Molecular Plant • Volume 2 • Number 1 • Pages 152–165 • January 2009 RESEARCH ARTICLE

Heat Shock Factors HsfB1 and HsfB2b Are Involved in the Regulation of *Pdf1.2* Expression and Pathogen Resistance in *Arabidopsis*

Mukesh Kumar[a], Wolfgang Busch[b], Hannah Birke[c], Birgit Kemmerling[d], Thorsten Nürnberger[d] and Friedrich Schöffl[c,1]

a Present address: Heinrich-Pette-Institut, Martinistrabe 52, D-20251 Hamburg, Germany
b Max-Planck-Institut für Entwicklungsbiologie, Abteilung Molekularbiologie, Speemannstrabe 37–39, D-72076 Tübingen, Germany
c Universität Tübingen, Zentrum für Molekularbiologie der Pflanzen (ZMBP)—Allgemeine Genetik, Auf der Morgenstelle 28, D-72076 Tübingen, Germany
d Universität Tübingen, Zentrum für Molekularbiologie der Pflanzen (ZMBP)—Biochemie der Pflanzen, Auf der Morgenstelle 5, D-72076 Tübingen, Germany

ABSTRACT In order to assess the functional roles of heat stress-induced class B-heat shock factors in *Arabidopsis*, we investigated T-DNA knockout mutants of *AtHsfB1* and *AtHsfB2b.* Micorarray analysis of double knockout *hsfB1/hsfB2b* plants revealed as strong an up-regulation of the basal mRNA-levels of the defensin genes *Pdf1.2a/b* in mutant plants. The *Pdf* expression was further enhanced by jasmonic acid treatment or infection with the necrotrophic fungus *Alternaria brassicicola*. The single mutant *hsfB2b* and the double mutant *hsfB1/B2b* were significantly improved in disease resistance after *A. brassicicola* infection. There was no indication for a direct interaction of Hsf with the promoter of *Pdf1.2*, which is devoid of perfect HSE consensus Hsf-binding sequences. However, changes in the formation of late HsfA2-dependent HSE binding were detected in *hsfB1/B2b* plants. This suggests that HsfB1/B2b may interact with class A-Hsf in regulating the shut-off of the heat shock response. The identification of *Pdf* genes as targets of Hsf-dependent negative regulation is the first evidence for an interconnection of Hsf in the regulation of biotic and abiotic responses.

Key words: Abiotic/environmental stress; transcriptional control and transcription factors; transcriptome analysis; defense responses; disease resistance; *Arabidopsis*.

INTRODUCTION

Environmental adaptation of plants depends on elaborate systems for stress sensing and signaling, common and stress-specific responses, and probably also on a hierarchical control of reactions. There is evidence that heat shock factors (Hsfs) play important roles in stress sensing and signaling of different environmental stresses and that different stresses, including also high temperature, induce reactive oxygen species (ROS) in plants (Dat et al., 1998). ROS, particularly H_2O_2, are important components in biotic and abiotic stress response and signaling mechanisms.

Hsfs recognize consensus binding motifs, so-called heat stress elements (HSE: 5′-nGAAnnTTCnnGAAn-3′ or 5′-nTTCnn-GAAnnTTCn-3′) conserved in promoters of heat-inducible genes of all eukaryotes. The classical target genes are the heat shock protein (Hsp) genes, which are (including the HSE motifs in the promoter region) highly conserved in eukaryotes (Wu, 1995). Hsfs display a modular structure with a highly conserved N-terminal DNA-binding domain (DBD), which is characterized by a central helix-turn-helix motif, and an adjacent bipartite oligomerization domain (HR-A/B) composed of hydrophobic heptad repeats. Hsf trimerization via the formation of a triple stranded alpha-helical coiled coil is a prerequisite for high-affinity DNA binding and subsequently for transcriptional activation of heat shock genes. Other functional domains of Hsf include clusters of basic amino acid residues (NLS) essential for nuclear import, leucine-rich export sequences in the HR-C region (NES) and—less conserved—a C-terminal activator domain (CTAD) rich in aromatic, hydrophobic, and acidic amino acids, the so-called AHA motifs (Nover et al., 1996, Döring et al., 2000). Sequence comparisons indicate that the combination of a C-terminal activator motif (AHA) with the consensus

[1] To whom correspondence should be addressed. E-mail friedrich.schoeffl@zmbp.uni-tuebingen.de, fax +49-7071-295042, tel. +49-7071-2978831.

doi: 10.1093/mp/ssn095
Received 9 September 2008; accepted 26 November 2008

sequence FWxx(F/L) (F/I/L) to an adjacent nuclear export signal (NES) represents a signature domain for many plant activator Hsfs (Koskull-Döring et al., 2007).

Based on structural characteristics and phylogenetic comparison, plant Hsfs are subdivided into three classes and several subgroups. Recent studies in *Arabidopsis* showed that different class A-Hsfs play important roles during early and late stages of stress response. Early Hsfs are considered to be constitutively expressed in the cell and may become activated immediately upon stress treatment. Late Hsf functions are considered for those Hsfs whose expression is significantly enhanced upon stress treatment, which suggests that these Hsfs require the action of early Hsfs for their own expression (Wunderlich et al., 2007).

Most research on Hsfs was focused on the functional characterization of class A-Hsfs of *Arabidopsis*, which comprises 15 different genes/proteins, some of which are known to function as transcriptional activators of stress target genes. Recent evidence obtained from the identification of class A-Hsf-knockout mutants and microarray expression profiling indicates that some early, like HsfA1a and HsfA1b (Busch et al., 2005), and the late HsfA2 share an overlapping set of target genes.

Much less is known about the function of class B-Hsfs, which differ from class A by a structural variation within the oligomerization domain and the lack of an AHA-motif that is required for transcriptional activation function of class A-Hsfs (Koskull-Döring et al., 2007). There are only five different class B-Hsf genes present in the *Arabidopsis* genome, two of which are considered to have early functions (B3 and B4), three are considered to act as late Hsfs (HsfB1, HsfB2a, HsfB2b) because their mRNA expression is significantly increased upon heat stress and there is evidence that this heat-induced expression requires the combined action of the early class A-HsfA1a and -A1b (Lohmann et al., 2004; Busch et al., 2005).

In contrast to Hsfs of class A, classes B- and C-Hsfs have no evident function as transcription activators on their own (Kotak et al., 2004; Czarnecka-Verna et al., 2000, 2004). There is evidence that tomato HsfB1 interacts with HsfA1a and may also be involved in CREB-dependent expression of housekeeping genes during recovery (Bharti et al., 2004). Interestingly, the orthologous *Arabidopsis* HsfB1 did not show such properties in the same assay systems (Bharti et al., 2004). In *Arabidopsis*, HsfB1 is the most strongly heat-induced class B-Hsf (Busch et al., 2005). Surprisingly, transgenic overexpression of *Hsf4* (synonymous for *HsfB1*) had no effect on the expression of heat shock proteins (Hsp) or the development of thermotolerance (Prändl et al., 1998). Since class B-Hsfs have the capacity to bind to similar or the same sites in the heat shock gene promoters as class A-Hsfs, it was proposed that they may act as repressors of target gene expression (Czarnecka-Verna et al., 2000, 2004).

In the present study, we investigated T-DNA knockout mutants of *HsfB1* and *HsfB2b* for understanding their functional roles during the later phases of heat shock response. *HsfB1*/*B2b* are the only two out of three class B-Hsfs, which are clear targets of class Hsf-dependent enhancement of expression and for which viable knockout mutants were available. Microarray analysis for the identification of putative target genes revealed that, in *hsfB1*/*hsfB2b* double knockout plants, the major targets are *Pdf1.2* genes, which are involved in immunity against infection by necrotrophic microorganisms. The identification of *Pdf* genes as targets of Hsf-dependent negative regulation is the first evidence for an interconnection of Hsf in the regulation of biotic and abiotic responses.

RESULTS

Isolation and Characterization of *hsfB1* and *hsfB2b* Knockout Mutants

In order to analyze the function of the heat shock-induced Hsf genes, *AtHsfB1* and *AtHsfB2b*, a loss-of-function strategy was employed. The T-DNA insertion lines Salk_012292 (insertion in *HsfB1*) and Salk_047291 (insertion in *HsfB2b*) were obtained from the stock collection of mutants available at the Signal Salk institute (http://signal.salk.edu/cgi-bin/tdnaexpress) and characterized. Homozygous mutants were identified via PCR screening using gene-specific and T-DNA-specific primers. The positions of the T-DNA insertions were confirmed and precisely mapped by DNA sequencing. In *HsfB1*, the insertion maps to the second exon 367 nucleotides upstream of the stop codon, in *HsfB2b* to a position 277 nucleotides downstream from the first exon border (Figure 1A). The homozygous mutants *hsfB1*$^{-/-}$ and *hsfB2b*$^{-/-}$ are designated as *hsfB1* and *hsfB2b*, respectively.

The expression of the respective Hsf in *hsfB1* and *hsfB2b* plants was investigated at the transcript level using qRT–PCR both at control temperature and after 1 h of heat stress in comparison to wild-type (WT) plants. It was shown that, at 22°C, *HsfB1* and *HsfB2b* are expressed at low levels (15% for *HsfB1* and 4% for *HsfB2b* compared to *actin2* = *100%*) in WT plants (Figure 1B). After heat stress, the transcript levels of *HsfB1* were increased in WT and *hsfB2b* plants (approximately 12- and 30-fold) but no *HsfB1* transcripts were detected in *hsfB1* plants with or without HS. *HsfB2b* mRNA levels were up-regulated in WT and *hsfB1* plants by factors of three to five-fold after HS but *HsfB2b* transcripts were not detected in *hsfB2b* plants with or without HS. These results indicate that the T-DNA insertions caused a complete knockout of the expression of the respective *Hsf* genes in *hsfB1* and *hsfB2b* single mutant plants.

However, there was no obvious phenotypic effect on morphology or thermotolerance associated with either of the single *hsfB1* or *hsfB2b* mutant lines. Therefore, we isolated a double knockout mutant *hsfB1*$^{-/-}$/*hsfB2b*$^{-/-}$ (abbreviated *hsfB1*/*B2b*). Following a crossing of the homozygous single knockout lines, we isolated a double knockout mutant homozygous for both Hsf mutations, in subsequent generations by PCR screening. The transcript levels of *HsfB1* and *HsfB2b* were determined in a time course experiment from 0 to 8 h heat stress in the *hsfB1*/*B2b* double mutant compared to WT plants using qRT–PCR. As previously shown by Lohmann et al. (2004),

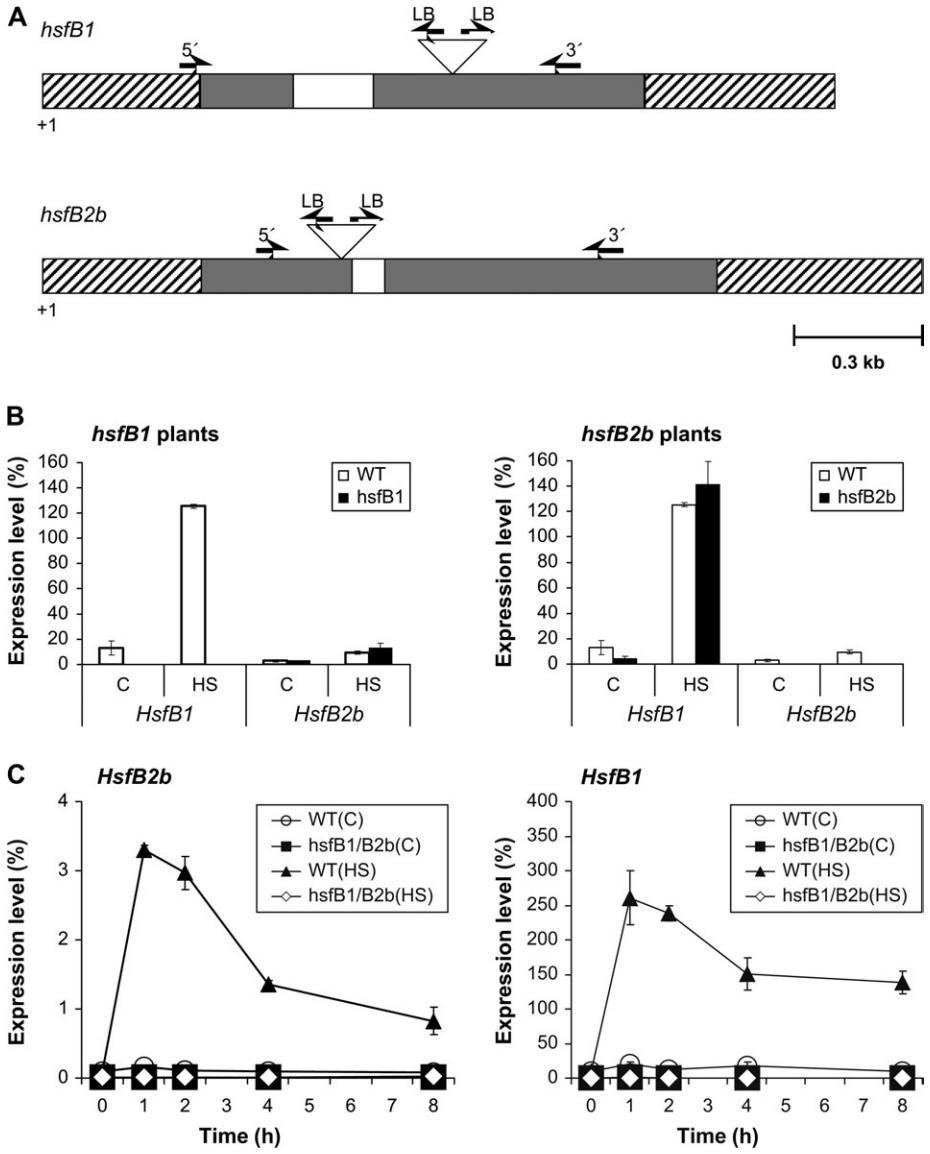

Figure 1. mRNA Quantification of *HsfB1* and *HsfB2b* in Single and Double Mutants.

(A) T-DNA insertion sites in *hsfB1* and *hsfB2b* lines as determined by sequencing PCR products obtained from genomic DNA of the tagged lines using either gene-specific 5′ or 3′ primers in combination with T-DNA left border (LB) primers. Triangles indicate the T-DNA elements. Exons are depicted as grey boxes, introns as open boxes, non-translated regions as hatched boxes. The position and direction of primers for *Hsf* genes or T-DNA sequences are marked by horizontal arrows. T-DNA elements and primers are not drawn to scale.

Poly $(A)^+$-RNA was isolated from leaves and analyzed by qRT–PCR for the mRNA levels of *HsfB1* and *HsfB1b* of wild-type (WT) and single mutant (*hsfB1* and *hsfB2b*) plants that had been subjected to heat stress (37°C, 1 h) or control temperature (22°C, 1 h) **(B)**, or double knockout (*hsfB1/B2b*) plants that had been subjected to heat stress (37°C) or control temperature (22°C) for 0–8 h **(C)**.

PCR levels were normalized with respect to *Actin2* mRNA (= 100%). Data points show means (n = 2) and range (B), and means (n = 3) and S.D. (C), respectively.

HsfB1 and *HsfB2b* mRNAs are expressed at low levels (20 and0.2%, respectively, compared to *actin2*) at 22°C in WT. The expression levels are significantly increased after heat stress (Figure 1C): for *HsfB1* to a high level (from 20 to 275% of *actin2*), for *HsfB2b* to a lower level (from 0.2 to 3.5% of *actin2*) after 1–2 h heat stress. No mRNAs were detected for either of the two Hsfs in the double knockout mutant *hsfB1/B2b*, neither at control temperature nor after heat stress. This indicates that *HsfB1/B2b* expression is completely eliminated in *hsfB1/B2b* plants. Despite this clear knockout effect at the level of gene expression, there was no obvious phenotypic difference with respect to growth, fertility and thermotolerance between *hsfB1/B2b*, single knockout, or WT plants.

In order to test whether the combined functions of HsfB1 and HsfB2b are generally required for late effects in heat shock gene

expression, we determined the mRNA expression profiles of a number of known Hsf target genes (*Hsp17.6*, *Hsp23.6*, *Hsp70*, *Hsp83.1*, *Hsp101*, and *GolS*) in a time course experiment from 0 to 8 h heat stress (Figure 2). Interestingly, the mRNA profiles, which differ individually from each other, are almost identical for WT and *hsfB1/B2b* double knockout plants. Hence, the deficiency of HsfB1 and HsfB2b has little if any effect on the expression profiles of typical Hsf-A1a/1b target genes (Lohmann et al., 2004; Panikulangara et al., 2004; Busch et al., 2005).

Expression Profiling: Identification of Putative HsfB1/HsfB2b Target Genes

In order to understand the functions of HsfB1 and HsfB2b, we tried to identify genes whose expression is affected in *hsfB1/B2b* plants, at either 22°C or after heat stress. Considering the heat-induced expressions of *HsfB1* and *HsfB2b*, it was implicated that the effect should become more obvious after longer times of heat stress. As suggested by Figure 2, we applied heat stress at 37°C for 2 h to detect differences in gene expression between WT and *hsfB1/B2b* plants.

Whole genome Affymetrix microarray mRNA hybridizations were performed using RNA samples isolated from leaves that had been subjected to either 2 h at 22°C (control) or heat stress temperature. By conducting a Welch's *t*-test (confidence 95%, Multiple Testing Correction Benjamini and Hochberg) on the normalized hybridization data of the two control mRNA samples (not subjected to heat stress), it was examined whether HsfB1 and HsfB2b exert an influence on basal gene expression. The datasets of genes expressed at 22°C in WT versus *hsfB1/B2b* plants were compared. Only 16 genes appeared to be differentially expressed by a factor of more than two (Figure 3; black spots, left-hand panel), two of them, *Pdf1.2a* and *Pdf1.2b*, were outstanding with respect to the extraordinary differences in expression levels, with a more than 15-fold increase in *hsfB1/B2b* plants (Table 1). The expression of *Pdf* genes is known to be induced in response to necrotrophic pathogens

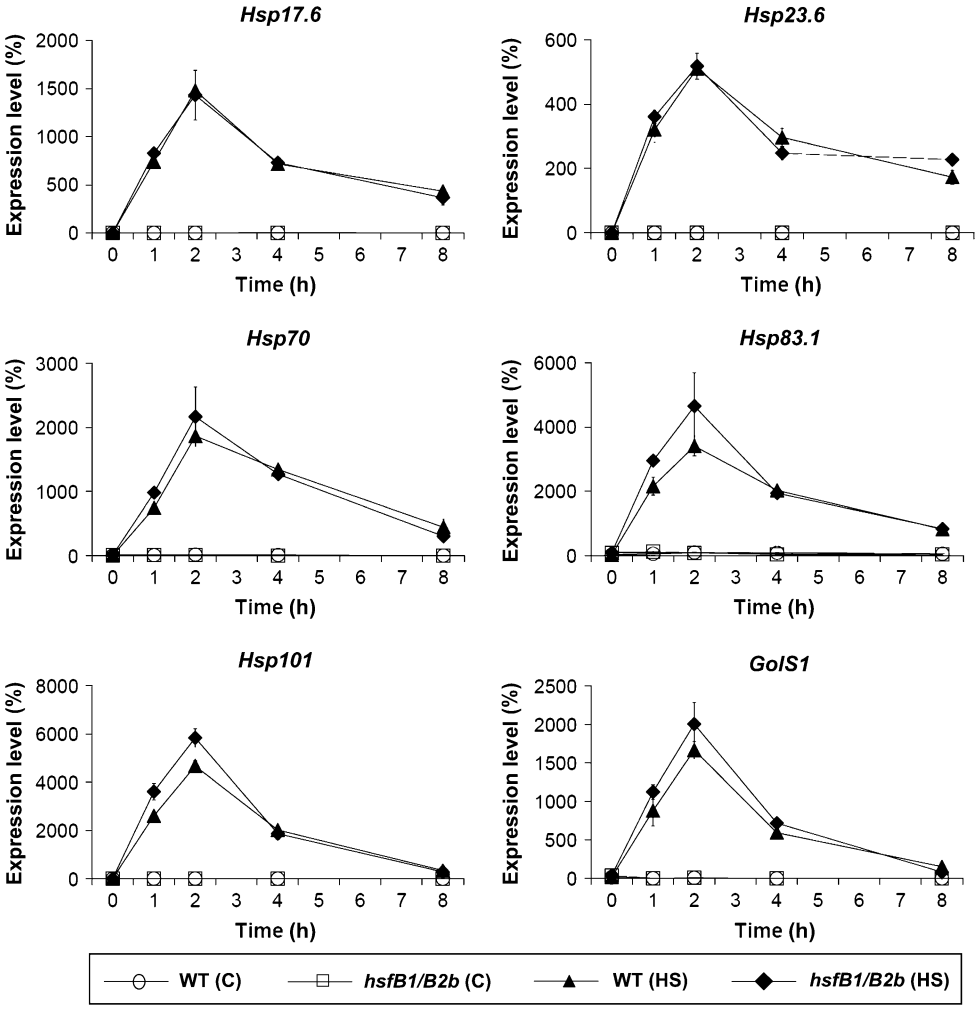

Figure 2. mRNA Expression Profiles of Heat Shock Factor Target Genes.

Levels of mRNAs of different *Hsp* and *GolS1* in wild-type (WT) and *hsfB1/B2b* double mutant plants during heat stress (HS). Poly $(A)^+$-RNA was isolated from leaves that had been heat-stressed at 37°C for the indicated times, converted into cDNA and subjected to real-time PCR. Relative amounts were calculated and normalized with respect to *Actin2* mRNA (= 100%). Data points show means and range (n = 3).

and the defensins exhibit protective functions. Only four of the differentially regulated genes are down-regulated in *hsfB1/B2b* plants by a factor of more than two. Among the down-regulated genes, the strongest effect was on *HsfB1* (18.44-fold), which is consistent with the knockout mutation in this gene. These data indicate that, at 22°C, the expression levels of >99.93% of all genes represented on the chip are identical in WT and *hsfB1/B2b* plants.

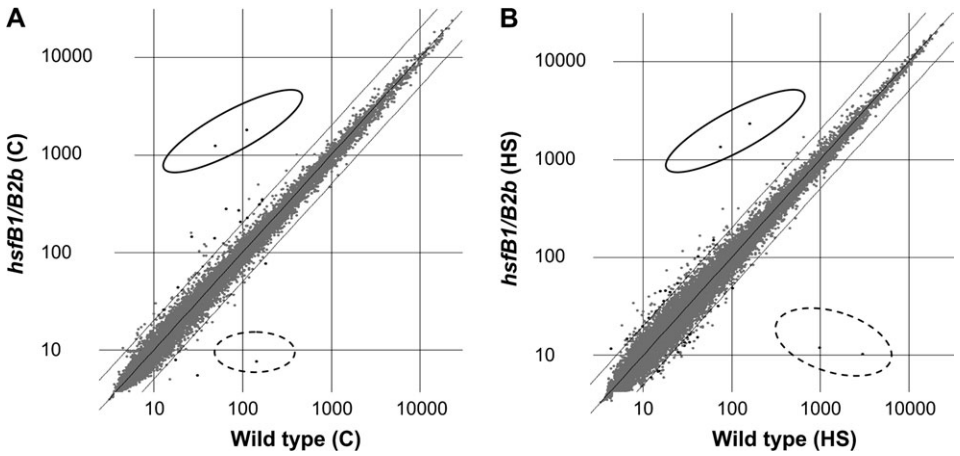

Figure 3. Comparison of Expression Characteristics Wild-Type versus *hsfB1/B2b* Double Mutant Plants.

Expression levels (relative units) of all genes (gray spots) detected by microarray hybridization; black spots indicate genes differentially expressed by the following criteria: more than two-fold difference, confidence 95%, multiple testing correction Benjamini and Hochberg. The data points for *Pdf1.2a* and *Pdf1.2b* are marked with a full circle; dashed circles mark the expression levels genes (*HsfB1* and *HsfB2b*) significantly down-regulated mutant plants.

(A) Control condition (room temperature: 22°C) comparison of wild-type (WT) and *hsfB1/B2b* double mutant.

(B) Heat shock (37°C) comparison of WT and *hsfB1/B2b* double mutant.

Table 1. List of Up-Regulated **(A)** and Down-Regulated **(B)** Genes in *hsfB1/B2b* Double Mutant Compared to WT at Control Temperature.

(A) Affymetrix identifier	WT (C)	WT (HS)	*hsfB1/B2b* (HS)	*hsfB1/B2b* (C)	$\frac{hsfB1/B2b^C}{WT^C}$ (FC)	AGI	Annotation
249052_at	48.86	73.46	1,346.00	1,242.00	25.43	At5g44420	Plant defensin protein, putative (PDF1.2a)
257365_x_at	110.70	156.80	2,341.00	1,802.00	16.28	At2g26020	Plant defensin protein, putative (PDF1.2b)
259169_at	26.62	27.75	116.00	145.70	5.47	At3g03520	Phosphoesterase family protein
245228_at	89.70	48.74	118.20	271.00	3.02	At3g29810	Phytochelatin synthetase-like protein
266841_at	64.77	17,191.00	17,090.00	280.30	3.00	At2g26150	Putative heat shock transcription factor, AtHsfA2
263374_at	48.03	7,591.00	7,769.00	141.00	2.94	At2g20560	DNAJ heat shock family protein
256518_at	18.43	1,518.00	1,415.00	44.14	2.40	At1g66080	Expressed protein
260025_at	93.37	8,128.00	8,786.00	207.30	2.22	At1g30070	SGS domain-containing protein
248258_at	164.30	759.50	990.50	352.00	2.14	At5g53400	Nuclear movement family protein
249867_at	12.72	59.00	98.56	25.98	2.04	At5g23020	2-isopropylmalate synthase-like protein
247771_at	153.00	527.90	606.70	311.70	2.04	At5g58590	Ran binding protein 1 homolog-like
262656_at	112.20	2,449.00	2,263.00	226.80	2.02	At1g14200	Zinc finger (C3HC4-type RING finger) family protein

(B) Affymetrix identifier	WT (C)	WT (HS)	*hsfB1/B2b* (HS)	*hsfB1/B2b* (C)	$\frac{hsfB1/B2b^C}{WT^C}$ (FC)	AGI	Annotation
246214_at	141.60	2,986.00	10.25	7.68	18.44	At4g36990	Heat shock transcription factor Hsf4
261662_at	30.64	6.71	6.59	5.50	5.58	At1g18350	MAP kinase kinase 5, putative
245449_at	179.90	176.20	126.30	76.94	2.34	At4g16870	Retrotransposon-like protein
247213_at	17.51	13.60	13.80	7.92	2.21	At5g64900	Expressed protein

FC, fold change; AGI, *Arabidopsis* genome initiative gene model (www.arabidopsis.org); HS, heat stress (37°C); C, control (22°C).

In a second comparative analysis, a *t*-test was applied for a dataset comprising the more than two-fold differences in RNA levels of heat-stressed WT versus heat-stressed *hsfB1/B2b* plants (Figure 3; right-hand panel). The scattering of the expression levels (gray spots) indicates the robustness of the analysis. The black spots mark the expression levels of 31 genes, which were differentially expressed; 15 genes showed significantly higher, and 16 genes lower levels in WT compared to *hsfB1/B2b* plants (Table 2). Thus, only a relatively small fraction (0.93%) of 3333 heat stress-affected genes (data not shown) can be attributed to the functions of HsfB1/B2B in WT. Interestingly, *Pdf1.2a* and *Pdf1.2b* appeared to be also up-regulated (fold changes of 14–18) in *hsfB1/B2b* plants; both are expressed at approximately the same high levels after heat stress as compared to control temperature. Among the genes down-regulated in *hsfB1/B2b* plants are, as expected,

Table 2. List of Down-Regulated **(A)** and Up-Regulated **(B)** Genes in *hsfB1/B2b* Double Mutant Compared to WT After Heat Stress.

(A) Affymetrix identifier	WT (C)	WT (HS)	*hsfB1/B2b* (HS)	*hsfB1/B2b* (C)	$\frac{WT^{HS}}{hsfB1/B2b^{HS}}$ (FC)	AGI	Annotation
246214_at	141.6	2,986	10.25	7.68	291.2	At4g36990	Heat shock transcription factor Hsf-B1
254878_at	16.5	980.1	11.9	8.95	82.37	At4g11660	Heat shock transcription factor-like protein, Hsf-B2b
256400_at	9.51	40.66	15.35	8.256	2.649	At3g06140	Zinc finger (C3HC4-type RING finger) family protein
253916_at	46.93	33.14	13.63	51.46	2.431	At4g27240	Zinc finger (C2H2 type) family protein
247141_at	45.29	28.95	12.47	51.44	2.321	At5g65560	Pentatricopeptide (PPR) repeat-containing protein
252268_at	10.41	24.49	10.71	12.5	2.288	At3g49650	Kinesin motor protein-related
246314_at	69.22	69.06	31.97	69.19	2.16	At3g56850	ABA-responsive element binding protein 3
254489_at	6.139	14.96	6.938	5.966	2.156	At4g20800	FAD-binding domain-containing protein
261381_at	14.19	28.85	13.53	11.06	2.132	At1g05460	RNA helicase SDE3 (SDE3)
263507_s_at	6.432	14.5	6.849	5.708	2.118	At2g07684	Hypothetical protein
246200_at	153.6	100.7	48.71	136.1	2.067	At4g37240	Expressed protein
263795_at	15.31	33.61	16.41	14.21	2.048	At2g24610	Cyclic nucleotide-regulated ion channel, putative
264689_at	68.18	30.83	15.11	65.42	2.04	At1g09900	Pentatricopeptide (PPR) repeat-containing protein
260198_at	6.09	12.08	5.945	6.849	2.032	At1g67635	Hypothetical protein
247922_at	64.07	23.14	11.4	64.12	2.03	At5g57500	Expressed protein

(B) Affymetrix identifier	WT (C)	WT (HS)	*hsfB1/B2b* (HS)	*hsfB1/B2b* (C)	$\frac{hsfB1/B2b^{HS}}{WT^{HS}}$ (FC)	AGI	Annotation
249052_at	48.86	73.46	1,346	1,242	18.33	At5g44420	Plant defensin protein, putative (PDF1.2a)
257365_x_at	110.7	156.8	2,341	1,802	14.93	At2g26020	Plant defensin protein, putative (PDF1.2b)
247529_at	515.7	15.3	45.02	545.7	2.943	At5g61520	Monosaccharide transporter STP3
247533_at	70.64	4.264	11.7	57.39	2.743	At5g61570	Protein kinase family protein
248450_at	27.4	17.55	45.88	25.66	2.614	At5g51290	Ceramide kinase-related
254300_at	47.41	41.1	102.7	57.52	2.498	At4g22780	Translation factor EF-1 alpha-like protein
259502_at	1,908	61.25	150.4	2,613	2.455	At1g15670	Kelch repeat-containing F-box family protein
250379_at	5.032	6.455	14.69	4.682	2.276	At5g11590	Transcription factor TINY
256940_at	15.97	24.15	54.48	22.56	2.256	At3g30720	Expressed protein
246405_at	1,008	19.82	44.24	865.6	2.232	At1g57630	Disease resistance protein (TIR class), putative
257129_at	9.129	16.92	35.97	9.101	2.125	At3g20100	Cytochrome p450 family
257876_at	10.42	11.45	24.13	14.04	2.106	At3g17130	Invertase/pectin methylesterase inhibitor family protein
256595_x_at	32.25	30.15	61.96	38.15	2.055	At3g28530	Expressed protein
251842_at	8.311	9.711	19.74	8.103	2.033	At3g54580	Proline-rich extensin-like family protein
251756_at	23.5	13.95	28.26	28.71	2.026	At3g55820	Hypothetical protein

FC, fold change; AGI, *Arabidopsis* genome initiative gene model (www.arabidopsis.org); HS, heat stress (37°C); C, control (22°C).

HsfB1 (fold-change 291) and *HsfB2b* (fold-change 82) mRNAs. This clearly proves the *Hsf*-gene knock character of the mutant line. All other differentially expressed genes, up- or down-regulated in *hsfB1/B2b* plants, show only relatively little differences in expression (fold change 2–3) compared to WT.

In order to test the reliability of the microarray data, we re-examined the mRNA levels of *Pdf1.2* and a number of other differentially expressed genes using qRT–PCR (Table 3). By and large, the results of qRT–PCR confirm the gene-chip hybridization data but, as expected, the PCR data of the strongly heat-induced genes such as *AtHsfA2* and *AtHsfB1* show a greater discrepancy in expression levels to the microarray data. Similar observations have been reported in the analysis of other *hsf* mutants (Busch et al., 2005).

Analysis of *Pdf1.2* Gene Promoter Region and Hsf–HSE Binding

Pdf1.2 genes are up-regulated in *hsfB1* and *hsfB1/B2b* plants and therefore we inspected the promoter region of *Pdf1.2a* and *Pdf1.2b.* Both genes are highly conserved in coding and in promoter regions. Using the internet tool http://arabidopsis.med.ohio-state.edu/AtcisDB/, several other putative binding motifs in the promoter sequence of *Pdf1.2a* and *Pdf1.2b* were identified. Both promoters share a number of common motifs, such as DPBF1 and -2, RAV1-A, BoxII, GATA box, GCC-box, and Ibox. The GCC-box mediates jasmonic acid-induced activation of Pdf1.2 expression in *Arabidopsis* (Brown et al., 2003).

However, there was no perfect match to the three-boxed HSE consensus sequences nGAAn/nTTCn/nGAAn, the preferred binding site for Hsfs. One variant HSE, representing only two boxes (nGAAn/nTTCn), could be detected in *Pdf1.2b* at position –629 to –619 upstream of the transcription start site, while this was absent in *Pdf1.2a*. However, another imperfect HSE (nGAAn/nATCn) that represents only one complete box consensus sequence was present in both promoters, at positions –594/–584 of *Pdf1.2a* and at positions –678/–668 in *Pdf1.2b*. Since both genes and promoters appear highly homologous and co-regulated, we concentrated further investigations only on the *Pdf1.2a* promoter.

Using EMSA, we were unable to identify any difference in the formation of binding-specific complexes in the *Pdf1.2a* promoter between WT and *hsf*-mutant plants, neither without nor after heat stress (not shown). This result suggests that no Hsf can directly bind to the promoter upstream region.

In a second approach, we assayed for any changes in late Hsf binding capacity in WT and *hsfB1/B2b* plants by using synthetic HSEs, which contain the perfect three-box consensus sequence nGAAn/nTTCn/nGAAn (Lohmann et al., 2004) as a probe. In a time course experiment covering up to 4 h heat stress prior to protein extraction, there was a reproducible difference in the EMSA profiles between WT and *hsfB1/B2b* plants (Figure 4). The *hsfB1/B2b* plants show an earlier onset (60 min), a prolonged appearance (240 min), and a stronger intensity of a signal that represents the major late Hsf–HSE binding complex. In WT, the same signal appears with a maximum after 2 h heat stress and is already diminished after 240 min.

Effects of Pathogen Infection on *Pdf* Expression and Pathogen Resistance in WT and *Hsf*-Knockout Plants

There is ample evidence that *Pdf1.2a* and *Pdf1.2b* are induced during pathogen attack or jasmonic acid application

Table 3. Comparison of Gene Expression Levels Determined by Gene Chip Hybridization and qRT–PCR.

Gene	WT (C)		WT (HS)		hsfB1/B2b (C)		hsfB1/B2b (HS)	
	RT–PCR[a]	GC[b]	RT–PCR[a]	GC[b]	RT–PCR[a]	GC[b]	RT–PCR[a]	GC[b]
Pdf1.2a	1.3	0.9	2.3	2.0	39.1	23.7	47.6	37.4
Pdf1.2b	1.4	2.1	2.1	4.2	36.2	34.4	61.8	65.1
AtHsfA2	1.8	1.2	1965.4	459.5	7.6	5.3	1922.9	475.7
DnaJ	1.3	0.9	267.1	202.8	2.9	2.7	293.1	215.8
Atmkk7	1.7	0.6	0.0	0.2	0.0	0.1	0.0	0.2
AtHsfB1	12.9	2.7	238.6	79.6	0.5	0.1	0.4	0.3
AtHsfB2b	0.1	0.3	3.0	26.2	0.0	0.2	0.0	0.3
Stp3	16.2	9.7	0.7	0.4	18.2	10.4	1.2	1.3
F7H2.2	16.9	36.1	0.7	1.6	22.4	49.7	1.1	4.2

a mRNA level determined quantitative PCR. Relative amounts were calculated and normalized with respect to *Act2* mRNA (= 100%). Data represents mean ± range (n = 2).
b Expression estimates based on the gene chip (GC) analysis; data represent normalized and averaged intensity values of the replicates relative to the signal of Act2 mRNA hybridization (= 100%).

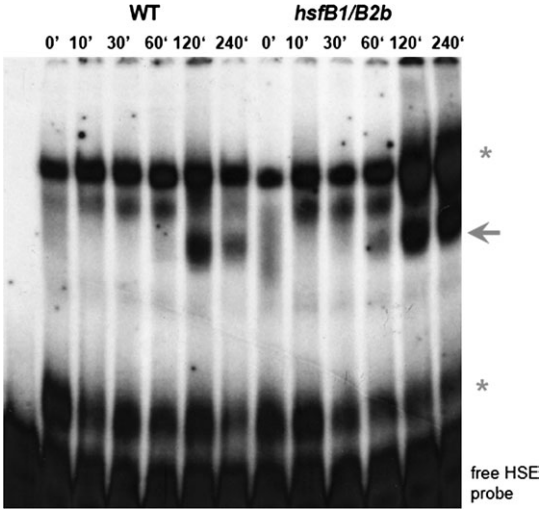

Figure 4. The Formation of Late Hsf-HSE Binding Complexes in Wild-Type and *hsfB1/hsfB2b* Plants.

Leaves of *Arabidopsis* wild-type (WT) (Col-0) and double *Hsf* mutant (*hsfB1/hsfB2b*) plants were incubated at 37°C for different periods of time (0, 10, 30, 60, 120, and 240 min). Total protein was extracted from each sample and subjected to EMSA using radioactive-labeled synthetic HSE consensus sequence as a probe. The first lane contains only the free probe without protein. The major late Hsf–HSE binding complex (Lohmann et al., 2004) is marked by an arrowhead; the unspecific constitutive binding complexes (Lohmann et al., 2004) are marked by asterisks.

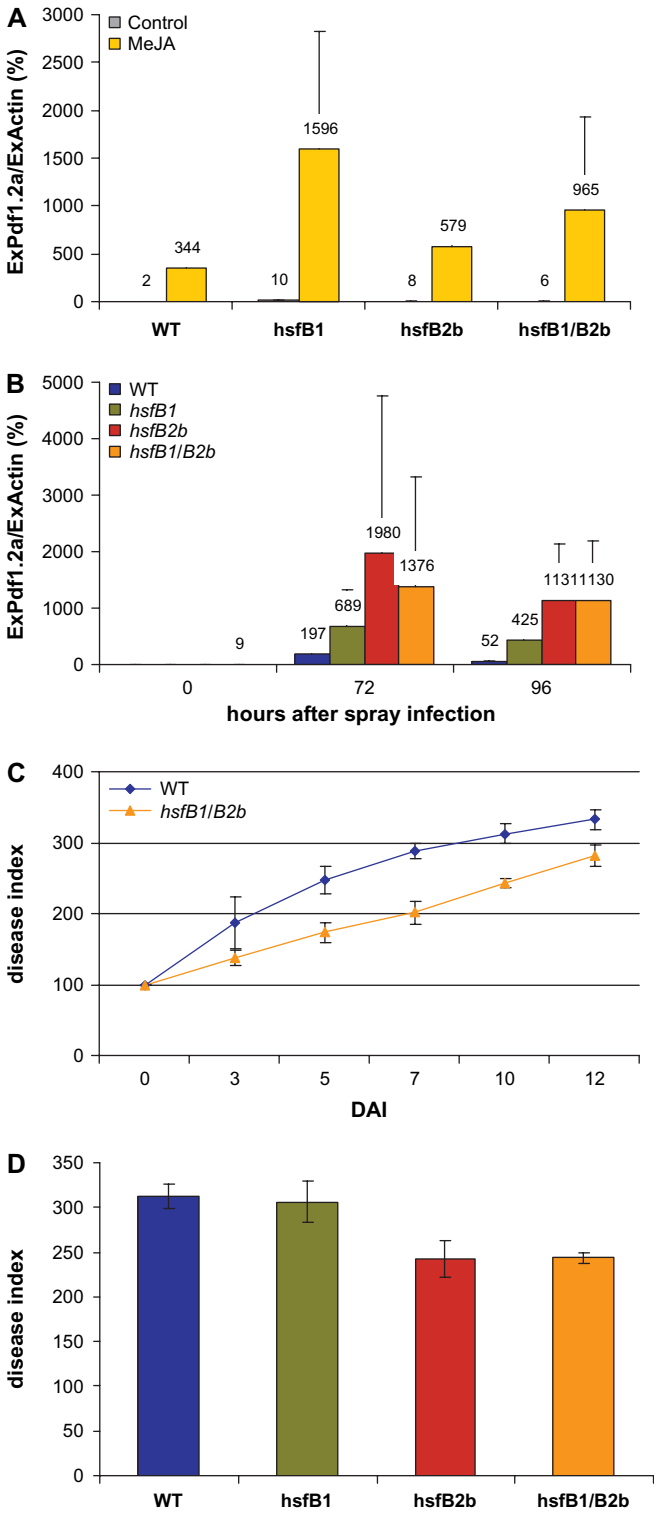

Figure 5. Effects MeJ and *A. brassicicola* Infection on *Pdf1.2a* Expression and Pathogen Resistance in *Arabidopsis* Wild-Type, Single, and Double Knockout *Hsf* Mutants.

Expression of *Pdf1.2a* was quantified after 6 h treatment with MeJ **(A)** or after 72 and 96 h post infection with *A. brassicicola* **(B)**. Data were normalized with respect to Actin 2 expression (= 100%), points show means and S.D. (n = 3).

(Penninckx et al., 1998). We tested whether *Pdf* genes are still responsive to jasmonic acid induction in double mutants (*hsfB1/B2b*) and single *Hsf*-mutant (*hsfB1* or *hsfB2b*) plants. Leaves of 5-week-old plants were sprayed with 100 μM MeJ and samples were collected after 6 h treatment for mRNA analysis. The results show (Figure 5A) that (1) *Pdf1.2a* mRNA is induced by MeJ to higher levels in *hsf* mutant plants, and (2) the induced levels in the mutants exceed the induced levels of WT plants.

We examined whether the infection with the necrotrophic fungus *Alternaria brassicicola* leads to enhanced induction of *Pdf1.2a* expression and an improved pathogen tolerance in *Hsf*-knockout plants. Leaves of 6-week-old plants were inoculated with *A. brassicicola* in a spray assay for the analysis of *Pdf1.2a* mRNA levels using qRT–PCR (Figure 5B) and in a spot assay (two 5-μL drops of 5×10^5 conidial spores suspended in water per leaf) for the determination of the disease index (Figure 5C). The results show that *Pdf1.2a* expression is significantly induced at 72 h post inoculation. This timing in *Arabidopsis* WT is in good agreement with published data (Schenk et al., 2003) showing a significant increase in mRNA levels 72 h after infection. The mRNA levels appeared to be induced to higher levels in the *Hsf* knockout plants compared to WT.

The disease resistance of WT and mutant lines was monitored on days 3, 5, 7, 10, and 12 after infection with *A. brassicicola* (Figure 5C). Infected leaves of double knockout plants (*hsfB1/hsfB2b*) provided significantly higher levels of tolerance (lower disease index) against *A. brassicicola* infection compared to WT plants. These differences in pathogen resistance can be seen from the beginning (3 days after infection (DAI)) throughout the whole experiment (12 DAI). Comparing the disease index of all four lines on day 10 after infection, there is a significant difference between *hsfB2b* and *hsfB1/hafB2b* plants, which were more resistant, compared to WT and single knockout *hsfB1* plants (Figure 5D).

DISCUSSION

Considering the complexity of the *Hsf* gene family, the isolation of knockout mutants for individual *Hsf* genes is crucial for the determination of their functional role and biological importance in plants. Although single and double mutations in *HsfB1* and *HsfB2b* lead to a complete loss of mRNA accumulation, there was no detectable effect on the heat stress response. This finding contrasts the functions of class A-Hsfs in

(C) Leaves of 6-week-old *Arabidopsis* wild-type (WT) (Col-0), and *hsfB1/B2b* plants were drop-inoculated with spores of *A. brassicicola*. The disease index was calculated 3, 5, 7, 10, and 12 d after inoculation (DAI). Data points show means and S.D. (n = 3–5).
(D) The disease index calculated on day 10 post inoculation of WT (Col-0), double knockout *hsfB1/B2b*, and single knockout mutants *hsfB1*, *hsfB2b* plants. Columns represent means and S.D. (n = 3–5). Significant differences are indicated by asterisks (α = 5).

which double knockout mutations in *hsfA1a*/*hsfA1b* caused a strong attenuation of the induction of a number of target genes including *Hsp17.6*, *Hsp70*, *Hsp83.1*, and *Hsp101* (Lohmann et al., 2004). In our present study, the class B-*Hsf* mutations *hsfB1*/*B2B* had no effect on the expression of these *HsfA1a*/*HsfA1b* target genes or any other known heat shock genes in *Arabidopsis*. Hence, HsfB1 and HsfB2b are not directly involved in the regulation of the onset of the heat shock response. This is reminiscent to tomato, in which, when the expression of *LbHsfB1* was compromised in a transgenic approach, plants showed no obvious effect on the heat shock response (Mishra et al., 2002).

Plant Defensin Genes Are De-Repressed in the *hsfB1/B2b* Mutant Plants

In order to understand the biological roles of HsfB1/B2b, we performed microarray expression analysis to identify possible target genes and pathways affected in *hsfB1*/*B2b* mutant plants. The total numbers of differentially expressed genes (control versus heat stress) in WT (3333 = 15%) and *hsfB1*/*B2b* plants (2875 = 13%) are relatively high but only 44 genes were identified as targets of the HsfB1/B2b-dependent transcriptional regulation. Comparable expression profiling results have been previously obtained by Busch et al. (2005) in a different genetic background of *Arabidopsis* (ecotype Wassilewskija), where a total of 112 class A-Hsf target genes has been identified.

Out of the 44 putative HsfB1/B2B-dependent target genes, 15 were differentially regulated (12 up and three down-regulated) at control temperature, 29 (15 up- and 14 down-regulated) upon heat stress in mutant plants compared to WT. The major effect was the enhanced expression (16 to 25-fold) of *Pdf1.2a* and *Pdf1.2b*, both at heat stress and control temperature conditions. *Pdf1.2a* and *Pdf1.2b* encode small polypeptides that protect plants against pathogen attack (Penninckx et al., 1996, 1998; Broekaert et al., 1995; Thomma et al., 2002).

The almost selective up-regulation of *Pdf1.2a* and *Pdf1.2b* does not exclude the importance of other differentially expressed genes with a fold change of two to three. Besides *Pdf1.2a* and *Pdf1.2b*, three other genes de-repressed in *hsfB1*/*B2b* plants are involved in plant defense: (1) *Stp3*, a green leaf-specific, low-affinity monosaccharide transporter (Buttner et al., 2000), (2) a kelch repeat-containing F-box family protein, which has been shown to be induced after pathogen attack (www.genevestigator.ethz.ch/), and (3) a TIR class disease resistance gene, which is highly induced by pathogens (www.genevestigator.ethz.ch/). These genes are down-regulated after heat stress in WT but heat stress has only little effect on the repression of these genes in *hsfB1*/*B2b* plants. Another gene, encoding a 2-isopropylmalate synthase-like protein (also known as methylthioalkymalate synthase-like protein), which might be involved in resistance to insect herbivores (Kroymann et al., 2003), shows an enhanced mRNA level (approximately two-fold) in *hsfB1*/*B2b* plants at control temperature.

Interestingly, among the other genes up-regulated in *hsfB1*/*B2b* plants is the class-A transcription factor *AtHsfA2* (approximately three-fold up-regulated basal level), which is the strongest heat-inducible class A-*Hsf* of *Arabidopsis* (Busch et al., 2005). It is proposed that AtHsfA2 forms a heteromeric complex with AtHsfA1a and AtHsfA1b to regulate the expression of target genes during early and late stages of the heat shock response (Schramm et al., 2006). AtHsfA2 appears to act dominantly as a late, and AtHsfA1a and AtHsfA1b as early Hsfs that share a number of common target genes including *Apx2*, *Hsp26.5*, *Hsp25.3*, *Hsp22.0*, *Hsp18.1*, *Hsp70*, *Hsp101*, and *DNAJ* (Wunderlich et al., 2007).

HsfB2b Is a Negative Regulator of Defensin Gene Expression and Pathogen Resistance

There is ample evidence for the involvement of jasmonic acid in the regulation of *Pdf1.2* expression and the protective role of defensins in plant pathogen resistance (Brown et al., 2003; Nandi et al., 2005; Thomma et al., 1998). Jasmonic acid application induces the expression of *Pdf1.2* genes. The *Arabidopsis* triple mutant, *fad3-2 fad7-2 fad8*, which is unable to accumulate jasmonic acid, is susceptible to infection by *Pythium* spp. (Vijayan et al., 1998). Moreover, the mutants in jasmonate signaling pathway such as *coi1* and *jar1* showed enhanced susceptibility to fungal pathogens, such as *A. brassicicola*, *Botrytis cinerea*, etc. (Penninckx et al., 1996; Thomma et al., 1998) and suppressed *Pdf1.2* expression (Penninckx et al., 1998).

MeJ treatment led to further enhanced *Pdf1.2* mRNA levels in *hsfB1*, *hsfB2b*, and in double knockout plants by a factor of approximately 100. There is a clear tendency that the mutant lines accumulate much higher mRNA levels compared to WT. This indicates that, in *Hsf*-mutant plants, the de-repression is not only restricted to the low basal levels of *Pdf1.2a* expression.

The effects of MeJ on *Pdf1.2a* expression are largely reproduced in experiments using biotic stress infection with *A. brassicicola* for induction. *Pdf1.2a* and *Pdf1.2b* are induced to very high levels when plants are infected with *A. brassicicola* compared to other pathogens (genevestigator data).

The results of our patho-assays are in good agreement with the hypothesis that defensin expression correlates with pathogen resistance. The single mutant *hsfB2b* and the double mutant *hsfB1*/*B2b* showed significantly improved resistance levels (lower disease index) compared to WT and, surprisingly, to *hsfB1* plants. The discrepancy between high levels of *Pdf1.2* mRNA and only WT-level of disease resistance suggests that other molecular processes of the pathogen response are negatively affected by HsfB2b and thus result in improved pathogen resistance in plants unable to express *HsfB2b*. HsfB2b acts as a negative regulator of *Pdf1.2* expression and pathogen resistance. The effect of *hsfB2b* is reminiscent to another recessive mutation, the overexpressor of cationic peroxidase 3 (*Ocp3*), which confers a constitutive expression of *Pdf1.2* that results in an enhanced resistance of *ocp3* plants to *B. cinerea* and *P. cucumerina* (Coego et al., 2005). The protective role of

defensin gene expression against fungal infections in plants has been further demonstrated for transgenic rice (Kanzaki et al., 2002) or potato (Khan et al., 2006) plants overexpressing the wasabi defensin gene.

HsfB1 and HsfB2b seem to exert similar effects on *Pdf* expression, suggesting that they cooperate in repressing the basal and pathogen-induced levels. The involvement of HsfB1 in *Pdf1.2* expression and pathogen response is strongly supported by expression profiling data (www.genevestigator.ethz.ch/at/). Many pathogens (*P. syringae*, *P. infestans*, *B. cinerea*, *A. brassicicola*) and a number of signaling mutants like *Cpr5* (Bowling et al., 1997; Clarke et al., 2001), and *nahG* (Delaney et al., 1994) affect the expression of both *Pdf* genes.

Functional Roles of HsfB1 and HsfB2b

The regulation of *Pdf1.2* expression through jasmonate signaling seems to require a region located at position –255 to –277 bp upstream of the *Pdf1.2a* transcription start site. This region includes the GCC-box, which is a common motif in the promoters of various genes encoding defense-related proteins, and has been identified as the specific binding site for members of the ERF subfamily of AP2/ERF transcription factors (Buttner and Singh, 1997; Ohme-Takagi and Shinshi, 1995; Zhou et al., 1997). It is possible that general transcription factors have general binding capability to different *cis*-regulatory elements and may activate gene expression through interactions with ERFs. Co-regulation of gene expression by the GCC-box and other promoter elements has been observed. For example, the GCC-box is required but not sufficient for high-level induction of the tobacco osmotin gene by ethylene (Raghothama et *al*., 1997). A G-box is also linked to jasmonate responsiveness in defense-associated genes in various plant species (Kim et al., 1992; Mason et al., 1993).

Perfect HSEs (three-box sequences), the regulatory elements for Hsf-dependent gene expression, are present neither in this region of the *Pdf1.2a* promoter nor in the introns or the other non-translated regions of the gene. An imperfect two-box variant HSE is unable to bind Hsf, as indicated by our negative results in detecting any changes in protein binding to promoter segments (not shown). This suggests that the negative effect of HsfB1/B2b on *Pdf* expression in WT must be indirect, possibly through interaction with other proteins in the chromatin or in the cell. According to the *Arabidopsis* Small RNA Project Database (http://asrp.cgrb.oregonstate.edu/; Gustafson et al., 2005), there are no microRNAs in *Arabidopsis thaliana* that target the transcripts of the *Pdf1.2*.

Using perfect synthetic HSE sequences as a probe, we were able to detect a positive effect on the formation of the late Hsf–HSE complex, which appears earlier, persists longer, and is more intense in *hsfB1*/*B2b* plants compared to WT. The formation of this complex requires HsfA2, the major heat stress- and high light-induced class A-Hsf in *Arabidopsis* (Wunderlich et al., 2007). A higher binding capacity of the HsfA2-dependent late complex in *hsfB1*/*B2b* plants suggests that HsfB1/B2b may interact with class A-Hsf in regulating the shut-off of the heat shock response. The absence of other HsfB1/B2b in knockout plants was not associated with the loss of HSE-binding capacity. This further supports the hypothesis that these Hsfs are not directly involved in binding to the HSE *in vivo*. It is conceivable that these class B-Hsf form heteromeric complexes with class A-Hsf with an increasing probability during the heat shock response. There is evidence that the tomato LpHsfB1 (ortholog of AtHsfB1) can interact with the class A-Hsf LpHsfA1a but also with other general factors like HAC1/CBP and thus specifying a co-activator function (Bharti et al., 2004). In a similar way, the *Arabidopsis* HsfB1 may interact with other Hsfs (possibly with HsfA2) and/or other general transcription factors or chromatin components for exerting a negative (repressive) role on target gene expression. Further studies identifying target genes during later stages of the heat shock response or during recovery and interaction partners of class B-Hsf will shed more light on the functions and molecular mechanism of Hsf-dependent repression of genes.

Biological Role of Limiting Pdf Expression

The identification of *Pdf* genes as targets of Hsf-dependent negative regulation is the first evidence for an interconnection of Hsf in the regulation of biotic and abiotic responses. The question arises as to why plant defensin expression is down-regulated by heat-inducible Hsfs. Regarding plant defense, a simple explanation may be that the pathogens are not capable of efficient infection at high temperature and thus plants do not require the expression of defensins for protection. This is consistent with our findings that *A. brassicicola* does not grow under heat stress temperature conditions, that a heat stress early after infection causes a delay in the development of disease symptoms, and that jasmonate induction of *Pdf1.2* expression is blocked by heat treatment (not shown). Moreover, higher levels of *Pdf1.2* expression may be detrimental to plant growth and development, which might become effective only on a long-term evolutionary scale. In eukaryotic cells, it has been demonstrated that beta-defensins have little effect on the epithelial cells at any concentration. In contrast, alpha-defensin promotes proliferation of the epithelial cells at low concentration but has a cytotoxic effect at high concentration and may have adverse effects on the host (Nishimura et al., 2004). In a similar way, *Arabidopsis* plants carrying the *iop1* mutation show much higher induced expression levels of *Pdf1.2*, but, during their entire lifespan, the *iop1* mutants stayed significantly smaller (Penninckx et al., 2003).

METHODS

Plant Material, Growth Condition, and Heat Treatment

Arabidopsis thaliana (Columbia-0) was used in experiments. Col-0 T-DNA insertion lines were obtained from the Signal Salk institute (http://signal.salk.edu/tdnaprimers.2.html).

Seeds were spread on soil or MS media and kept in the dark at 4–8°C for 2 d to achieve a uniform germination. Ten DAG

(days after germination), seedlings were singled out in individual pots and grown in soil with a light/dark cycle of 16/8 h at 22°C, 60% relative humidity and a light intensity of 5000 lumen m^{-2}. All experiments were carried out with 5-week-old plants unless specified.

For heat treatment (HS), fully expanded leaves were collected and pooled, incubated at 37°C (for 1, 2, 4, or 8 h, depending on the experiment performed) in pre-warmed SIB (1 mM potassium phosphate, pH 6, 1% (W/V) sucrose), then placed in a shaking water bath with 40 oscillations per minute. As a control (C), leaf pools were incubated under the same experimental conditions but at room temperature (22°C). After heat treatment, excess SIB was rinsed off with sterile water; leaf tissue was blotted dry with filter paper and immediately frozen in liquid nitrogen. Samples were stored at –70°C for future use.

RNA Isolation, Labeling, and Microarray Hybridization

Frozen leaf tissue (–80°), which had been collected from 45 individual plants per biological replicate, was crushed by using mortar and pistil and RNA was extracted with the Plant RNeasy Mini Kit (Qiagen, Hilden, Germany). A total of 5 μg RNA was used as starting material for double-stranded cDNA synthesis using the Superscript Choice System (Invitrogen, Karlsruhe, Germany) and an oligo dT-T7 primer (Genset, Paris, France). Biotinylated cRNA was synthesized from cDNA template using BioArray High Yield Transcript Labeling Kit (Enzo, Farmingdale, NY, USA). RNeasy columns (Qiagen) were employed to clean the biotinylated cRNA. The fragmentation of cRNA was performed according to GeneChip protocol (Affymetrix, Santa Clara, CA, USA).

The fragmented cRNAs were hybridized to *Arabidopsis* ATH 1-12151 (Affymetrix Gene-Chips), which represents 22 810 probe sets. GeneChip arrays were hybridized according to the manufacturer's protocol (Affymetrix). Hybridized GeneChips were scanned with the GeneChip Scanner 3000 (Affymetrix). Samples were quality-controlled by examining of 3′ to 5′ intensity ratios of control genes.

Analysis of Expression Data

Data were imported in Genespring 7.2 (Silicon Genetics, Redwood City, CA, USA) and retransformed in linear values. Data for every condition were available in two replicates of each experiment. Raw data were quantile-normalized and expression estimates were calculated by gcRMA (Wu et al., 2004) implemented in R. Statistical analysis of data was carried out on gene sets after eliminating all genes that showed less then two-fold change between conditions, by Welch's *t*-test to compare the different conditions and to find differentially expressed genes (selected *P*-value 0.05); the Benjamini and Hochberg False Discovery Rate Multiple Testing Correction was used to reduce the detection of false positives. The data are deposited in a MIAME compliant fashion in the ArrayExpress database (www.ebi.ac.uk/microarray/) under accession E-MEXP-1725.

Isolation of mRNA and Preparation of cDNA for Quantitative Real-Time PCR

This method has been used for the verification of knockout mutants and the expression profiles of individual Hsf target genes. Poly $(A)^+$-RNA was isolated from 60–80 mg frozen leaf material using the chemagic mRNA Direct kit (Chemagen) and the amounts were quantified using the dye RiboGreen. cDNA was synthesized from 100 ng of mRNA using the iScript™ cDNA synthesis kit (Biorad). The amount of cDNA was quantified using PicoGreen. Quantitative RT–PCR was performed in triplicates using undiluted (1 ng) and 1/8 and 1/64 diluted cDNA as template. Real-time PCR was performed in a 50-μl reaction volume. The primers shown in Table 4 were used.

Isolation of mRNA and Preparation of cDNA for Quantitative Real-Time PCR

This method was used for determining the Pdf1.2-mRNA expression after MeJ treatment or Alternaria spray assay. Poly $(A)^+$-RNA was isolated from 50–70 mg frozen leaf material using the chemagic mRNA Direct kit (Chemagen). A total volume of 15 μl was used for cDNA synthesis using the iScript™cDNA kit (Biorad). Quantitative RT–PCR was performed with three dilutions (1, 1/8, and 1/64) of cDNA and in duplicate samples. As a control, undiluted mRNA was used. The reaction volume

Table 4. Primers Used for Real-Time PCR.

Target gene	Primer sequence
Actin2	5′-AAGCTGGGGTTTTATGAATGG-3′
(At3g18780)	5′-TTGTCACACACAAGTGCATCAT-3′
Hsp17.6	5′-GGTGAGTGGCAAAAGACAGA-3′
(At1g59860)	5′-AAACTTCCCCATCCTCCTCT-3′
Hsp23.6	5′-CGATGAGATTAAGGCGGAGA-3′
(At4g25200)	5′-TCGACGTTTTTAGTTGATCTCG-3′
Hsp70	5′-AGGAGCTCGAGTCTCTTTGC-3′
(At2g32120)	5′-AGGTGTGTCGTCATCCATTC-3′
Hsp83.1	5′-GCTGCTAGGATTCACAGGATG-3′
(At5g52640)	5′-TCCTCCATCTTGCTCTCTTCA-3′
Hsp101	5′-TAACGGGCCAAAGAGAAGTG-3′
(At1g74310)	5′-CACACGTTGGAGGTCAAGACT-3′
AtHsfB1	5′-GGACCGGGATGAAAAGAATTA-3′
(At36990)	5′-CACGCTGGTTTGAACAGTCTT-3′
AtHsfB2b	5′-TGGAGGAGAATAACTCCGGTAA-3′
(At11660)	5′-ATGCAATGGGGATTCAGTAACA-3′
GolS1	5′-AGCTTAGCCACAATATAATCATCG-3′
(At1g56600)	5′-ATCCTCCAAAACCCATAAAAATTA-3′
Pdf1.2a	5′-CCAAGTGGGACATGGTCAG-3′
(At5g44420)	5′-ACTTGTGTGCTGGGAAGACA-3′
Pdf1.2b	5′-GGTACTTGGTCAGGAGTTTGC-3′
(At2g26020)	5′-ACTTGTGAGCTGGGAAGACA-3′

was 30 μl. mRNA expression level of *Pdf1.2a* was analyzed with respect to *Actin2.* The following primers were used:

Actin2-F3 (5′-AAGCTGGGGTTTTATGAATGG-3′),
Actin2-R3 (5′-TTGTCACACACAAGTGCATCAT-3′),
Pdf1.2a_for (5′-TGCTTTCGACGCACCGGC-3′),
Pdf1.2a_rev (5′-TGTAAAATACACACGATTTAGCACC-3′)

Pathogen Treatment: Spot Assay

Plants were grown in soil with a light/dark cycle of 8/16 h at 22°C, 100% relative humidity and a light intensity of 5000 lumen m^{-2}. Pathogen (*Alternaria brassicicola*) treatment was carried out by apoplectic puncturing 6-week-old plants. Care was taken to choose leaves with approximately equal size and having the same developmental stage in WT as well as in mutants.

Two leaves per plant were chosen for fungal inoculation. Plants were inoculated at a concentration of 5×10^5 conidial spores per milliliter. Each leaf was inoculated with two 5-μL drops of a suspension in water. Inoculated plants were incubated at 100% RH and 22°C for control conditions and 35°C for heat stress treatment, respectively. The spread of fungal colonization on each leaf was analyzed 3, 5, 7, 10, and 12 DAI. As a control, one plant per line was treated with water.

Detection of Pathogen Colonization on Inoculated Plants and Disease Index

Pathogen-inoculated leaves were bonitated according to the disease symptoms shown in Table 5.

The disease index (DI) for each DAI was calculated from the results of bonitation by the following formula:

$$DI = \left(\sum \text{Bonitation scores}/\text{Number of inoculated leaves}\right)^{*}100.$$

Methyl Jasmonate Treatment

Five-week-old plants were sprayed with methyl jasmonate (MeJ) solution (100 μM). Mock inoculations were only sprayed with water.

Table 5. Pathogen Inoculated Leaves Bonitated According to Disease Symptoms.

Score	Symptoms
1	No symptom
2	Little brownish at point of inoculation
3	Dark-brown spot at point of inoculation
4	Necrotic spots spreading around the point of inoculation
5	Maceration
6	Sporulation
7	Leaf detachment

Combined heat stress and MeJ treatment was carried out as follows: plants were first sprayed with MeJ and then kept in a growth chamber (set at 37°C, light/dark cycle of 16/8 h, relative humidity 60%, light intensity 5000 lumen m^{-2}). As a control, water-sprayed plants were kept in a growth chamber at 22°C (light/dark cycle of 16/8 h, relative humidity 60%, light intensity 5000 lumen m^{-2}).

Pathogen Treatment: Spray Assay

Plants were grown in soil with a light/dark cycle of 8/16 h at 22°C, 100% relative humidity and light intensity 5000 lumen m^{-2}. For pathogen treatment (*Alternaria brassicicola*), 6-week-old plants were sprayed with a concentration of 5×10^5 conidial spores per milliliter. Leaf material was collected immediately after spray infection and after 72 and 96 h post inoculation, respectively.

Isolation of Single and Double Hsf Mutants

Individual T-DNA insertion lines for *AtHsfB1* and *AtHsfB2b* were obtained from the Salk Institute. Suitable primers for testing the T-DNA insertion (as described by the Salk website: http://signal.salk.edu/tdnaprimers.2.html) were generated and used in PCR reactions with genomic DNA as the template. Using 5′ and 3′ gene-specific primers, WT plants were identified and eliminated from segregating population. Mutants were identified by specific PCR fragments obtained using a T-DNA and a gene-specific primer.

To generate a double *Hsf* mutant, 8–9-week-old single homozygous mutant plants were selected for pollination. For ♀ (*hsfB2b* mutant plants), closed flower buds were selected. Flowers were opened with a pin set and anthers were removed. Flowers were then left opened for 2 d before pollination with pollen from *hsfB1*. After 2 d, healthy and non-pollinated stigmas were chosen for fertilization. For ♂ (*hsfB1* mutant plants), fully mature anthers with ripened pollens were chosen.

The resulting F1 heterozygous population was selfed to get homozygous *hsfB1/B2b* double mutant plants. WT plants were identified using gene-specific primers for *hsfB1* and *hsfB2b*. Accordingly, the double mutation was identified with T-DNA left border primer and the gene-specific primer (Table 6).

Electrophoretic Mobility Shift Assay (EMSA)

The Hsf binding to DNA has been tested essentially as described by Lohmann et al. (2004), and the same synthetic HSE sequences (5′-CCA**GAA**GC**TTC**CA**GAA**GCC) were used as a probe. Unspecific binding complexes are discriminated

Table 6. Double Mutations Identified with T-DNA Left Border Primer and the Gene-Specific Primers.

AtHsfB1 (*At36990*)	5′-AAAAGTTCGCCGGAGATGACG-3′
	5′-GTCGCAACCTTCGCACTCACT-3′
AtHsfB2b (*At11660*)	5′-CACAGAGGTCAATTCCGACGC-3′
	5′-CTTCTTCCTCTGCAGCACCCA-3′
T-DNA (Lba1)	5′-ATGGTTCACGTAGTGGGCCATC-3′

according to Lohmann et al. (2004) by the ability to be also formed with mutated HSE, while specific binding complexes are formed only with perfect consensus HSE sequences but not with mutated HSE.

FUNDING

The research was funded by grants of the Deutsche Forschungsgemeinschaft (SFB446, project A2).

ACKNOWLEDGMENTS

We thank Dr Jasmin Doll and Markus Wunderlich (ZMBP-Allgemeine Genetik, Universität Tübingen) for advice and fruitful discussions. No conflict of interest declared.

REFERENCES

Bharti, K., Koskull-Döring, von P., Bharti, S., Kumar, P., Tintschl-Körbitzer, A., Treuter, E., and Nover, L. (2004). Tomato heat stress transcription factor HsfB1 represents a novel type of general transcription coactivator with a histone-like motif interacting with the plant CREB binding protein ortholog HAC1. Plant Cell. **16,** 1521–1535.

Bowling, S.A., Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X. (1997). The cpr5 mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell. **9,** 1573–1584.

Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., and Osborn, R.W. (1995). Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant Physiol. **108,** 1353–1358.

Brown, R.L., Kazan, K., McGrath, K.C., Maclean, D.J., and Manners, J.M. (2003). A role for the GCC-box in jasmonate-mediated activation of the *PDF1.2* gene of *Arabidopsis*. Plant Physiol. **132,** 1020–1032.

Busch, W., Wunderlich, M., and Schöffl, F. (2005). Identification of novel heat shock factor dependent genes and biochemical pathways in *Arabidopsis thaliana*. Plant J. **41,** 1–14.

Buttner, M., and Singh, K.B. (1997). *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), an ethylene-inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. Proc. Natl Acad. Sci. U S A. **94,** 5961–5966.

Buttner, M., Truernit, E., Baier, K., Scholz-Starke, J., Sontheim, M., Lauterbach, C., Huss, V.A.R., and Sauer, N. (2000). AtSTP3, a green leaf-specific, low affinity monosaccharide H^+-symporter of *Arabidopsis thaliana*. Plant Cell Environ. **23,** 175–184.

Clarke, J.D., Aarts, N., Feys, B.J., Dong, X., and Parker, J.E. (2001). Constitutive disease resistance requires EDS1 in the *Arabidopsis* mutants *cpr1* and *cpr6* and is partially EDS1-dependent in *cpr5*. Plant J. **26,** 409–420.

Coego, A., Ramirez, V., Gil, M.J., Flors, V., Mauch-Mani, B., and Vera, P. (2005). An *Arabidopsis* homeodmain transcription factor, *overexpressor of cationic peroxidase 3*, mediates resistance to infection by necrotrophic pathogens. Plant Cell. **17,** 2123–2137.

Czarnecka-Verner, E., Pan, S., Salem, T., and Gurley, W.B. (2004). Plant class B Hsfs inhibit transcription and exhibit affinity for TFIIB and TBP. Plant Mol. Biol. **56,** 57–75.

Czarnecka-Verner, E., Yuan, C.-X., Scharf, K.-D., English, G., and Gurley, W.B. (2000). Plants contain a novel multi-member class of heat shock factors without transcriptional activator potential. Plant Mol. Biol. **43,** 459–471.

Dat, J.F., Foyer, C.H., and Scott, I.M. (1998). Changes in salicylic acid and antioxidants during induction of thermotolerance in mustard seedlings. Plant Physiol. **118,** 1455–1461.

Delaney, T.P., et al. (1994). A central role of salicylic acid in plant disease resistance. Science. **266,** 1247–1250.

Döring, P., Treuter, E., Kistner, C., Lyck, R., Chen, A., and Nover, L. (2000). The role of AHA motifs in the activator function of tomato heat stress transcription factors HsfA1 and HsfA2. Plant Cell. **12,** 265–278.

Gustafson, A.M., Allen, E., Givan, S., Smith, D., Carrington, J.C., and Kasschau, K.D. (2005). ASRP: the *Arabidopsis small RNA project database*. Nucleic Acids Res. **33,** D637–D640.

Kanzaki, H., Nirasawa, S., Saitoh, H., Ito, M., Nishihara, M., Terauchi, R., and Nakamura, I. (2002). Overexpression of the wasabi defensin gene confers enhanced resistance to blast fungus (*Magnaporthe grisea*) in transgenic rice. Theor. Appl. Genet. **105,** 809–814.

Khan, S.R., Nishihara, M., Yamamura, S., Nakamura, I., and Mii, M. (2006). Transgenic potatoes expressing wasabi defensin peptide confer partial resistance to gray mold (*Botrytis cinerea*). Plant Biotech. **23,** 179–183.

Kim, S.R., Choi, J.L., Costa, M.A., and An, G. (1992). Identification of G-box sequence as an essential element for methyl jasmonate response of potato proteinase inhibitor II promoter. Plant Physiol. **99,** 627–631.

Koskull-Döring, P., Scharf, k-D., and Nover, L. (2007). The diversity of plant heat stress transcription factors. Trends Plant Sci. **12,** 452–457.

Kotak, S., Port, M., Ganguli, A., Bicker, F., and Koskull-Döring, P. (2004). Characterization of C-terminal domains of *Arabidopsis* heat stress transcription factors (Hsfs) and identification of a new signature combination of plant class A Hsfs with AHA and NES motifs essential for activator function and intracellular localization. Plant J. **39,** 98–112.

Kroymann, J., Donnerhacke, S., Schnabelrauch, D., and Mitchell-Olds, T. (2003). Evolutionary dynamics of an *Arabidopsis* insect resistance quantitative trait locus. Proc. Natl Acad. Sci. U S A. **100,** 14587–14592.

Lohmann, C., Eggers-Schumacher, G., Wunderlich, M., and Schöffl, F. (2004). Two different heat shock transcription factors regulate immediate early expression of stress genes in *Arabidopsis*. Mol. Gen. Genom. **271,** 11–21.

Mason, H.S., DeWald, D.B., and Mullet, J.E. (1993). Identification of a methyl jasmonate-responsive domain in the soybean *vspB* promoter. Plant Cell. **5,** 241–251.

Mishra, S.K., Tripp, J., Winkelhaus, S., Tschiersch, B., Theres, K., Nover, L., and Scharf, K.D. (2002). In the complex family of heat stress transcription factors, HsfA1 has a unique role as master regulator of thermotolerance in tomato. Genes Develop. **16,** 1555–1567.

Nandi, A., Moeder, W., Kachroo, P., Klessig, D.F., and Shah, J. (2005). *Arabidopsis* ssi2-conferred susceptibility to Botrytis cinerea is dependent on EDS5 and PAD4. Molec. Plant Microbe Interact. **18,** 363–370.

Nishimura, M., Abiko, Y., Kurashige, Y., Takeshima, M., Yamazaki, M., Kusano, K., Saitoh, M., Nakashima, K., Inoue, T., and Kaku, T. (2004). Effect of defensin peptides on eukaryotic

cells: primary epithelial cells, fibroblasts and squamous cell carcinoma cell lines. J. Dermatol. Sci. **36,** 87–95.

Nover, L., Scharf, K.D., Gagliardi, D., Vergne, P., Czarnecka-Verner, E., and Gurley, W.B. (1996). The Hsf world: classification and properties of plant heat stress transcription factors. Cell Stress Chaperones. **1,** 215–223.

Ohme-Takagi, M., and Shinshi, H. (1995). Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. Plant Cell. **7,** 173–182.

Panikulangara, T.J., Eggers-Schumacher, G., Wunderlich, M., Stransky, H., and Schöffl, F. (2004). *Galactinol synthase 1*, a novel heat-inducible and Hsf-target gene responsible for heat-induced synthesis of Raffinose Family Oligosaccharides in *Arabidopsis*. Plant Physiol. **136,** 3148–3158.

Penninckx, I.A., Eggermont, K., Schenk, P.M., van den Ackerveken, G., Cammun, P.B., and Thomma, B.P. (2003). The *Arabidopsis* mutant iop1 exhibits induced over-expression of the plant defensin gene PDF1.2 and enhanced pathogen resistance. Mol. Plant Pathol. **4,** 479–486.

Penninckx, I.A., Eggermont, K., Terras, F.R., Thomma, B.P., De Samblanx, G.W., Buchala, A., Metraux, J.P., Manners, J.M., and Broekaert, W.F. (1996). Pathogeninduced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid independent pathway. Plant Cell. **8,** 2309–2323.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. Plant Cell. **10,** 2103–2113.

Prändl, R., Hinderhofer, K., Eggers-Schumacher, G., and Schöffl, F. (1998). Hsf3, a new heat shock factor from *Arabidopsis thaliana*, derepresses the heat shock response and confers thermotolerance when overexpressed in transgenic plants. Mol. Gen. Genet. **258,** 269–278.

Raghothama, K.G., Maggio, A., Narasimhan, M.L., Kononowicz, A.K., Wang, G., D'Urzo, M.P., Hasegawa, P.M., and Bressan, R.A. (1997). Tissue-specific activation of the osmotin gene by ABA, C2H4 and NaCl involves the same promoter region. Plant Mol. Biol. **34,** 393–402.

Schenk, P.M., Kazan, K., Manners, J.M., Anderson, J.P., Simpson, R.S., Wilson, I.W., Somerville, S.C., and Maclean, D.C. (2003). Systemic gene expression in *Arabidopsis* during an incompatible interaction with *Alternaria brassicicola*. Plant Physiol. **132,** 999–1010.

Schramm, F., Ganguli, A., Kiehlmann, E., Englich, G., Walch, D., and Koskull-Döring, P. (2006). The heat stress transcription factor HsfA2 serves as a regulatory amplifier of a subset of genes in the heat stress response in *Arabidopsis*. Plant Mol. Biol. **60,** 759–772.

Thomma, B.P.H.J., Camme, B.P.A., and Thevissen, K. (2002). Plant defensins. Planta. **216,** 193–202.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammune, B.P.A., and Broekaert, W. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc. Natl Acad. Sci. U S A. **95,** 15107–15111.

Vijayan, P., Shockey, J., Levesque, C.A., Cook, R.J., and Browse, J. (1998). A role for jasmonate in pathogen defence of *Arabidopsis*. Proc. Natl Acad. Sci. U S A. **95,** 7209–7214.

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

Wu, Z., Irizarry, R.A., Gentleman, R., Murillo, F.M., and Spencer, F.A. (2004). A model based background adjustment for oligonucleotide expression arrays (Working Paper 1, Dept. of Biostatistics Working Papers: Johns Hopkins University).

Wunderlich, M., Doll, J., Busch, W., Kleindt, C.K., Lohmann, C., and Schöffl, F. (2007). Heat shock factors: regulators of early and late functions in plant stress response. Plant Stress. **1,** 16–22.

Zhou, J.M., Tang, X.Y., and Martin, G.B. (1997). The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis-related genes. EMBO J. **16,** 3207–3218.