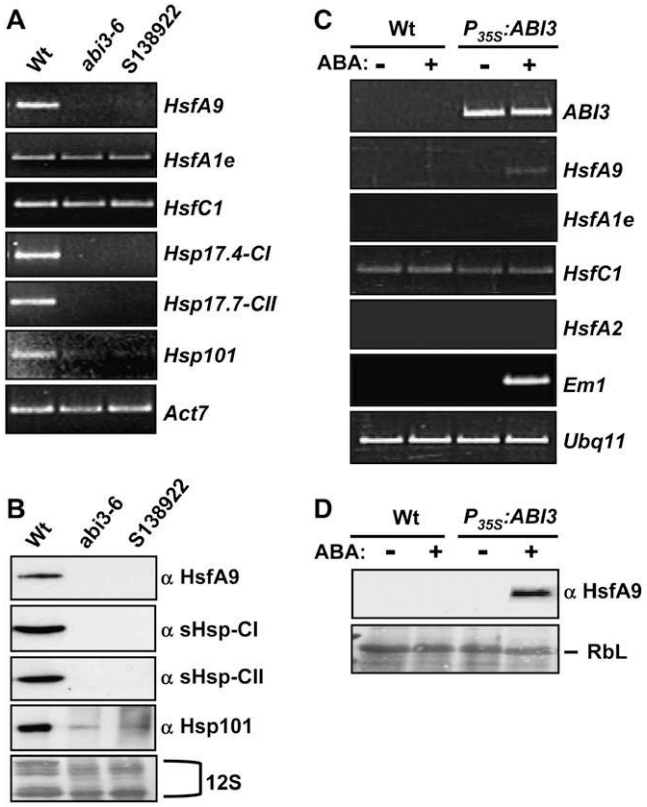

Figure 4. *HsfA9* and *Hsp* Expression Are Dependent on ABI3 in Planta.

(A) Transcript levels of *HsfA9*, *HsfA1e*, *HsfC1*, and different *Hsp* genes were analyzed by RT-PCR from mRNA extracted from seeds 26 d after pollination from wild-type plants and the two ABI3 mutant lines (*S138922* and *abi3-6*; for details and references, see Supplemental Table 1 online) as indicated. As a control, the transcript levels of *Actin7* (*Act7*) were analyzed.

(B) The corresponding immunoblot analysis to **(A)**. As a control, the 12S storage proteins (12S) are shown on the Ponceau-stained membrane. For further details, see Figure 2B and Methods.

(C) Transcript levels of selected *Hsfs*, *ABI3*, and *Em1* analyzed by RT-PCR from mRNA extracted from wild-type seedlings and a transgenic line ectopically overexpressing ABI3 (P_{35S}*:ABI3*). Seeds were grown for 20 d on Murashige and Skoog (MS) medium, and subsequently seedlings were transferred to MS medium containing 50 μM ABA for 4 d. As a control, *Ubiquitin11* (*Ubq11*) levels were analyzed in all samples.

(D) The corresponding immunoblot analysis to **(C)** performed as in **(B)**.

addition to the available *abi3-6* null allele (Nambara et al., 1994), we characterized the Salk line *S138922*, which carries a T-DNA insertion in the first exon of the *ABI3* gene (see Supplemental Figures 3B and 3C online). Similar to other *ABI3* mutants, the *S138922* line has desiccation-intolerant seeds that remain green due to nondegradation of chlorophyll (see Supplemental Figure 3B online). RT-PCR analysis showed that the *S138922* line is a null mutant that contains no detectable *ABI3* transcript (see Supplemental Figure 3C online). We next tested whether the expression of *HsfA9* and *Hsps* in dry green seeds is affected in the *ABI3* knockout lines. Using RT-PCR analysis, we could not detect any transcripts encoding *HsfA9*, *Hsp17.4-CI*, and *Hsp17.7-CII* and drastically reduced levels of *Hsp101* in dry green seeds of both *ABI3* knockout lines (Figure 4A). This situation is also reflected at the protein level; neither HsfA9 nor the analyzed sHsp was expressed at detectable levels, and the level of Hsp101 was strongly reduced (Figure 4B). Interestingly, in contrast with *HsfA9*, the transcript levels of other Hsfs that are expressed in seeds such as *HsfA1e* and *HsfC1* were not affected (Figure 4A). It has been suggested that *ABI4* and *ABI5* genes, which encode an AP2 and a bZIP transcription factor, respectively, function in a combinatorial network together with ABI3 to control seed development and ABA response (Söderman et al., 2000). Therefore, we analyzed described mutant lines of *ABI4* and *ABI5* (for details and references, see Supplemental Table 1 online) for *HsfA9* and *Hsp* expression in seeds. Immunoblot analysis revealed that none of these mutants is defective for the expression of *HsfA9* and *Hsp* genes (see Supplemental Figure 3D online). Summarizing our results on ABA-insensitive mutant lines, we conclude that only ABI3, but not ABI4 and ABI5, controls *HsfA9* and *Hsp* gene expression in seeds.

Plants Ectopically Expressing *ABI3* Induce *HsfA9* in the Presence of ABA, whereas Endogenous ABA Content Seems Not to Play a Major Role in Its Seed-Specific Expression

Because of the pleiotropic nature of the *ABI3* mutants (*abi3-6* and *S138922*), we analyzed the expression of *HsfA9* in a plant line ectopically expressing *ABI3* (Parcy et al., 1994). To avoid possible interference with other endogenous factors from seeds, we analyzed the expression of *HsfA9* in 24-d-old seedlings. The 20-d-old wild-type and transgenic seedlings were transferred onto plates with or without 50 μM ABA for 4 d. Addition of ABA alone had no influence on the expression of *HsfA9* in wild-type seedlings (Figures 4C and 4D). However, *HsfA9* transcript and HsfA9 protein accumulated in samples from ABA-treated seedlings ectopically expressing *ABI3* (Figures 4C and 4D). The expression of *Hsps* in the vegetative tissue has already been documented in the microarray data of a *VP1* (the homologue of *ABI3* from maize [*Zea mays*]) overexpression line in *abi3* null background when subjected to exogenous supply of ABA (Suzuki et al., 2003). Expression of the *Em1* gene, encoding a seed-specific small hydrophilic protein, was also monitored as a positive control (Parcy et al., 1994). The results presented here demonstrate that *HsfA9* expression can indeed be induced by increased ABA level, but ABI3 is essential for this response. This effect could also be reconstructed in transiently transformed protoplasts, where addition of ABA led to a twofold higher GUS activity in samples expressing plasmid-borne *ABI3*, whereas ABA alone had no effect (see Supplemental Figure 4 online). In contrast with the induction of *HsfA9*, we could not monitor any effect of ABI3 with or without exogenous supply of ABA on the expression of other developmentally regulated or heat stress–induced Hsfs, (i.e., *HsfA1e*, *HsfC1*, and *HsfA2*) (Figure 4C). These data suggest that the expression of *HsfA9* might be subjected to an ABA-modulated ABI3-dependent regulation in seeds. The existence of a possible role of endogenous ABA in the control of *HsfA9* and *Hsps* expression was further investigated using several mutant lines that were deficient for the ABA level in

seeds (*aba1*, *aba2*, and *aba3*; for details, see Supplemental Table 1 online). Immunoblot analysis indicates that all of these mutants accumulate HsfA9 and Hsp proteins at levels comparable to the wild type (see Supplemental Figure 5 online). The same observation has been documented already for Hsp17.4-CI accumulation in dry seeds of the *aba1* mutant (Wehmeyer et al., 1996). In addition to these results, we could detect a higher protein level of HsfA9 in the ecotype Columbia (Col) in comparison to Landsberg *erecta* (L*er*) correlating with similar levels of Hsps (see Supplemental Figure 5 online).

Analysis of HsfA9-Dependent *Hsp* Expression in Vegetative Tissues

To gain insight into the function of HsfA9, we generated plants ectopically expressing *HsfA9* with a 3HA tag at the C terminus under the control of the constitutive 35S promoter of *Cauliflower mosaic virus* (*CaMV35S*) (P_{35S}*:HsfA9-3HA*). We monitored the expression profiles of *Hsp* genes in leaf samples from three independent lines of unstressed 4-week-old plants (P_{35S}*:HsfA9-3HA* lines 1, 2, and 3) and compared these with untreated and heat-stressed wild-type plants of the same age (Figures 5A and 5B). Transgenic plants with ectopic *HsfA9* expression showed constitutive expression of *Hsp* genes in leaves under nonstress conditions (Figures 5A and 5B). These results demonstrate that HsfA9 needs no other seed-specific factors to induce chromatin-embedded genes encoding Hsp17.4-CI, Hsp17.7-CII, and Hsp101 (Figures 5A and 5B). Surprisingly, we could also detect expression of other *Hsp* genes that are not highly expressed in seeds, such as *Hsp17.6A-CI* and *Hsp17.6-CII* (Figure 5B).

The expression of *Hsps* during seed development could be attributed to the transcriptional activity of either HsfA9 or to ABI3 and ABA signaling. To address this issue, we established a dual activator-reporter–based transient *GUS* system in tobacco mesophyll protoplasts (Figure 5C). We cloned *HsfA9-3HA* under control of its own inducible promoter in a plant expression vector (P_{HsfA9}*:HsfA9-3HA*) and analyzed its activator potential either alone or in combination with ABI3 and ABA on reporter constructs containing 0.5 to 1 kb of upstream sequences of selected *Hsp* promoters in fusion to GUS (P_{Hsp}*:GUS*; see Methods for details). As shown in Figure 5C, cotransformation of P_{HsfA9}*:HsfA9-3HA* and the P_{Hsp}*:GUS* reporter constructs showed no GUS activity and no detectable HsfA9-3HA protein level. However, the presence of ABI3 and ABA lead to the expression of HsfA9-3HA from the P_{HsfA9}*:HsfA9-3HA* construct and a 10- to 20-fold increase in GUS activity from the cotransformed P_{Hsp}*:GUS* constructs (Figure 5C). The induced expression of P_{HsfA9}*:HsfA9-3HA* by ABI3 and ABA is in accordance with our results where ABI3 showed an activation of the P_{HsfA9}*:GUS* reporter construct that was enhanced by ABA (see Supplemental Figure 4 online). Furthermore, in the absence of P_{HsfA9}*:HsfA9-3HA*, ABI3 showed no induction of any *Hsp* promoter-driven *GUS* reporter (Figure 5C). Accordingly, the data obtained from transgenic plants ectopically expressing *HsfA9* (Figure 5A) and the dual activator-reporter–based transient *GUS* reporter system strongly suggest that HsfA9 is an essential transcription factor for the expression of *Hsp* genes during seed development (Figure 5C).

Heat Stress Leads to Hsp Accumulation in Mature Siliques of the *abi3-6* Mutant

The influence of *ABI3* on HsfA9 and Hsp accumulation during seed development prompted us to examine the expression of *HsfA9* and *Hsp* genes in heat-stressed siliques from wild-type plants and the *abi3-6* mutant line. Siliques at three different developmental stages were heat-stressed at 38°C for 2 h. As shown in Figure 6A, *HsfA9* transcripts could not be detected in control or heat-stressed silique samples of the *abi3-6* mutant line. In contrast with this, we could detect the synthesis of *Hsp17.4-CI*, *Hsp17.6A-CI*, *Hsp17.6-CII*, *Hsp17.7-CII*, and *Hsp101* transcripts at comparable levels in the heat-stressed samples from both wild-type and *abi3-6* mutant siliques (Figure 6A). Similar to Hsps, the expression profile of *HsfA2*, a strictly heat stress–induced Hsf (Schramm et al., 2006), was unaltered in heat stress siliques samples of wild-type plants and the *abi3-6* mutant line (Figure 6A). Transcripts of the developmentally regulated late-embryogenesis abundant genes *Em1* and *Em6* were found in wild-type samples but were absent in the *abi3-6* mutant and were not affected by heat stress (Figure 6A). The corresponding immunoblot analysis correlates well with the results obtained by RT-PCR analysis (Figure 6B): (1) HsfA9 is absent in the *abi3-6* mutant and is not inducible by heat stress; (2) Hsp17.4-CI, Hsp17.7-CII, and Hsp101 seem to be regulated by the HsfA9-dependent pathway but are also induced by heat stress, as indicated by the accumulation of high levels under heat stress in both the wild type and the *abi3-6* mutant. These results indicate that HsfA9 is essential for the developmental regulation of *Hsp* expression in seeds but that an HsfA9-independent pathway controls the expression of *Hsp* genes in response to heat stress.

DISCUSSION

HsfA9 Is Specifically Expressed in Seeds and Regulated by ABI3

HsfA9 is an exceptional candidate among all the 21 members of the *Arabidopsis* Hsf family, being strictly and exclusively developmentally regulated during the seed maturation phase. In the publicly available AtGenExpress microarray database, we found that besides *HsfA9*, other Hsfs are also expressed at the transcript level during seed development (e.g., *HsfA1e* and *HsfC1*; see Figure 1A). However, in contrast with *HsfA9*, these *Hsfs* are also expressed at other stages of development and/or induced by abiotic stresses (see Supplemental Figure 1 and 2 online). The specialized role of HsfA9 is in agreement with studies of a sunflower homologue, Ha-HsfA9 (Almoguera et al., 2002). However, Ha-*HsfA9* regulation during embryo development in sunflower has not been documented. We show by several lines of evidence that *HsfA9* expression is controlled by developmentally regulated *ABI3* activity. First, ABI3 activates an *HsfA9* promoter *GUS* fusion construct (P_{HsfA9}*:GUS*) in transient assays in protoplasts, and this activity is independent of the plant species from which the protoplasts are derived (*Arabidopsis* versus tobacco). Second, *ABI3* mutants (*abi3-6* and *S138922*) lack detectable levels of both HsfA9 transcript and protein. Third, ectopic expression of the *ABI3* gene led to accumulation of HsfA9 in

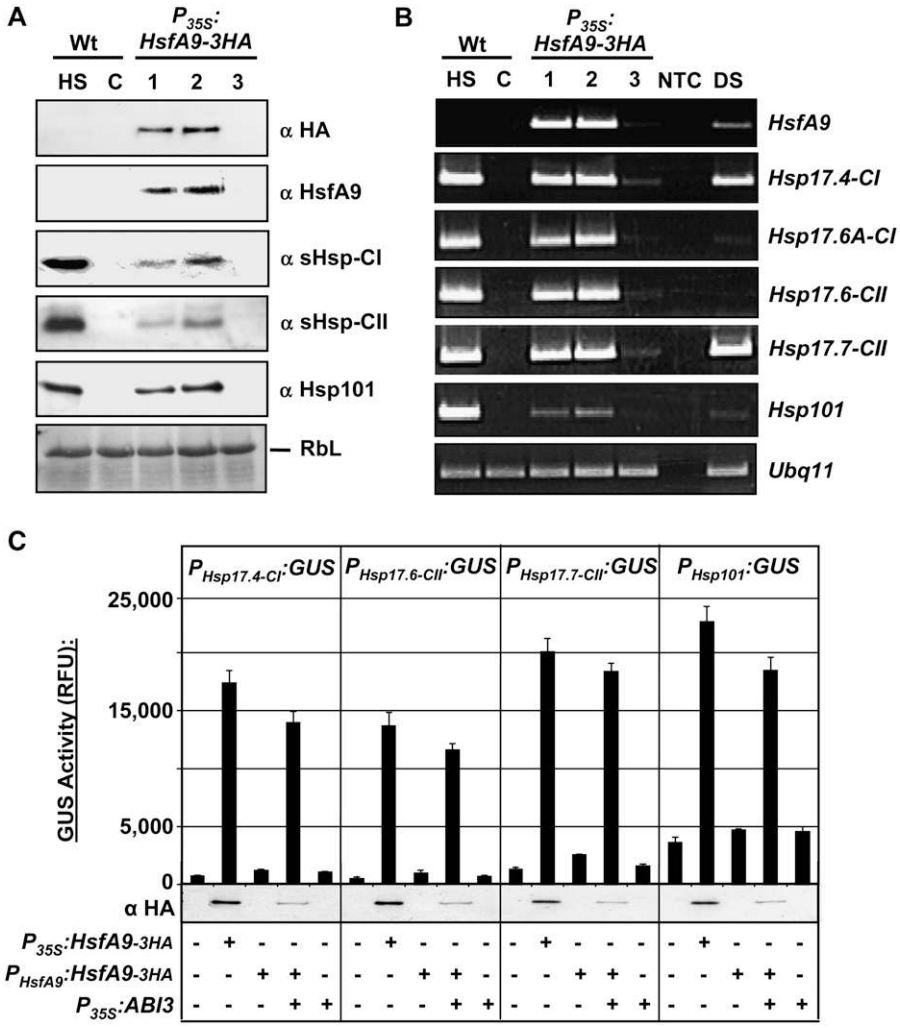

Figure 5. HsfA9 Activates *Hsp* Promoters in Planta and in Transient Reporter Assays.

(A) Analysis of HsfA9 and Hsp protein levels by immunoblot analysis from samples extracted from wild-type leaves and three transgenic lines (numbered 1, 2, and 3) ectopically overexpressing HsfA9 (P_{35S}*:HsfA9*). As a control, the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RbL) is shown on the Ponceau-stained membrane. As further controls, wild-type samples were kept at room temperture (C) or heat-stressed (HS) for 2 h at 38°C prior to extraction. For further details, see Figure 2B and Methods.

(B) The corresponding RT-PCR analysis to **(A)**. As a control, *Ubiquitin11* (*Ubq11*) levels were analyzed in all samples. NTC, no template control; DS, dry seed sample as described in Figure 2.

(C) The activator potential of HsfA9 and ABI3 (together with 5 μM ABA) was tested in tobacco mesophyll protoplasts by transient cotransformation of expression constructs for *ABI3* or *HsfA9-3HA* under control of the constitutive *35SCaMV* promoter (P_{35S}*:ABI3* and P_{35S}*:HsfA9-3HA*) or in the case of P_{HsfA9}*:HsfA9-3HA*, under control of its own inducible promoter with reporter constructs containing 0.5- to 1-kb promoter fragments of selected *Hsp* genes fused to *GUS* (P_{Hsp}*:GUS*; for details, see Methods) as indicated above the corresponding samples. The resulting GUS activities (relative fluorescence units [RFU]) are presented with error bars for the standard deviation of three independent replicates. As shown below, expression of the HsfA9-3HA protein was monitored by immunoblot analysis of the corresponding samples using an antibody against the 3HA-tag (α HA).

vegetative tissues in the presence of ABA. Taken together, these results strongly suggest that the accumulation of *HsfA9* transcripts during seed maturation is controlled by ABI3, and ABA may be involved in this process. The activation potential of ABI3 on P_{HsfA9}*:GUS* was abolished by deletion of the RY/Sph motif (Figure 3C). This observation is consistent with the previous reports where deletion of an RY/Sph motif in the promoter of a *legumin* gene of *V. faba* (Bäumlein et al., 1986, 1991a, 1991b; Fiedler et al., 1993), the promoter of a *napin* gene of *Brassica napus* (Ellerström et al., 1996), and the *C1* promoter of *Z. mays* (Suzuki et al., 1997) abolished most of the seed-specific promoter activity. Interestingly, FUS3 was inactive on P_{HsfA9}*:GUS* (Figure 3B); however, both B3 domain transcription factors (ABI3 and FUS3) have been shown to recognize the same RY/Sph core motif (Reidt et al., 2000; Mönke et al., 2004). This discrepancy might be due to the RY/Sph flanking sequence, which may allow

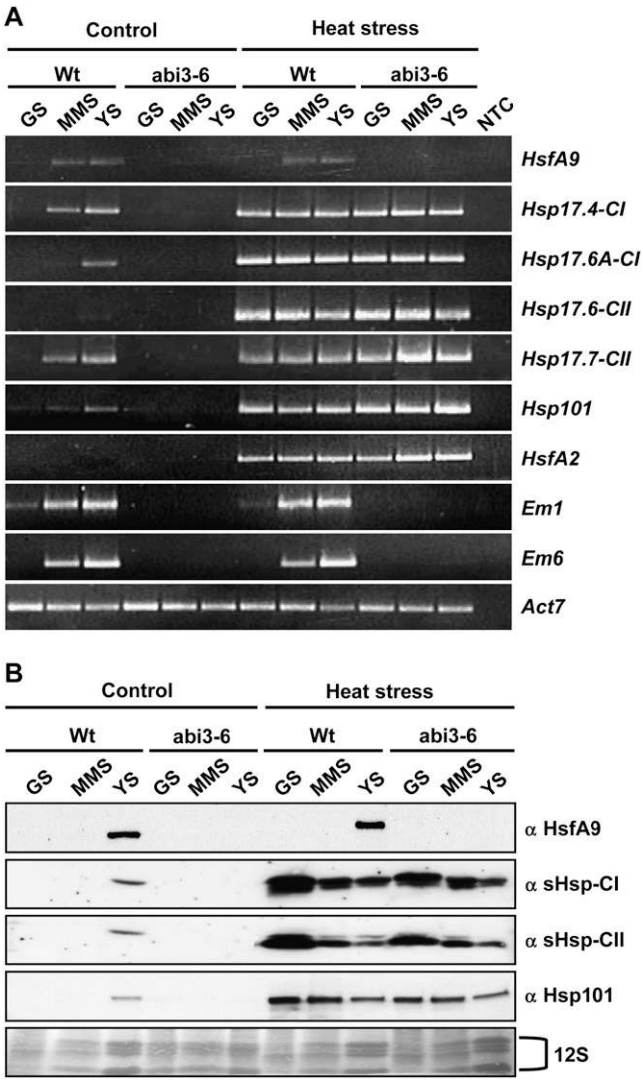

Figure 6. Wild-Type and *abi3-6* Plants Respond Similarly to Heat Stress.

(A) Transcript levels of *HsfA9* and *Hsps* analyzed by RT-PCR from mRNA extracted from green siliques (GS), mid-mature siliques (MMS), or yellow siliques (YS) from wild-type plants and the *abi3-6* mutant. Prior to extraction, samples were kept at room temperature as a control or heat-stressed for 2 h at 38°C as indicated. As controls, the transcript levels of *Actin7* (*Act7*), *HsfA2*, *Em1*, and *Em6* were analyzed in all samples. NTC, no template control.

(B) The corresponding immunoblot analysis to **(A)**. For further details, see Figure 2B and Methods.

only ABI3 but not FUS3 to interact with the component of transcription machinery. Furthermore, using transgenic and knockout approaches, the overlapping and independent function of *ABI3* and *FUS3* has been suggested (To et al., 2006), which might explain why *HsfA9* is specifically regulated by *ABI3* but not by *FUS3*. Our results also exclude the involvement of two other transcription factors encoded by ABA-insensitive loci (*ABI4* and *ABI5*) for the regulation of HsfA9 in seeds. However, we also cannot exclude the possibility that ABI3 indirectly regulates the expression of HsfA9 via an unknown protein that binds to the RY/Sph element during seed development. Interestingly, the transcript levels of *HsfA1e* and *HsfC1* were not affected in the *ABI3* mutants (Figure 4A); in addition, ectopically expressed *ABI3* did not induce the expression of *HsfA1e* and *HsfC1* (Figure 4C). These results indicate that, indeed, the action of *ABI3* is specific for the *HsfA9* promoter not only on plasmid-borne reporter assays but also in planta, where the *HsfA9* promoter is present in a chromatin-bound state.

Crosstalk among ABA-Dependent and ABA-Independent Developmental Cues with ABI3 for the Regulation of *HsfA9* and *Hsp* Genes

The involvement of ABA in seed desiccation tolerance has been a matter of controversy. Transcripts encoding several storage proteins and late embryogenesis abundant proteins, thought to be involved in providing desiccation tolerance, could be induced by exogenous supply of ABA in cultured embryos (Rock and Quatrano, 1995; Ingram and Bartels, 1996; Merlot and Giraudat, 1997). Surprisingly, maize *vp* and *Arabidopsis aba1* biosynthetic

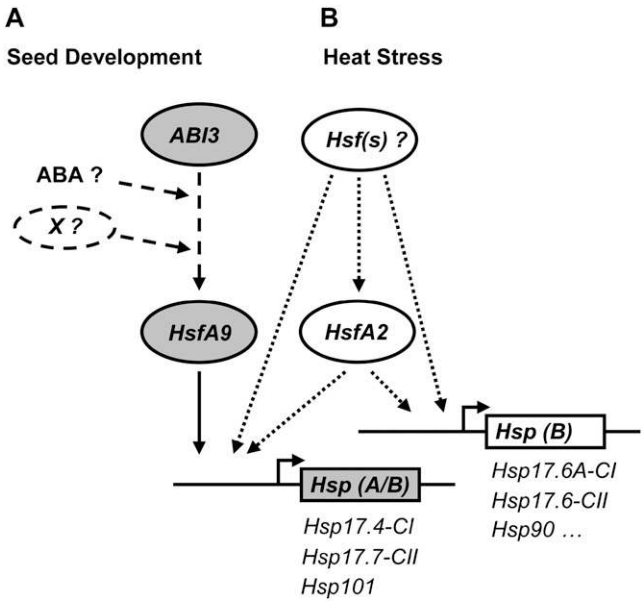

Figure 7. Model for the Regulation of *Hsp* Genes in Seeds and the Role of Hsfs.

(A) Developmental expression of the *HsfA9* gene is likely transcriptionally regulated by the seed-specific transcription factor ABI3. An additional effect by ABA or a yet unknown discrete developmental signal is shown. HsfA9 in turn acts as the transcriptional regulator of the developmentally expressed subset of *Hsps*.

(B) Independent of HsfA9, the same subset of *Hsps* is transcriptionally induced by heat stress as are many other *Hsp* genes that are not developmentally regulated. This pathway is probably regulated only by *Hsf* genes, either directly or indirectly by, for example, HsfA2, which itself is heat stress–induced.

mutants do not display a significant reduction in the accumulation of storage protein mRNA or in desiccation tolerance (Koornneef et al., 1989; Paiva and Kriz, 1994; Parcy et al., 1994). Characterization of an ectopically expressing *ABI3* line (Parcy et al., 1994) for HsfA9 expression suggested that either ABI3 functionally interacts with the ABA signaling cascade or a component of the ABA cascade might modify ABI3 itself in a vegetative tissue. Surprisingly, the developmental regulation of the HsfA9 and Hsp protein level was unaltered in dry seeds of ABA-deficient mutants (see Supplemental Figure 5 online; Wehmeyer et al., 1996). The same observation has been reported for cruciferin *CRC* and napin *At2S3* transcript levels, where ABI3 permits the expression of these genes in an ABA-dependent manner in a vegetative tissue (Parcy et al., 1994; Kagaya et al., 2005a, 2005b), but their expression was almost unaltered in the siliques of the *aba-1* mutant, which contains <5% of the wild-type amount of endogenous ABA (Karssen et al., 1983; Parcy et al., 1994). As previously discussed for seed storage protein encoding transcripts (Giraudat et al., 1994; Parcy et al., 1994; Merlot and Giraudat, 1997), the possibility of residual ABA content in ABA-deficient mutants, which may be sufficient to ensure wild-type expression of *HsfA9* and *Hsp* genes, cannot be excluded. Alternatively, it cannot be ruled out that expression of *HsfA9* and *Hsp* genes might conceivably be triggered by an increase in ABA sensitivity in embryonic cells. Furthermore, the involvement of a distinct developmental signal other than endogenous ABA content for the accumulation of HsfA9 and Hsps during seed development cannot be ignored, whereas the function of ABI3 is indispensable.

HsfA9 Regulates Expression in a Specific Subset of *Hsp* Genes

Previous investigation of the *abi3-6* mutant allele showed that the *ABI3* gene plays a critical role in the regulation of *Hsp17.4-CI* (Wehmeyer et al., 1996; Wehmeyer and Vierling, 2000). We show that not only *Hsp17.4-CI* but also other developmentally regulated Hsps (i.e., *Hsp17.7-CII* and *Hsp101*) also require HsfA9 for full activation. In contrast with the activity of HsfA9, we did not observe any activation potential of ABI3 on P_{Hsp}*:GUS* constructs in transient reporter assays (Figure 5C). The absence of HsfA9 and Hsps in the *ABI3* mutant lines (*abi3-6* and *S138922*) further argues for the involvement of HsfA9 in the expression of *Hsp* genes during seed development. This observation is consistent with previous studies of sunflower Ha-*Hsp17.7G4*, where *GUS* fused to the Ha-*Hsp17*.7G4 promoter could be activated by Ha-HsfA9 in transiently transformed sunflower embryos (Almoguera et al., 2002). Furthermore, ectopic expression of *HsfA9* in leaf tissues resulted in constitutive expression of genes encoding Hsps (Figures 5A and 5B). This strongly suggests that HsfA9 is a tissue-independent, potent transcriptional activator of *Hsp* genes, as are HsfA1a/HsfA1b (Busch et al., 2005), and HsfA9 can operate without seed-specific coregulators and/or developmentally tuned posttranslational modifications. The absence of expression of other *Hsp* genes in seeds such as *Hsp17.6A-CI* and *Hsp17.6-CII* (cf. Figures 5B and 1B) suggests that chromatin architecture might play an essential role in determining the subset of *Hsp* genes transcribed under developmental control.

Regulatory Pathways for Hsp Expression during Heat Stress and Development Are Distinct

Genetic and molecular analysis of knockout mutants of the constitutively expressed *HsfA1a*/*HsfA1b* (Busch et al., 2005) and the heat stress–induced *HsfA2* (Schramm et al., 2006) have revealed that these Hsfs play an important role in regulation of *Hsp* genes in vegetative tissue under heat stress conditions. Here, we provide evidence for the involvement of Hsfs in two distinct regulatory pathways: developmentally regulated versus heat stress–induced. Irrespective of the presence or absence of HsfA9, the same subset of *Hsp* genes was transcriptionally induced by heat stress in addition to many other *Hsp* genes that are not developmentally regulated (Figure 6). This heat-induced transcriptional response is probably regulated by other *Hsf* encoding genes, either directly (e.g., HsfA1a/A1b) or indirectly (e.g., by HsfA2, which itself is heat stress–induced). In this respect, it would be intriguing to investigate the role of other Hsfs, which are present on the transcript level during these seed stages (e.g., HsfA1e and HsfC1; Figure 1). The results presented here indicate that although other Hsfs may coexist with HsfA9, they seem not to play a dominant role in the developmental induction of *Hsp* genes. However, it will be crucial to analyze the corresponding knockout lines and particularly the heat stress response in their seeds to characterize the role of these Hsfs in the Hsf/Hsp network. The accumulation of nondevelopmentally regulated Hsps in heat-stressed silique samples is in accordance with previous observations, where it was shown that the nondevelopmentally regulated class II sHsps, as well as plastid- and endoplasmic reticulum–localized Hsps, accumulate in heat stress–treated pea (*Pisum sativum*) embryos (De Rocher and Vierling, 1994).

Based on these results, we propose a model in which the seed-specific expression of *HsfA9* is controlled by ABI3, directly or via an unknown protein in developing seeds (Figure 7A). *HsfA9* in turn activates the expression of downstream genes, such as *Hsp17.4-CI*, *Hsp17.7-CII*, and *Hsp101*. So far, we cannot completely exclude the role of ABA and a discrete developmental signal that cooperates with ABI3 for the regulation of *HsfA9* and *Hsp* genes until the expression of these genes in the absolute null ABA background has been worked out. However, in developing siliques, heat stress can activate or induce other nondevelopmentally regulated Hsfs (e.g., HsfA1a/A1b), leading ultimately to Hsp synthesis either directly or via heat stress–induced HsfA2 (Figure 7B). The different pathways of *Hsp* induction during development and/or heat stress demonstrate that there is a fine-tuned mechanism of crosstalk within the Hsf/Hsp network that is required to balance the protein homeostasis under a changing cellular environment.

Evolutionarily Conserved Mechanism of *HsfA9* and *Hsp* Expression during Seed Development

Our investigation of HsfA9 and Hsp abundance during the late seed maturation phase emphasizes the important role of these genes in seed development. The expression profile of *HsfA9* and *Hsp* genes during seed development coincides with the acquisition of dormancy and desiccation tolerance, and based on

facts already discussed, we hypothesized that the action of these genes may be important for one of these processes. We have so far been unable to rescue homozygous seeds for a T-DNA insertion line for *HsfA9* and also did not succeed in obtaining seeds representing knockdown expression of *HsfA9* by RNA interference approach to decipher further the important role of *HsfA9* and *Hsp* genes during seed development. In parallel to *HsfA9*, Ha-*HsfA9* from sunflower has been shown to be exclusively expressed in embryos (Almoguera et al., 2002). However, despite the apparent functional similarity, the amino acid sequences of *Arabidopsis* and sunflower HsfA9 are poorly conserved (see Supplemental Figure 6 online). In addition to sunflower, analysis of the available EST databases (http://www.ncbi.nlm.nih.gov/BLAST/) identified potential HsfA9 homologues from tomato, potato (*Solanum tuberosum*), maize, barley (*Hordeum vulgare*), rice (*Oryza sativa*), and coffee (*Coffea arabica*) (see Supplemental Figure 6 online). Phylogenetic analysis based on the DNA binding domain revealed that *Arabidopsis* HsfA9 is not closely related to other HsfA9 homologues (see Supplemental Figure 7 online). However, in a phylogenetic analysis of all Hsf members of *Arabidopsis*, tomato, and rice, HsfA9 of *Arabidopsis* and tomato clearly forms a separate branch, placing HsfA9 in a unique position within the plant Hsf family (Baniwal et al., 2004). Interestingly, RT-PCR analysis of mRNA from mature coffee beans and dry seeds of tomato detected transcripts of these HsfA9 homologues, while *HsfA9* transcripts were absent in leaf tissues of coffee and tomato (S. Kotak, unpublished data). Similar to HsfA9, a growing body of evidence suggests that Hsps are also developmentally regulated not only in *Arabidopsis* and sunflower but also in several other plants during embryogenesis. Hernandez and Vierling (1993) immunodetected class I Hsp in mature, field-grown seeds of a variety of legumes, including pea, soybean (*Glycine max*), cowpea (*Vigna unguiculata*), and acacia (*Acacia constricta*). Other than Fabaceae, occurrence of Hsp during seed development has also been reported in Poaceae and Solanaceae families (zur Nieden et al., 1995; Guan et al., 2004). In the view of a recent publication, where overexpression of Ha-*HsfA9* in tobacco seeds has been shown to enhance the accumulation of Hsp, which ultimately improved the seed longevity (Prieto-Dapena et al., 2006) and our data presented in this manuscript, it is tempting to speculate about the evolutionary specification of one Hsf member within the complex family of 20 to 30 Hsfs as the key regulator for the expression of *Hsp* in seeds of many plant species.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* ecotypes Col, L*er*, *C24*, and Wassilewskija were used as wild types for developmental experiments as indicated. The *abi3-6* mutant (Nambara et al., 1994) was provided by E. Nambara (RIKEN Institute, Japan), and the line ectopically expressing ABI3 (Parcy et al., 1994) was obtained from F. Parcy (Physiologie Cellulaire Végétale, Centre National de la Recherche Scientifique, France). The ABA-deficient mutants *aba1-3*, *aba2-1*, and *aba3-1* were obtained from the ABRC. *abi3-6* (Nambara et al., 1994) and *S138922* desiccation-intolerant homozygous seeds were maintained by propagation of green seeds. Plants were grown routinely in a greenhouse (22°C with a 16-h photoperiod) on soil. For aseptic growth, seeds were surface sterilized according to Finkelstein and Somerville (1990) and germinated on agar plates (4.6 g/L) with MS salt, 10 g/L sucrose, and 3 g/L Gelrite (Merck), pH 5.8. ABA (A1049; Sigma-Aldrich) was diluted from a 10 mM stock solution prepared in methanol; equivalent volumes of methanol were included in the ABA-free controls. *Arabidopsis* cell suspension cultures were maintained and grown as described (Forreiter et al., 1997). ABA treatment of in vitro–grown plantlets was performed as described by Parcy et al. (1994). The *Arabidopsis ABI3* T-DNA insertion line (*S138922*; Col-0 ecotype) was obtained from the collection of the SIGnAL project (Salk Institute Genomic Analysis Laboratory; http://signal.salk.edu/tabout.html) ordered via the Nottingham Arabidopsis Stock Centre (NASC). Information about the T-DNA insertion was obtained from the Salk Institute Genomic Analysis Laboratory website (http://signal.salk.edu). The T-DNA insertion sites were confirmed by PCR using the T-DNA left border primer (5′-TGGTTCACG-TAGTGGGCCATCG-3′) and *ABI3*-specific primer (5′-GCGGTGGTGAT-TGCATTGATA-3′). Direct heat stress to the siliques was imposed by increasing the temperature of the climate chamber to 38°C for 2 h. High humidity was maintained during heat stress to prevent transpiration cooling. The *Arabidopsis* seed development profile was established as previously described (Wehmeyer et al., 1996). For imbibition experiments, seeds were imbibed under continuous light without stratification according to Kushiro et al. (2004).

Plant Transformation

A full-length cDNA clone of HsfA9 was first cloned into a modified pRT plant expression vector (Töpfer et al., 1988) with a 3HA tag at the C terminus. The whole cassette having *CaMV35S-HsfA9-3HA* was digested with *Kpn*I and *Sac*I and cloned into pBIN19 followed by transformation into Col wild-type plants by the floral dipping method (Clough and Bent, 1998). A total of 15 independent lines (T1 generation) were selected on MS plates containing 30 μg/mL kanamycin. Three independent T1 lines (*P_{35S}:HsfA9-3HA*) were selected for experiments.

RNA Isolation and RT-PCR

Total RNA was isolated from 50 mg of seeds according to a method described by Vicient and Delseny (1999) followed by purification with the QIAquick RNeasy purification kit (Qiagen). For RT-PCR analysis, 1 μg of total RNA was reverse transcribed using an oligo(dT) primer and MMLV reverse transcriptase (MBI Fermentas). PCR was performed with one-eighth of the first-strand reaction mix, with gene-specific primers (see Supplemental Table 2 online). PCR conditions were 95°C for 120 s, followed by 27 to 30 cycles of 95°C for 30 s (denaturation), 60°C for 30 s (annealing), 72° for 60 s (elongation), and finally 72° for 5 min. *Act7*, *Hsp17.4-CI*, *Hsp17.6-CI*, *Hsp17.6-CII*, *Hsp17.7-CII*, and *Hsp101* were amplified for 27 cycles. The *ABI3* and *HsfA9* transcripts were amplified for 30 cycles.

Protein Isolation and Immunoblotting

Total protein from seeds and siliques was extracted in lysis buffer (Scharf et al., 1998a) and separated from insoluble material by centrifugation two times at 10,000 rpm for 10 min. The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories) according to the manufacturer's protocol. Samples were separated on 12% SDS-PAGE. Gels were processed for immunoblots by electroblotting to nitrocellulose (Mishra et al., 2002). Rabbit antisera against *Arabidopsis* Hsp17-CI/CII and Hsp101 were described previously (Wehmeyer et al., 1996; Hong and Vierling, 2001). For protein gel blot analysis of HsfA9, a glutathione *S*-transferase–tagged C-terminal fragment (amino acids 166 to 331; see Kotak et al., 2004) expressed in *Escherichia coli* and purified on GST-Sepharose (Amersham Biosciences) was used for immunization of

guinea pigs (Eurogentec). The polyclonal antiserum was used at 1:2500 dilution. As secondary antibodies, a 1:3000 dilution of anti-guinea pig Ig-conjugated to horseradish peroxidase was used (Sigma-Aldrich).

Transient Expression Assay Using *Arabidopsis* Cell Culture and Tobacco Mesophyll Protoplasts

Transient expression assays were performed using *Arabidopsis* suspension cell culture and tobacco (*Nicotiana tabacum*) mesophyll protoplasts as described (Forreiter et al., 1997; Lyck et al., 1997; Scharf et al., 1998a), with slight modifications. Protoplasts were isolated and transformed by polyethylene glycol–mediated transformation at room temperature (25°C) under dark conditions. Plant expression vectors used are based on the pRT series of vectors (Töpfer et al., 1988; Döring et al., 2000). The expression vector for *HsfA9* used in transient reporter assays was constructed with a PCR-amplified cDNA fragment (forward 5′-GGTCATGACGGCAATTCCAAACGTCG-3′ and reverse 5′-ATACATGCGGCCGCTCTACTCTATCTCTATCC-3′) by ligation into the 5′ *Nco*I and 3′ *Not*I sites of pRT103 (Töpfer et al., 1988). The activator plasmids of *ABI3*, *FUS3*, and *LEC1* were used as described previously (Reidt et al., 2000). The ~1-kb promoter region of *HsfA9* (1017 bp) and ~0.5- to 1-kb promoter regions (referring to the ATG) of *Hsp17.4-CI* (998 bp), *Hsp17.7-CII* (498 bp), *Hsp17.6-CII* (1017 bp), and *Hsp101* (1000 bp) were PCR-amplified from genomic DNA with gene-specific primers (see Supplemental Table 2 online), introducing 5′ *Hin*dIII and 3′ *Xho*I sites and inserted in fusion to the coding region of a GUS gene in pBT2gus (Töpfer et al., 1988). The RY/Sph deletion construct $P_{HsfA9\Delta RY}$*:GUS* with a deletion of 8 bp (CATGCATG) was constructed as described (QuikChange site-directed mutagenesis kit; Stratagene) using the following primers: forward 5′-GCTTTCCCTAAGCGACACTTGTCCTAAATCAAATTCAATGGACGACCG-3′ and reverse 5′-CGGTCGTCCATTGAATTTGATTTAGGACAAGTGTCGCTTAGGGAAAGC-3′.

Microarray Analysis

For expression profiles of selected genes from the AtGenExpress microarray database, the signal intensities were gcRMA-normalized and averaged (available at http://www.weigelworld.org/resources/microarray/AtGenExpress) and visualized as heat maps (with GeneSpring version 7.2) with retransformed linear signal intensities. For description of the samples and further details, see http://www.weigelworld.org/resources/microarray/AtGenExpress and Schmid et al. (2005).

Further details about the seed stages presented in Figure 1 can be found at http://www.genomforschung.uni-bielefeld.de/GF-research/AtGenExpress-SeedsSiliques.html.

Phylogenetic Analysis

Protein sequences of HsfA9 homologues from various plant species were aligned using ClustalW (Thompson et al., 1997). Phylogenetic dendrograms were constructed using the minimum evolution method (MEGA version 3.1) (Kumar et al., 2004) with a nearest-neighbor-joining tree as starting point and 2000 bootstrap replicates.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *HsfA9* (At5g54070), *HsfA2* (At2g26150), *ABA1* (At5g67030), *ABA2* (At1g52340), *ABA3* (At1g16540), *ABI3* (At3g24650), *ABI4* (At2g40220), *ABI5* (At2g36270), *FUS3* (At3g26790), *LEC1* (At1g21970), *Hsp17.4-CI* (At3g46230), *Hsp17.6A-CI* (At1g59860), *Hsp17.6-CII* (At5g12020), *Hsp17.7-CII* (At5g12030), *Hsp101* (At1g74310), *EM1* (At3g51810), *EM6* (At2g40170), *HsfA2* (At2g26150), and *Act7* (At5g09810)

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Selected Microarray Expression Profiles of the AtGenExpress Developmental Series.

Supplemental Figure 2. Selected Microarray Expression Profiles of the AtGenExpress Abiotic Stress Series.

Supplemental Figure 3. Analysis of *ABI* Mutant Lines.

Supplemental Figure 4. Influence of ABA on the Induction of *HsfA9* in Transient Reporter Assays.

Supplemental Figure 5. Analysis of ABA-Deficient Mutant Lines.

Supplemental Figure 6. Comparison of the Amino Acid Sequences of Putative HsfA9 Proteins from Different Plants.

Supplemental Figure 7. Neighbor-Joining Analysis of HsfA9 Homologues.

Supplemental Table 1. Overview of *ABI* and ABA Mutant Lines.

Supplemental Table 2. Oligonucleotides Used for RT-PCR.

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