A Novel Transcriptional Cascade Regulating Expression of Heat Stress Proteins during Seed Development of Arabidopsis

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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, 1998; 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...
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 ABSCI-SIC 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; Schöfl 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).
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 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).

**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; Reindt 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 (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 (Bämlein et al., 1991a) was used as a positive control for the seed-specific transcriptional activators ABI3 and FUS3, respectively (Reindt et al., 2000). As shown in Figure 3B,
ABI3 led to an activation of the $P_{HsfA9}$ 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 $P_{HsfA9}$ 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
null mutant that contains no detectable expression of Supplemental Figure 3C online. We next tested whether the ABI3 gene (see Supplemental Figure 3B online). RT-PCR analysis showed that the line has desiccation-intolerant seeds that remain green due to nondegradation of chlorophyll (see Supplemental Figure 3B online). Similar to other ABI3 insertion in the first exon of the ABI3 null allele (Nambara et al., 1994), we characterized the Salk line with 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-C1, 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 Shsps were 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 ABI3 in the control of HsfA9 and Hsps expression was further investigated using several mutant lines that were deficient for the ABA level in

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

(A) Transcript levels of HsfA9, HsfA1e, 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 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.

(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 (P35S-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).
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 (Ler) 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 HsfA9 to gain insight into the function of HsfA9, we generated plants ectopically expressing HsfA9 under the control of the constitutive 35S promoter of HsfA9. We monitored the expression profiles of Hsp genes in leaf samples from three independent lines of unstressed 4-week-old plants (P35S: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, Hsp17.6A-CI, and Hsp17.6-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 (P35S: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 (P35S:GUS; see Methods for details). As shown in Figure 5C, cotransformation of P35S:HsfA9-3HA and the P35S: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 P35S:HsfA9-3HA construct and a 10- to 20-fold increase in GUS activity from the cotransformed P35S:GUS constructs (Figure 5C). The induced expression of P35S:HsfA9-3HA by ABI3 and ABA is in accordance with our results where ABI3 showed an activation of the P35S:GUS reporter construct that was enhanced by ABA (see Supplemental Figure 4 online). Furthermore, in the absence of P35S: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, 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 silique 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, Hsp17.6-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 (P35S: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
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 PHsfA9: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 PHsfA9: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...
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
mutants do not display a significant reduction in the accumulation of storage protein mRNA or in desiccation tolerance (Koomneef 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 Ai2S3 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_{Hsp101-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 siliques 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 (Coffeea 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 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.

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 Geltrite (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’-TGTTTACG-TAGTTGGCCATCG-3’) and ABI3-specific primer (5’-GGGTTGGGTAT-TGCATTGATA-3’). Direct heat stress to the silique 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 PstI and SacI 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 (P35S-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 Vicent 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°C for 60 s (elongation), and finally 72°C for 5 min. Act7, Hsp17.4-CI, Hsp17.6-CII, Hsp17.6-CIII, 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 twice 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 (Forrester 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 pRT103 (Töpfer et al., 1988) plasmid. The activator plasmids of HsfA9, 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-Cil (498 bp), Hsp17.6-Cil (1017 bp), and Hsp101 (1000 bp) were PCR-amplified from genomic DNA with gene-specific primers (see Supplemental Table 2 online), introducing 5′ HindIII and 3′ XhoI sites and inserted in fusion to the coding region of a GUS gene in pBT2gus (Töpfer et al., 1988). The activator plasmids of ABI3, FUS3, and LEC1 were used as described previously (Reidt et al., 2000). The 5′-GCCCTTCTGACACTTGTTCTAATCAATTGCAATGC-3′ and reverse 5′-GCTTTCCCTAAGCGACACTTGTCCTAAATCAAATTCAATGGACGAGA-3′ were used.

**Microarray Analysis**

For expression profiles of 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 3.1) (Kumar et al., 2004) with a nearest-neighbor-joining tree as the root. Phylogenetic dendrograms were constructed using the minimum evolution method (MEGA version 3.1) (Kumar et al., 2004) with a nearest-neighbor-joining tree as the root. Phylogenetic dendrograms were constructed using the minimum evolution method (MEGA version 3.1) (Kumar et al., 2004) with a nearest-neighbor-joining tree as the root.

**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 the root 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 (At1g20160), Hsp17.4-CI (At3g48620), Hsp17.6-CI (At1g58860), Hsp17.7-Cil (At5g12020), Hsp17.7-Cil (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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A Novel Transcriptional Cascade Regulating Expression of Heat Stress Proteins during Seed Development of Arabidopsis

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