

The xenobiotic β -aminobutyric acid enhances Arabidopsis thermotolerance

Laurent Zimmerli^{1,2,t,*}, Bi-Huei Hou¹, Chia-Hong Tsai³, Gabor Jakab^{2,†}, Brigitte Mauch-Mani² and Shauna Somerville¹

¹Department of Plant Biology, Carnegie Institute, Stanford, CA 94305, USA,

²Department of Science, Laboratory of Molecular and Cellular Biology, University of Neuchâtel, 2009 Neuchâtel, Switzerland, and

³Department of Life Science, Institute of Plant Biology, National Taiwan University, Taipei, Taiwan

*For correspondence (fax +888 2 23918940; e-mail lauzim2@ntu.edu.tw).

†Present address: Department of Life Science, Institute of Plant Biology, National Taiwan University, Taipei, Taiwan.

*Present address: Department of Plant Physiology, Institute of Biology, University of Pecs, PO Box 266, H-7601 Pecs, Hungary.

Summary

The non-protein amino acid β -aminobutyric acid (BABA) primes Arabidopsis to respond more quickly and strongly to pathogen and osmotic stress. Here, we report that BABA also significantly enhances acquired thermotolerance in Arabidopsis. This thermotolerance was dependent on heat shock protein 101, a critical component of the normal heat-shock response. BABA did not enhance basal thermotolerance under a severe heat-shock treatment. No roles for the hormones ethylene and salicylic acid in BABA-induced acquired thermotolerance were identified by mutant analysis. Using global gene expression analysis, transcript levels for several transcription factors and DNA binding proteins regulating responses to the stress hormone abscisic acid (ABA) were found to be elevated in BABA-treated plants compared with water-treated plants. The role of ABA in BABA-induced thermotolerance was complex. BABA-enhanced thermotolerance was partially compromised in the ABA-insensitive mutant, *abi1-1*, but was augmented in *abi2-1*. In an unrelated process, BABA, like ABA, inhibited root growth, and the level of inhibition was roughly additive in roots treated with both compounds. Root growth of both *abi1-1* and *abi2-1* was also inhibited by BABA. Unexpectedly, *abi1-1* and *abi2-1* root growth was inhibited more strongly by combined ABA and BABA treatments than by BABA alone. Our results, together with previously published data, suggest that BABA is a general enhancer of plant stress resistance, and that cross-talk occurs between BABA and ABA signalling cascades. Specifically, the BABA-mediated accumulation of ABA transcription factors without concomitant activation of a downstream ABA response could represent one component of the BABA-primed state in Arabidopsis.

Keywords: β -aminobutyric acid, acquired thermotolerance, abscisic acid, root growth, salicylic acid, microarray.

Introduction

The effects of heat stress on plants are significant. High temperatures alter membrane properties (Sangwan *et al.*, 2002), and also reduce or inactivate enzyme activity through protein denaturation (Kampinga *et al.*, 1995). Above-normal temperatures can induce programmed cell death (Swidzinski *et al.*, 2002; Vacca *et al.*, 2004). To survive, sessile organisms such as plants need to sense these environmental changes and respond appropriately. The term 'basal thermotolerance' describes the inherent resistance to temperatures above that optimal for growth (Lindquist, 1986). The adaptive response, acquired thermotolerance, protects

plants first exposed to a preliminary mild heat stress against a second otherwise lethal high-temperature treatment (Hong and Vierling, 2000; Queitsch *et al.*, 2000). For example, the Columbia (Col-0) accession of Arabidopsis will normally survive exposure to 45°C for 2 h following prior exposure to a mild heat stress of 38°C for 90 min (Hong and Vierling, 2000; Queitsch *et al.*, 2000). Adaptation to heat stress involves the induction of heat-shock proteins (HSPs), active oxygen species, salicylic acid (SA) and abscisic acid (ABA) signalling (Larkindale *et al.*, 2005). These multiple responses suggest that many interconnected processes are

involved in resistance to heat stress. Plants at various growth stages respond differently to heat stress, suggesting a link between development and thermotolerance (Hong *et al.*, 2003; Larkindale *et al.*, 2005).

One of the most studied responses to heat stress is the accumulation of HSPs (Gurley, 2000; Vierling, 1991). These proteins are thought to act as molecular chaperones that prevent aggregation of proteins unfolded by heat treatment (Miernyk, 1999). A functional HSP101 is necessary for acquired thermotolerance, as loss-of-function mutants in *Arabidopsis* (*hot1*) (Hong and Vierling, 2000, 2001) and in maize (Nieto-Sotelo *et al.*, 2002) and antisense RNA inhibition mutants (Queitsch *et al.*, 2000) of this gene are unable to acclimatize to heat stress.

Oxidative stress, for example treatment with H₂O₂, induced thermotolerance in potato microplant tissues (Lopez-Delgado *et al.*, 1998). In mustard seedlings, H₂O₂ levels, catalase activity and antioxidant pools were altered during heat acclimatization (Dat *et al.*, 1998a,b). Recently, Larkindale *et al.* (2005) showed that antioxidant mutants are partially altered in thermotolerance, adding to the body of evidence suggesting a role for the oxidative response during heat stress.

In addition to its role in systemic acquired resistance (Durrant and Dong, 2004), SA is known to increase the heat-shock survival rates of plants such as mustard and *Arabidopsis* (Clarke *et al.*, 2004; Dat *et al.*, 1998b; Larkindale and Knight, 2002). Transgenic *NahG* plants, which catabolize SA to catechol and do not accumulate SA (Lawton *et al.*, 1995), display reduced thermotolerance. Taken together, these data suggest that SA is important for the survival of heat stress (Clarke *et al.*, 2004; Larkindale *et al.*, 2005).

The plant hormone ABA regulates plant development and stress adaptation. Plant responses to cold, salt and drought are modulated through ABA signalling (Chandler and Robertson, 1994; Verslues and Zhu, 2005; Zhu, 2002). Recently, a role for ABA in plant-pathogen interactions has been suggested (Anderson *et al.*, 2004; Mauch-Mani and Mauch, 2005). Although less well documented, ABA is thought to enhance resistance to heat stress. In *Arabidopsis*, ABA treatment protected wild-type plants from heat stress, and mutants defective in ABA signalling were generally less resistant to heat stress (Larkindale and Knight, 2002; Larkindale *et al.*, 2005). However, the precise role of this plant hormone in heat-stress resistance is unknown. Numerous mutants with altered ABA responsiveness or ABA biosynthesis have been isolated. Among mutants insensitive to ABA, *ABA-insensitive 1* (*abi1-1*) and *2* (*abi2-1*) are characterized by both reduced seed dormancy and diminished sensitivity to the inhibitory effect of ABA on germination (Finkelstein and Somerville, 1990; Koornneef *et al.*, 1984). The dominant *abi1-1* and *abi2-1* mutants are also insensitive to ABA inhibition of seedling growth and display abnormal stomatal regulation (Finkelstein and Somerville, 1990;

Koornneef *et al.*, 1984; Schnall and Quatrano, 1994). The *ABI1* and *ABI2* genes encode serine/threonine protein phosphatases 2C (Leung *et al.*, 1994; Meyer *et al.*, 1994; Leung *et al.*, 1997). Plants expressing loss-of-function alleles of *ABI1* and *ABI2* are hypersensitive to ABA, suggesting that they are negative regulators of ABA responses (Gosti *et al.*, 1999; Merlot *et al.*, 2001; Yoshida *et al.*, 2005).

The non-protein amino acid β -aminobutyric acid (BABA) is a xenobiotic compound that, when supplied to the root system via watering, can be translocated to all aerial parts of the *Arabidopsis* plant (Jakab *et al.*, 2001). BABA protects plants against pathogens through potentiation of SA- and/or ABA-dependent defence mechanisms (Prime-A-Plant Group, 2006; Ton and Mauch-Mani, 2004; Zimmerli *et al.*, 2000, 2001). Recently, BABA was shown to enhance resistance to drought and high-salinity stresses in *Arabidopsis* by priming both ABA accumulation and the expression of stress-regulated genes (Jakab *et al.*, 2005). When compared with non-treated controls, stomata from BABA-treated plants exhibited reduced stomatal conductance during drought stress. Together, these results suggest that BABA acts at multiple levels to enhance drought resistance (Jakab *et al.*, 2005). To broaden our knowledge of BABA effects on plant resistance to abiotic stresses, we tested BABA-induced resistance to heat stress in *Arabidopsis*. The data presented here on heat-stress protection, together with previous results on osmotic and biotic stresses, provide evidence that BABA is a general enhancer of stress resistance in *Arabidopsis*.

Results

BABA increases acquired thermotolerance in Arabidopsis

To test the effect of BABA on the basal tolerance to heat shock, 10-day-old Col-0 plants grown on medium supplemented with 0.5 mM BABA were transferred to 45°C for various periods of time. BABA-treated plants were not statistically significantly more tolerant to exposure to 45°C for 60 or 75 min than untreated control plants. Most plants died after treatment at 45°C for 90 min whether or not they had been treated with BABA (Figure 1a). To test whether BABA could prime the *Arabidopsis* response to heat stress, and thus increase the adaptive response to heat stress, Col-0 plants were first treated at 38°C for 45 min and then at 45°C for 90 min. The 45 min acclimatization period is too short to allow *Arabidopsis* plants to reach full acclimatization, and only about 30% of the water-treated pre-conditioned plants survived this treatment regime (Figure 1b,c). By contrast, more than 80% of pre-conditioned, BABA-treated plants survived (Figure 1b,c). Thus, BABA treatment produced a statistically significant enhancement of acquired thermotolerance, but had no significant effect on basal thermotolerance under these experimental conditions.

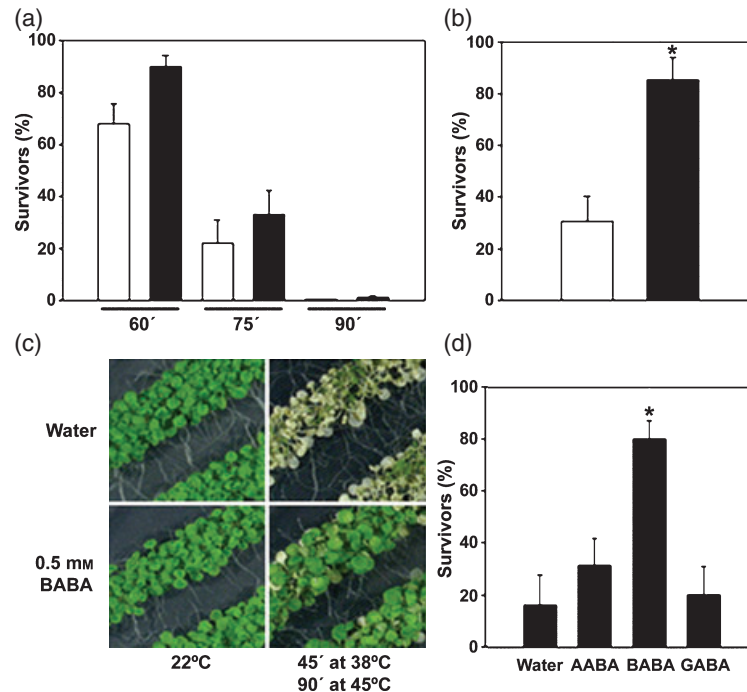


Figure 1. BABA enhances acquired but not basal tolerance to severe heat stress.

(a) Basal thermotolerance. Col-0 plants were grown on medium supplemented (closed bars) or not (water control, open bars) with BABA at a final concentration of 0.5 mM for 10 days at 22°C and directly moved to 45°C for the indicated number of min. Survivors showing no necrosis on true leaves were counted 4 days after the heat-shock treatment and expressed as the percentage of plants per plate prior to the heat-shock treatment. Data represent the means and SE of four independent experiments grouped together ($n > 12$ plates consisting of approximately 150 seedlings per plate). The percentage of survivors for BABA-treated plants and water-treated controls at each time point were not significantly different by an unpaired Student's t -test ($P > 0.01$).

(b) Acquired thermotolerance. Plants were treated as in (a) except that they were conditioned by a 45 min exposure to 38°C prior to heat-shock treatment at 45°C for 90 min (see details in Experimental procedures). Data represent the mean percentage survivors and SE of four independent experiments grouped together ($n > 10$ plates consisting of approximately 150 seedlings per plate). The asterisk denotes a statistically significant difference between BABA- and water-treated plants by an unpaired t -test ($P < 0.01$).

(c) Symptoms of heat-shocked plants. Representative plants 4 days after heat-shock treatment. Plants were grown and heat-treated as in (b).

(d) Isomer specificity. Plants were grown on medium containing either α -aminobutyric acid (AABA), β -aminobutyric acid (BABA) or γ -aminobutyric acid (GABA) at a final concentration of 0.5 mM, and heat-treated as in (b). Data represent the mean percentage survivors and SE of three independent experiments grouped together ($n > 9$ plates consisting of approximately 150 seedlings per plate). The asterisk denotes a statistically significant difference between chemical- and water-treated plants by an unpaired t -test ($P < 0.01$).

To test the isomer specificity of this effect, α - and γ -aminobutyric acids were evaluated for their ability to enhance acquired thermotolerance. At concentrations of up to 0.5 mM, neither isomer was able to enhance the acquired thermotolerance of Col-0 (Figure 1d). This demonstrates a remarkable selectivity of Arabidopsis towards aminobutyric acid isomers. Collectively, these results suggest that BABA is not acting as a compatible solute, but rather that this compound affects heat-stress acclimatization mechanisms (Chen and Murata, 2002).

A functional HSP101 is necessary

HSP101 is a well-characterized component of the heat-shock response, and plays a critical role in thermotolerance in Arabidopsis (Hong and Vierling, 2000, 2001; Queitsch *et al.*, 2000). To determine whether the BABA effect on thermotolerance acts via HSP101, we monitored the thermotoler-

ance of the HSP101-defective mutant *hot1-1* (Hong and Vierling, 2000). BABA treatment did not protect *hot1-1* plants from a 90 min heat shock of 45°C (Figure 2a) or 43.5°C (Supplementary Figure S1), even with a pre-conditioning treatment of 38°C for 45 min. These data suggest that BABA acts via activation of the classical heat-stress responses. We did not observe an earlier or stronger induction of the *HSP101* gene in BABA-treated heat-stressed plants (data not shown). Thus, unlike the priming effect of BABA on defence responses against pathogens and salt stress, BABA does not appear to enhance thermotolerance by potentiating *HSP101* expression (Ton *et al.*, 2005; Zimmerli *et al.*, 2000).

The role of SA signalling in BABA-induced acquired thermotolerance

SA signalling in response to pathogens is primed by BABA treatment (Zimmerli *et al.*, 2000, 2001), and the SA pathway

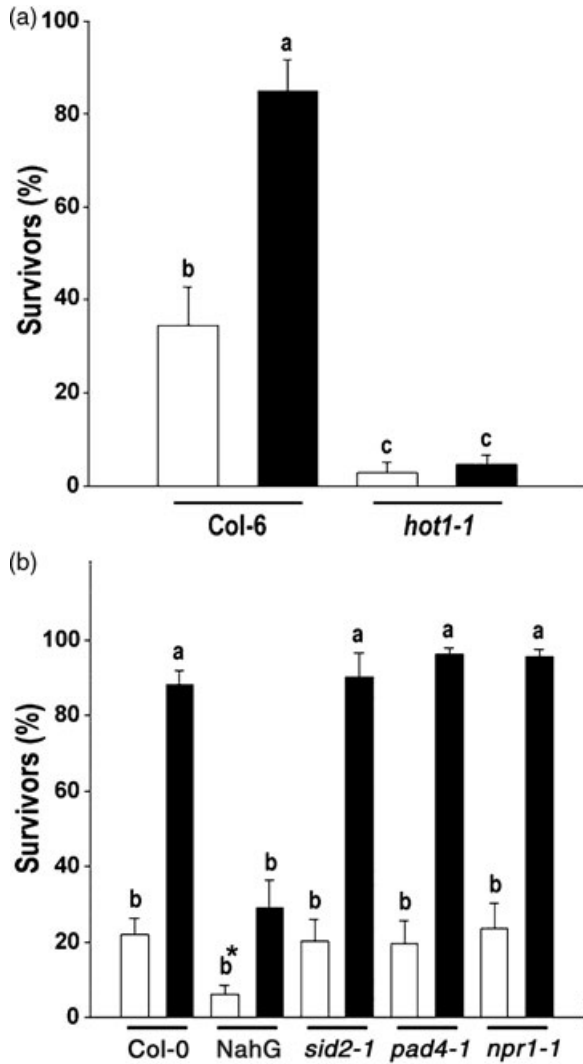


Figure 2. BABA-dependent acquired thermotolerance is compromised in *HSP101*-deficient mutant and *NahG* transgenic lines, but not in SA-deficient and SA signalling mutants.

(a) Role of HSP101. Ten-day-old Col-6 (wild-type) and *hot1-1* plants were grown at 22°C on half-strength MS plates containing either 0.5 mM BABA (closed bars) or no BABA (open bars). Heat shock was imposed by shifting plants to 38°C for 45 min, followed by a 90 min treatment at 45°C (see details in Experimental procedures). Seedlings were then returned to 22°C, and the percentage of survivors (i.e. plants with no visible necrosis on true leaves) was evaluated 4 days later. Data represent the means and SE of four independent experiments ($n > 12$ plates consisting of approximately 150 seedlings per plate). Different letters indicate statistically significant differences (two-way ANOVA followed by analysis of the combinations by the Tukey method, $P < 0.01$).

(b) Role of the SA pathway. Col-0 wild-type controls, *NahG* transgenics, and *sid2-1*, *pad4-1* and *npr1-1* mutants were grown as in (a) on half-strength MS plates containing either no (open bars) or 0.5 mM BABA (closed bars). Heat-shock treatments were as described in (a). Data represent the means and SE of four independent experiments ($n > 12$ plates consisting of approximately 150 seedlings per plate). Different letters indicate statistically significant differences (two-way ANOVA followed by analysis of the combinations by the Tukey method, $P < 0.01$). The asterisk indicates a statistically significant difference between BABA- and water-treated *NahG* plants at $0.05 > P > 0.01$ (two-way ANOVA followed by analysis of the combinations by the Tukey method).

has been implicated in basal and acquired thermotolerance (Clarke *et al.*, 2004; Larkindale *et al.*, 2005). To evaluate the role of this signal transduction pathway on BABA-enhanced acquired thermotolerance, we assessed the acquired thermotolerance of two genotypes that do not accumulate SA [i.e. the SA-biosynthesis mutant *sid2-1* (Nawrath and Metraux, 1999) and the *NahG* transgenic line (Lawton *et al.*, 1995)], and of two SA-signalling mutants [i.e. *pad4-1* (Glazebrook *et al.*, 1997) and *npr1-1* (Cao *et al.*, 1994)]. With the exception of the *NahG* transgenic line, these genotypes resembled the wild-type in their level of BABA-mediated acquired thermotolerance (Figure 2b). The discrepancy between *NahG* transgenics and *sid2* mutant responses to non-host pathogens (Lu *et al.*, 2001; Zimmerli *et al.*, 2004) has been ascribed to the production of H₂O₂ by catechol (van Wees and Glazebrook, 2003) or other SA-independent responses (Heck *et al.*, 2003). These data suggest that SA signalling does not play a critical role in BABA-induced resistance to heat stress.

BABA broadly alters gene expression

To evaluate the cellular effects of BABA, we investigated its ability to alter gene expression in Arabidopsis using a cDNA microarray representing roughly half of the Arabidopsis genome. Gene expression was monitored 24 h after BABA treatment. Seven hundred and sixty-one genes exhibited statistically significant changes of expression [analysed using Significance Analysis of Microarray (SAM) software (<http://www-stat.stanford.edu/~tibs/SAM/>); Tusher *et al.*, 2001) and had average log₂ (ratio) values with an absolute value ≥ 1 in BABA-treated plants compared to water-treated controls (Supplementary Table S1). Most of the differentially expressed genes were induced by BABA treatment (678 genes), while 83 genes were repressed. Gene ontology (GO) vocabulary was used to sort the genes in Supplementary Table S1 by molecular function (Figure 3) (Berardini *et al.*, 2004). Between the lists of up- and downregulated genes, the distribution of genes among GO categories was broadly similar for most categories (Figure 3). However, among downregulated genes, the 'structural molecule activity' and 'other molecular functions' terms were over-represented compared with the upregulated genes, while the terms 'kinase activity' and 'transcription factors' were either absent or rare (Figure 3). By contrast, GO terms such as 'transferase activity' and 'hydrolase activity' were over-represented in among the upregulated genes compared with the downregulated genes (Figure 3).

Approximately 13% (90/678) of the genes with elevated expression in BABA-treated plants were involved in protein metabolism or turnover mechanisms (Supplementary Table S2). For example, 11 transcripts with a putative myristoylation site preferentially accumulated after BABA treatment (Supplementary Table S2). Myristoylation is a post-transla-

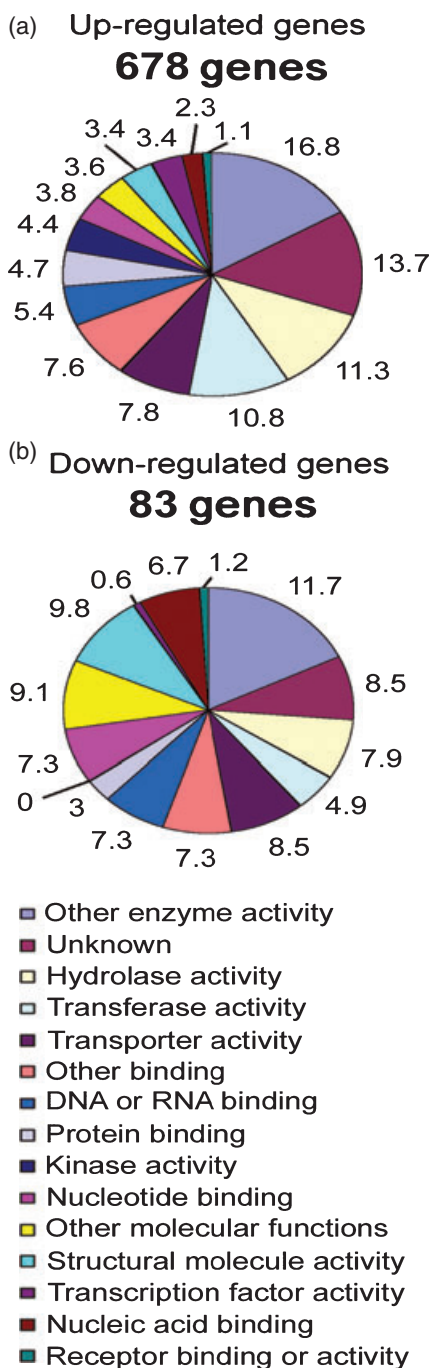


Figure 3. Molecular functions of 761 BABA-responsive genes.

Genes that were differentially expressed in BABA- and water-treated plants (false discovery rate $\leq 0.35\%$) and that had average \log_2 (BABA-treated/water-treated) values with an absolute value ≥ 1 were categorized by the GO parameter 'molecular function' (<http://www.arabidopsis.org/tools/bulk/go/index.jsp>).

(a) Distribution (as percentage) of molecular functions represented among the 678 upregulated genes.

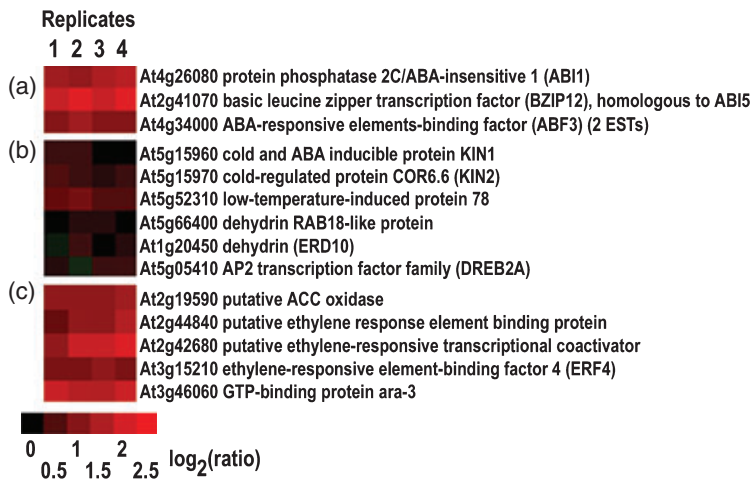
(b) Distribution (as percentage) of molecular functions represented among the 83 downregulated genes.

tional protein modification that plays a critical role in membrane targeting and signal transduction in plant responses to environmental stress (Nimchuk *et al.*, 2000). Eighteen genes encoding proteins involved in the ubiquitination pathway were upregulated by BABA (Supplementary Table S2). Ubiquitin-mediated protein degradation is an important regulatory component of many basic biological processes such as transcription, signal transduction and receptor desensitization (Hershko and Ciechanover, 1998). In addition, 18 mRNAs defined by the GO term 'proteolysis or peptidolysis' accumulated to higher levels after BABA treatment (Supplementary Table S2). A total of 43 genes coding for putative kinases or phosphatases were also significantly upregulated by BABA (Supplementary Table S2). Together, these data suggest that BABA treatment activates multiple protein degradation, modification and maturation mechanisms.

Another 12.4% (84/678) of genes with elevated expression in BABA-treated plants encode proteins that regulate transcription or signalling pathways (Supplementary Tables S3 and S4). Enhanced accumulation of transcripts involved in calcium-mediated signalling was observed (Supplementary Table S3). Small GTP-binding proteins function as molecular switches in numerous signalling processes (Vernoud *et al.*, 2003); five genes encoding such proteins were upregulated by BABA (Supplementary Table S3). The accumulation of mRNA for a putative enhancer of GTPase activity was also elevated (Supplementary Table S3, At1g08340). Sixty-one genes associated with the regulation of transcription were induced (Supplementary Table S4). This strong induction of genes involved in enzymatic reactions, regulation of protein degradation and modification, signalling cascades and transcription mechanisms suggests that BABA profoundly alters the Arabidopsis transcriptional response.

BABA activates the accumulation of transcripts involved in both stress signalling and Arabidopsis developmental responses

The expression of 46 and 30 genes, respectively, classified in the GO biological process categories 'response to stress' and 'developmental processes' was induced by BABA (Supplementary Tables S5 and S6). As ABA and ethylene modulate both plant stress, including heat stress, and developmental responses (Himmelbach *et al.*, 2003; Wang *et al.*, 2002), we surveyed the upregulated genes from Supplementary Table S1 for those associated with ABA or ethylene signalling and response. Among the ABA-signalling genes represented on the microarray slides, BABA upregulated expression of the phosphatase *ABI1* (Figure 4a, At4g26080; Supplementary Table S1, gene 310). Expression of the transcription factor *ABI3* was statistically significantly enhanced, but with a \log_2 (ratio) value of 0.95 (data not shown). To confirm and complement our microarray data,



we designed specific primers for *ABI1–5*, and analysed their expression by RT-PCR. *ABI1* and *ABI3* transcripts accumulated to significantly higher levels after BABA treatment (Supplementary Table S7). RT-PCR analyses revealed that BABA also upregulated *ABI2* and *ABI5* expression (Supplementary Table S7). In addition, the microarray analysis revealed that mRNAs for two other ABA-responsive transcription factors accumulated after BABA treatment, a homologue to *ABI5*, *EEL/DPBF4* (Figure 4a, At2g41070; Supplementary Table S1, gene 5) (Bensmihen *et al.*, 2002) and *ABF3/DPBF5* (Figure 4a, At4g34000; Table S1, gene 540) (Kang *et al.*, 2002). As components of the ABA/stress signal transduction pathway were induced by BABA, we analysed the expression of some downstream ABA-regulated genes. Of six downstream ABA-responsive genes present on the microarray (*KIN1*, At5g15960; *KIN2*, At5g15970; *RD29A/COR78*, At5g52310; *RAB18*, At5g66400; *ERD10*, At1g20450; *DREB2A*, At5g05410), only *KIN2* and *RD29A/COR78* were significantly upregulated, but only by a modest amount (Figure 4b; $\log_2(\text{ratio})$ values of 0.59 and 0.76, respectively, for *KIN2* and *RD29A/COR78*). Therefore, the BABA-mediated activation of some ABA-signalling intermediates might not be sufficient to induce a general ABA response.

BABA also significantly altered the expression level of six genes implicated in signalling by the stress hormone ethylene (Figure 4c) (Wang *et al.*, 2002). The expression values for a gene encoding the ACC oxidase 1 (At2g19590, Supplementary Table S1, gene 443), two ethylene-response factors (ERFs) (At2g44840 and At3g15210, Supplementary Table S1, genes 464 and 527, respectively), a putative ethylene-responsive transcriptional co-activator (At2g42680, Supplementary Table S1, gene 161) and a GTP-binding protein involved in ethylene signalling (At3g46060, Supplementary Table S1, gene 124) were upregulated, and one ERF (At5g61590, Supplementary Table S1, gene 739) was downregulated by BABA. ACC oxidase 1 catalyses the last step of ethylene biosynthesis, the conver-

Figure 4. Expression of ABA- and ethylene-signalling and -response genes by BABA treatment.

The $\log_2(\text{BABA-treated/water treated})$ values are colour coded as indicated by the scale shown. Data from each of the four replicates are shown. Supplementary Table S1 lists the numerical values.

- (a) Genes encoding ABA *trans*-acting factors.
(b) ABA downstream marker genes.
(c) Genes involved in the ethylene response.

sion of ACC to ethylene (Schaller and Kieber, 2002), and ERFs are either activators or repressors of the ethylene-signalling cascade (Fujimoto *et al.*, 2000). The BABA-mediated induction of ABA- and ethylene-signalling genes could potentially explain the observed upregulation of stress- and development-related genes.

BABA-induced acquired thermotolerance in ABA- and ethylene-signalling mutants

The ABA pathway is implicated in heat-stress resistance (Larkindale and Knight, 2002; Larkindale *et al.*, 2005), and BABA induced the expression of ABA-responsive signalling components, suggesting that BABA may act through ABA to confer enhanced thermotolerance (Figure 4a and Supplementary Table S7). We therefore evaluated BABA-enhanced acquired thermotolerance in two ABA-insensitive mutants, *abi1-1* (Leung *et al.*, 1994; Meyer *et al.*, 1994) and *abi2-1* (Koornneef *et al.*, 1984). As previously observed, *abi1-1* was less resistant to heat stress (Figure 5a) (Larkindale and Knight, 2002; Larkindale *et al.*, 2005). Under our standard conditions, water-treated *abi2-1* plants demonstrated a wild-type level of acquired thermotolerance (Figure 5a). Surprisingly, BABA-treated *abi2-1* plants were found to be more resistant to a prolonged heat stress than wild-type controls. If acclimatized, BABA protected about 30% of the wild-type plants and approximately 80% of the *abi2-1* plants to a treatment of 45°C for 2 h (Figure 5b). None of the water-treated wild-type or *abi2-1* mutants survived this protracted heat stress. Thus, acquired thermotolerance in *abi2-1* was more responsive to BABA than in *abi1-1*.

To test whether BABA increased acquired thermotolerance in Arabidopsis through the accumulation of ABA, we tested BABA-mediated acquired thermotolerance in *aba2-1*, a mutant that is defective in the biosynthesis of ABA (Leon-Kloosterziel *et al.*, 1996). The response of this mutant was indistinguishable from that of the wild-type (Figure 5c). ABA

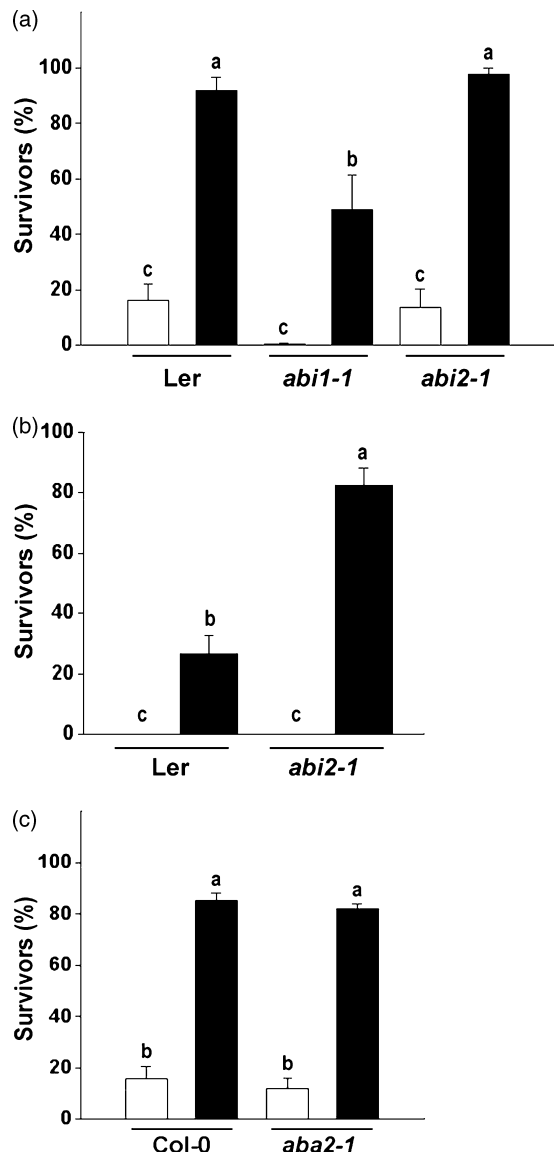


Figure 5. BABA-mediated acquired thermotolerance in ABA-insensitive and ABA biosynthesis mutants.

(a) Acquired thermotolerance to 90 min exposure to 45°C in water-treated (open bars) and BABA-treated (closed bars) *Ler* (wild-type control), *abi1-1* and *abi2-1* plants. Plants were grown for 10 days at 22°C, conditioned at 38°C for 45 min, and then exposed to 45°C for 90 min (see details in Experimental procedures). Each measurement represents the mean and SE of the percentage of survivors for three independent experiments ($n = 9$ plates consisting of approximately 150 seedlings per plate).

(b) Acquired thermotolerance to 120 min exposure to 45°C. Plants were treated as in (a), but were exposed to a heat-shock treatment at 45°C for 120 min following a conditioning treatment. Each measurement represents the mean and SE of the percentage of plants per plate that survived the heat-shock treatment for three independent experiments ($n = 9$ plates of approximately 150 seedlings per plate).

(c) Acquired thermotolerance to 90 min exposure to 45°C in water-treated (open bars) and BABA-treated (closed bars) *Col-0* (wild-type control) and *aba2-1* mutant. Plants were treated as in (a). Each measurement represents the mean and SE of the percentage of survivors on a plate for three independent experiments ($n = 12$ plates consisting of approximately 150 seedlings per plate). Different letters indicate statistically significant differences (two-way ANOVA followed by analysis of the combinations by the Tukey method, $P < 0.01$).

accumulation does not appear to play a critical role in BABA-mediated acquired thermotolerance in *Arabidopsis*.

BABA induced the upregulation of some ethylene-signaling elements (Figure 4c). We thus tested BABA-mediated acquired thermotolerance in *ein2-1*, a mutant that is insensitive to ethylene (Guzmán and Ecker, 1990). When treated with BABA, this mutant was as resistant as wild-type controls to heat stress (Supplementary Figure S2a). This suggests that ethylene perception is not required for BABA-induced resistance to heat stress in *Arabidopsis*.

BABA alters *Arabidopsis* root growth

To assess whether BABA mimics ABA in other ABA-sensitive processes, we determined whether BABA was also able to inhibit root growth (Ton and Mauch-Mani, 2004). Quantification of *Arabidopsis* root growth revealed that 0.25 mM BABA restricted root growth by about 30% when compared to non-treated *Col-0*. In this accession, the level of inhibition was similar to the effect of 3 μ M ABA (Figure 6). The *Ler* accession was similarly sensitive to BABA, but was more sensitive to ABA inhibition of root growth (Figure 6). ABA and BABA together had a partially additive effect on wild-type *Col-0* and *Ler*, roots suggesting that BABA acts independently of ABA to inhibit root growth (Figure 6). Although ABA inhibits seed germination (Finkelstein and Somerville, 1990), BABA did not mimic this ABA-mediated phenotype (Supplementary Figure S3). This observation shows that BABA phenocopies only some of the ABA effects on *Arabidopsis* physiology and development.

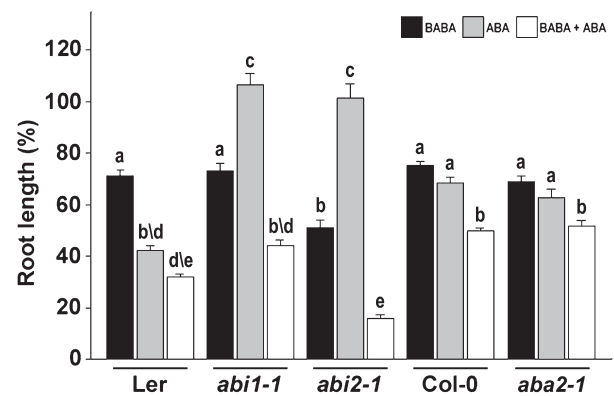


Figure 6. BABA- and ABA-mediated inhibition of root growth in ABA-insensitive and ABA biosynthesis-deficient mutants.

Five-day-old plants were transferred to fresh media containing 3 μ M ABA, 0.25 mM BABA or both chemicals. New root growth was evaluated 5 days later, and is expressed as the mean and SE of the percentage of growth on the control medium (no ABA or BABA). Each measurement is the mean of three independent experiments each consisting of at least ten replicates. Different letters indicate statistically significant differences (two-way ANOVA followed by analysis of the combinations by the Tukey method, $P < 0.01$). The mutants insensitive to ABA (*abi1-1* and *abi2-1*) are in the *Ler* background. The ABA biosynthesis-deficient *aba2-1* mutant is in the *Col-0* background.

To further investigate the possible relationship between ABA and BABA signalling, we analysed the root-growth response of *abi1-1*, *abi2-1* and *aba2-1*. As expected, the roots of ABA-insensitive mutants were insensitive to ABA treatment (Figure 6). Although BABA-treated *abi1-1* and wild-type roots were inhibited to a similar extent, *abi2-1* roots were hypersensitive to BABA, displaying a 50% reduction in root growth (Figure 6). In these two ABA-insensitive mutants, BABA seemed to act synergistically with ABA, inhibiting root growth substantially more than BABA treatment alone (Figure 6). BABA-mediated root-growth inhibition in the *aba2-1* mutant was similar to that in wild-type Col-0 (Figure 6). Thus, BABA-mediated root-growth inhibition appears to be independent of ABA accumulation.

The mutant *ein2-1* was used to test the role of ethylene signalling in the BABA-mediated inhibition of root growth. A block in ethylene signalling did not alter BABA inhibition of root growth (Supplementary Figure S2b). As for heat-shock resistance, ethylene signalling may not be critical for the inhibition of root growth by BABA.

Discussion

In this study, we show that BABA treatment enhances resistance to heat stress via its effect on acquired thermotolerance. No statistically significant enhancement of basal thermotolerance was observed under the heat-shock treatments used in these experiments. This requirement for thermo-acclimatization distinguishes BABA-induced resistance to heat stress from BABA-induced resistance to pathogens, salt and drought stresses, where no conditioning pre-treatment is required (Jakab *et al.*, 2005; Ton and Mauch-Mani, 2004; Ton *et al.*, 2005; Zimmerli *et al.*, 2000, 2001). Heat shock provokes a rapid and intense stress that may overwhelm BABA-induced resistance, which could explain why BABA treatment did not significantly enhance basal thermotolerance. Under less severe heat-shock treatments, it may be possible to observe an effect of BABA on basal thermotolerance.

NahG transgenics, and, to a lesser extent, the *npr1-1* mutant, are either defective solely in basal thermotolerance (Clarke *et al.*, 2004) or in both basal and acquired thermotolerance (Larkindale *et al.*, 2005), depending on the type of heat-shock assay employed. These observations implicate SA signalling in heat-stress resistance. Our data confirmed that *NahG* plants were compromised, although only partially, in their ability to develop acquired thermotolerance. However, in our experimental system, *npr1-1* exhibited wild-type levels of acquired thermotolerance. The *npr1-1* phenotype is subtle at the seedling stage (Larkindale *et al.*, 2005), and our bioassay may lack the sensitivity required to detect a small alteration in thermotolerance. Under our conditions, other mutants defective in SA signalling, and the SA biosynthesis mutant *sid2-1* had wild-type levels of acquired

thermotolerance. Together, these data suggest that SA signalling plays at best a minor role in acquired thermotolerance. Except *NahG* transgenics, all SA signalling and SA biosynthesis mutants tested demonstrated wild-type levels of BABA-mediated Arabidopsis thermotolerance. As for acquired thermotolerance, SA signalling may not be critical during BABA-induced resistance to heat stress.

Confirming published data (Hong and Vierling, 2000; Larkindale and Knight, 2002; Larkindale *et al.*, 2005), water-treated *hot1-1* and to a lesser extent *abi1-1* mutants were unable to mount an effective response to heat stress (Figures 2a and 5a). The loss or reduction of BABA-enhanced acquired thermotolerance observed in these mutants could reflect their intrinsic low acquired thermotolerance levels. Alternatively, *HSP101* or *ABI1* may represent elements involved in BABA-induced resistance to heat stress. In our standard heat-shock bioassay, water-treated *abi2-1* demonstrated a wild-type level of acquired thermotolerance. In contrast to our results, Larkindale *et al.* (2005) showed that *abi2-1* was, like *abi1-1*, more sensitive to heat stress than wild-type. Plant developmental stage plays an important role in heat-shock resistance (Larkindale *et al.*, 2005), and the difference in the age of plants exposed to heat stress [7 days in the study by Larkindale *et al.* (2005) versus 10 days in this study] may account for the difference in results. A second difference in protocols is that Larkindale *et al.* (2005) allowed the plants to recover at 22°C for 120 min between the conditioning treatment and the 45°C heat-stress treatment, while the plants in our study were moved immediately to the 45°C heat-stress treatment. This recovery period could be specifically deleterious to *abi2-1* mutants.

Defence-responsive genes are induced earlier in BABA-treated plants exposed to biotic and abiotic stresses (Jakab *et al.*, 2005; Ton *et al.*, 2005; Zimmerli *et al.*, 2000, 2001). A functional *HSP101* was necessary for BABA-induced resistance to heat stress; however, priming of *HSP101* expression was not observed in BABA-treated Arabidopsis. We cannot exclude the possibility that BABA may act post-transcriptionally to regulate *HSP101*. Alternatively, a variety of defence mechanisms have been implicated in adaptive responses to heat stress (Larkindale *et al.*, 2005), and BABA may prime some of these heat-stress repair mechanisms, even though the expression of *HSP101* is not primed. As discussed below, incomplete activation of the ABA response pathway may be a component of BABA-induced priming of heat-stress responses.

The hormones ABA and ethylene participate in signalling cascades regulating both development and responses to stress (Himmelbach *et al.*, 2003; Koornneef *et al.*, 2002; Wang *et al.*, 2002). Genes coding for ABA- and ethylene-signalling components were upregulated by BABA treatment, although only ABA appeared to affect BABA-induced resistance to heat stress. Like BABA, ABA treatment induced the accumulation of numerous transcripts involved in the

regulation of transcription, and regulated proteolysis and protein de/phosphorylation (Hoth *et al.*, 2002). However, two previous ABA expression profiles (Hoth *et al.*, 2002; Sanchez *et al.*, 2004) showed no substantial overlap with the genes upregulated by BABA in our study (Supplementary Table S1). BABA and ABA may therefore upregulate different sets of genes encoding proteins with similar functions.

ABF3 was one of the few genes that was both upregulated by BABA and is known to be involved in ABA signalling (Kang *et al.*, 2002). This gene encodes a transcription factor that binds to ABA-responsive elements (Kang *et al.*, 2002). Constitutive over-expression of *ABF3* in Arabidopsis results in ABA-mediated enhanced drought tolerance and altered expression of ABA/stress-regulated genes such as *ABI1* and *RAB18* (Kang *et al.*, 2002). BABA treatment elicited a subset of these responses in Arabidopsis. In BABA-treated plants, the induction of downstream ABA marker genes was not observed (Figure 4b) (Ton and Mauch-Mani, 2004); however, *ABI1* gene expression was elevated and drought tolerance was enhanced (Figure 4a and Supplementary Table S7) (Jakab *et al.*, 2005). The relatively low level of *ABF3* induction (\log_2 ratio = 1.22) could account for the limited induction of typical ABA responses in BABA-treated plants. In support of this idea, a transgenic line expressing *ABF3* at a low level displayed no significant upregulation of *RAB18* (Kang *et al.*, 2002). In addition to *ABF3*, mRNAs of other ABA transcription factors such as *ABI3*, *ABI5* and *EEL/DPBF4* accumulated to higher levels after BABA treatment, as did mRNAs encoding the phosphatases *ABI1* and *ABI2*. The BABA-mediated upregulation of such ABA-signalling intermediates could lead to faster ABA signalling upon stress perception. Supporting this hypothesis, constitutive over-expression of *ABRE1* (i.e. *ABF2*), another ABA-responsive transcription factor, does not directly lead to over-expression of *RD29B*, a downstream marker gene, in the absence of ABA treatment (Fujita *et al.*, 2006). However, upon ABA treatment, *ABRE1*-over-expressing transgenics do accumulate more *RD29B* mRNA than wild-type plants (Fujita *et al.*, 2006). Similarly, ectopic expression of *ABI3* enhances Arabidopsis freezing tolerance by accelerating the plant acclimatization response to cold stress (Tamminen *et al.*, 2001). Typically, downstream defence responsive genes such as *RAB18* were not constitutively upregulated in *ABI3*-over-expressing plants, but their expression levels upon stress perception were enhanced (Tamminen *et al.*, 2001). Accumulation of ABA transcription and signalling factors in BABA-treated Arabidopsis might thus prime Arabidopsis for ABA accumulation and ABA-dependent defence mechanisms during salt and drought stress (Jakab *et al.*, 2005), and ABA-dependent callose deposition during pathogen attack (Kaliff *et al.*, 2007; Ton and Mauch-Mani, 2004). This pattern of activation of early ABA-signalling steps, without induction of late ABA responses, may represent a key feature of BABA-primed Arabidopsis.

ABA also regulates plant developmental processes, such as root growth and germination (Koorneef *et al.*, 2002). Like ABA, BABA was found to inhibit Arabidopsis root growth. As inhibition of root development by both chemicals was additive, and the roots of ABA-signalling mutants were sensitive to BABA, we conclude that BABA and ABA act through separate signalling cascades to restrict root growth. However, the synergistic inhibition of root growth by BABA and ABA in the ABA-insensitive mutants, *abi1-1* and *abi2-1*, suggests that some cross-talk occurs between these two signalling pathways (Figure 6). Ectopic expression of *ABI3* in an *abi1-1* mutant background partially reverses the insensitivity of *abi1-1* to ABA-mediated stomatal regulation and root-growth inhibition (Parcy and Giraudat, 1997). Similarly, the BABA-mediated upregulation of ABA transcription factors might account for the restored ABA root-growth sensitivity of BABA-treated *abi1-1* and *abi2-1* mutants. The fact that BABA-induced resistance to heat stress was diminished in *abi1-1* but enhanced in *abi2-1* mutants also indicates the complexity of the interconnections between BABA and ABA effects on Arabidopsis.

BABA is a potent enhancer of defences against pathogens, and increases tolerance to salt, drought and heat stress (Jakab *et al.*, 2005; Ton and Mauch-Mani, 2004; Ton *et al.*, 2005; Zimmerli *et al.*, 2000, 2001). BABA-induced resistance is believed to act by potentiating or priming defence responses (Jakab *et al.*, 2001, 2005; Ton and Mauch-Mani, 2004; Ton *et al.*, 2005; Zimmerli *et al.*, 2000, 2001). The absence of a measurable effect of BABA on basal thermotolerance, as well as the absence of an effect by α - and γ -aminobutyric acid, supports the idea that BABA does not act directly as a compatible osmolyte. Although the direct target or mode of action of BABA in plants is unknown at this time, its ability to prime endogenous defences against a range of stresses suggests that responses to these stresses share a common underlying biochemical, physiological or signalling process that is targeted by BABA.

van Hulten *et al.* (2006) have demonstrated improved seed yields and relative plant growth rates following pathogen attack in plants primed to respond defensively by BABA treatment compared with plants in which defence responses were directly activated. These results highlight the reduction of plant fitness associated with the constitutive activation of defences, and suggest that priming provides a mechanism for plants to balance the fitness costs of defence with the costs of an imposed stress. Thus, a better understanding of the molecular basis of BABA-dependent priming in Arabidopsis is of major interest. This study suggests that BABA alters Arabidopsis stress responses through the manipulation of key ABA-signalling elements without activating a full ABA response. Notably, BABA treatments do not induce ABA accumulation (Jakab *et al.*, 2005). Such a partial activation of ABA signalling may represent one aspect of the primed state, and hence a common compo-

ment of defence responses associated with BABA-induced resistance to multiple stresses.

Experimental procedures

Plant materials, growth conditions and chemical treatments

Arabidopsis thaliana (L. Heynh.) wild-type Columbia (Col-0), Columbia *glabrous1* (Col-6) and Landsberg *erecta* (Ler) were used in this study. We also used the following mutants and transgenic plants: *npr1-1* (Cao *et al.*, 1994), *sid2-1* (Nawrath and Metraux, 1999), *pad4-1* (Glazebrook *et al.*, 1997), *NahG* (Lawton *et al.*, 1995), *ein2-1* (ABRC ID CS3071; Guzmán and Ecker, 1990), *aba2-1* (ABRC ID CS156; Leon-Kloosterziel *et al.*, 1996), *abi1-1* (Ler) (Koornneef *et al.*, 1984; Leung *et al.*, 1994; Meyer *et al.*, 1994), *abi2-1* (Ler) (NASC ID CS23; Koornneef *et al.*, 1984), *hot1-1* (Col-6) (Hong and Vierling, 2000) (all Col-0 background except as noted).

Ten-day-old plants were grown aseptically on plates containing inorganic MS salts (Murashige and Skoog, 1962) at half concentration (Sigma, <http://www.sigmaaldrich.com/>), 3 mM MES (Sigma) and supplemented with the indicated chemicals. pH was adjusted to 5.7 with 5 M KOH before autoclaving. The medium was solidified with 0.6% Phytoagar (Sigma) for heat-shock and germination assays, or 1% Phytoagar for vertical root-growth experiments. The plants were grown at 22°C in continuous light prior to heat treatments and root-growth assays. For the microarray experiments, Col-0 plants were grown in ProMix HP (Premier Horticulture; <http://www.premierhort.com>) for 14 days, and the soil was drenched with a 0.25 mM BABA solution 1 day prior to harvesting samples for RNA preparation. These plants were grown in a growth chamber at 21°C with a 14 h photoperiod. All plants were grown at a light intensity of about 100–150 $\mu\text{E m}^{-2} \text{sec}^{-1}$. α -, β - and γ -aminobutyric acids (mixed isomers, Sigma) were diluted in water at the indicated concentrations. ABA (mixed isomers) was diluted from a 10 mM stock solution prepared in methanol, and equivalent methanol volumes were added in the ABA-free controls.

Heat-shock treatments

Seeds were surface-sterilized in 70% ethanol for 4 min, followed by 6 min in 1.5% w/v hypochlorite and 0.02% w/v Triton X-100, then rinsed five times in sterile water before plating. One to two hundred surface-disinfested seeds were plated in rows on half-strength MS plates (30 ml per plate) (9 cm diameter, VWR International; <http://www.vwr.com>). Plants were grown as described above for 10 days. For basal thermotolerance assays, plates were moved directly from 22 to 45°C and held at 45°C for the indicated period of time. Acquired thermotolerance was evaluated by moving the plates from 22 to 38°C for 45 min. The growth chamber containing the plants was then allowed to heat up over 10 min to 45°C. The plants were kept at 45°C for the remaining time (i.e. 80 min for a treatment of 90 min). All heat-shock treatments were performed in the dark. After heat stress, the plants were returned to 22°C in continuous light, and evaluation of viability was assessed 4 days after the heat-shock treatment. Seedlings were also photographed after 4 days. Plants were considered as survivors if no necroses were visible on true leaves when observed at 100 × magnification with a stereo microscope.

Root-growth assay

Root-growth sensitivity to ABA and/or BABA was evaluated by transferring 5-day-old seedlings on plates containing half-strength

MS medium to plates containing the indicated concentrations of ABA and/or BABA. Plates were inclined at 85° during the growing process. Primary root length was measured after 5 days. Results are expressed as the percentage of growth observed in control untreated plants.

Germination assay

Col-0 seeds were surface-sterilized as described above, and plated on half-strength MS medium containing the indicated concentrations of ABA and/or BABA. The plates were incubated for 4 days at 4°C in the dark. Seeds were then placed in continuous light at 22°C for the indicated periods of time. The number of germinated seeds (with fully emerged radicle tip) was expressed as a percentage of the total number of seeds plated.

RNA isolation and microarray preparation

RNA isolation and microarray preparation were performed as described by Zimmerli *et al.* (2004). RNA samples from four independent biological replicates of BABA-treated and water-treated plants were prepared. The Y2001 AFGC DNA microarray contains 11 500 cDNA clones (Newman *et al.*, 1994; White *et al.*, 2000) and 3000 gene-specific amplicons. The Arabidopsis Functional Genomics Consortium web site (http://arabidopsis.org/info/2010_projects/comp_proj/AFGC/index.html) (Wu *et al.*, 2001) provides additional information about Y2001 AFGC microarrays.

Microarray data analysis

The scanning of microarray slides and the spot intensity quantifications were performed as described previously (Ramonell *et al.*, 2002). After spots flagged as bad had been removed from the data sets, we used the default normalization provided by the Stanford microarray to normalize the Cy3 (channel 1) and Cy5 (channel 2) intensities for each spot (Gollub *et al.*, 2003). We removed data points with net (Cy3) or normalized net (Cy5) spot intensities ≤ 350 . A one-class analysis was performed using the Significance Analysis of Microarrays program (SAM; <http://www-stat.stanford.edu/~tibs/SAM>) to identify genes that were differentially expressed between water- (control) and BABA-treated plants (Tusher *et al.*, 2001). The SAM parameters were $\Delta = 1.92$, with a false discovery rate of 0.35%. From 2432 genes selected by this analysis, a subset of 761 genes with an absolute average \log_2 (ratio) values ≥ 1 were selected for further analysis. The final list is presented in Supplementary Table S1. Known and putative functions, putative protein localizations and gene ontologies for selected genes are given in Supplementary Tables S2, S3, S4, S5 and S6 as retrieved from the Arabidopsis Information Resource (TAIR) (<http://www.arabidopsis.org>).

RT-PCR analysis

Total RNA isolated as described above for microarray data analysis was used for cDNA synthesis by Superscript III RNase H reverse transcriptase (Invitrogen, <http://www.invitrogen.com/>) according to the manufacturer's instructions. Gene-specific primers were designed to amplify 470, 226, 176, 207 and 252 bp fragments of *ABI1*, *ABI2*, *ABI3*, *ABI4* and *ABI5*, respectively. These primers spanned introns to differentiate products amplified from cDNA from any product amplified from contaminating genomic DNA. A 630 bp fragment of *ACTIN2* was also reverse-transcribed in the same reaction, and amplified separately.

Polymerase chain reaction cycle number and template amounts were optimized for all fragments amplified to yield products in the linear range. Primer sequences were as follows: *ABI1*, forward 5'-GATGCTCTGCGATGGTGATAC-3' and reverse 5'-CATCTCACACGTTCTTCATC-3'; *ABI2*, forward 5'-TGGAGTGACTTCGATTGTGG-3' and reverse 5'-TATCTCCTCCGTCAAAGCCAG-3'; *ABI3*, forward 5'-GGAACATGCGCTACAGTTT-3' and reverse 5'-TCCGCTCGGTTGTCTTACTT-3'; *ABI4*, forward 5'-GATGGGACAATTCACACC-3' and reverse 5'-CCACCGAACAGCTAGAGAG-3'; *ABI5*, forward 5'-AGTTCAGGCAGGTGTTGCT-3' and reverse 5'-CTCGGGTTCCTCATCAATGT-3'; *ACTIN2*, forward 5'-GTTGGTGATGAAGCACAATCCA-3' and reverse 5'-CTGGAACAAGACTTCTGGGCATCT-3'. PCR conditions used for comparison of transcription levels were 50 ng of template cDNA denatured at 94°C for 2 min, followed by various numbers of cycles of 94°C for 20 sec, 62°C for 30 sec and 72°C for 60 sec. PCR cycle numbers were as indicated in Supplementary Table S7. Reactions were followed by a 10 min incubation at 72°C, before separation of PCR products by electrophoresis in a 1.2% w/v agarose/Tris-acetate EDTA (TAE) gel. All fragments were sequenced on an ABI Prism 310 Sequencer (Applied Biosystems, <http://www.appliedbiosystems.com/>) according to the manufacturer's instructions for labelling and sequencing. PCR products were visualized by ethidium bromide staining of the gel, and were quantified using MACBASE 2.0 software (Fujifilm; <http://www.fujifilm.com>) after imaging using GelDoc 2000 (Bio-Rad, <http://www.bio-rad.com/>).

Quantification of HSP101 expression

HSP101 expression was quantified by RT-PCR as described above. Gene-specific primers (forward 5'-TCGTTACATAACTGGTCGGCATT-3' and reverse 5'-GGTCATCAAGCTCTTCCGCACC-3') were designed to amplify a 218 bp fragment. At each time point, 25 and 30 PCR cycles were performed at an annealing temperature of 56°C. The plants were selected for RNA extraction at 0, 12.5, 15, 17.5 and 20 min after shifting from 22 to 38°C.

Accession numbers

Microarray data are publicly available via the Stanford microarray database (<http://genome-www5.stanford.edu/>) under experiment IDs 33704, 33705, 33706 and 33707 (BABA 1–4) (Gollub *et al.*, 2003). These microarray data have also been deposited in Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE9515 (Barrett *et al.*, 2005).

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Defective BABA-mediated acquired thermotolerance in the *hot1-1* mutant exposed to a mild heat-shock treatment.

Figure S2. Responses of the ethylene-insensitive *ein2-1* mutant to BABA treatment.

Figure S3. BABA does not inhibit seed germination.

Table S1. log₂ (BABA-treated/water-treated) values for clones selected by SAM analysis and with an average absolute value ≥ 1. Columns BABA 1 to BABA 4 represent four independent biological replicates.

Table S2. BABA upregulated genes from Supplementary Table S1 encoding proteins putatively involved in protein modification or catabolism.

Table S3. BABA upregulated genes from Supplementary Table S1 encoding proteins putatively involved in signal transduction.

Table S4. BABA upregulated genes from Supplementary Table S1 encoding proteins putatively involved in transcriptional regulation.

Table S5. BABA upregulated genes from Supplementary Table S1 encoding proteins putatively involved in stress responses.

Table S6. BABA upregulated genes from Supplementary Table S1 encoding proteins putatively involved in developmental processes.

Table S7. Expression of *ABI* genes in BABA-treated and water-treated plants.

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