Article

Transcription factor BES1 interacts with HSFA1 to promote heat stress resistance of plants

Pablo Albertos[1], Gönül Dündar[1], Philipp Schenk[1], Sergio Carrera[1], Philipp Cavelius[1], Tobias Sieberer[2] & Brigitte Poppenberger[1,*]

Abstract

Heat stress is a major environmental stress type that can limit plant growth and development. To survive sudden temperature increases, plants utilize the heat shock response, an ancient signaling pathway. Initial results had suggested a role for brassinosteroids (BRs) in this response. Brassinosteroids are growth-promoting steroid hormones whose activity is mediated by transcription factors of the BES1/BZR1 subfamily. Here, we provide evidence that BES1 can contribute to heat stress signaling. In response to heat, BES1 is activated even in the absence of BRs and directly binds to heat shock elements (HSEs), known binding sites of heat shock transcription factors (HSFs). HSFs of the HSFA1 type can interact with BES1 and facilitate its activity in HSE binding. These findings lead us to propose an extended model of the heat stress response in plants, in which the recruitment of BES1 is a means of heat stress signaling cross-talk with a central growth regulatory pathway.

Keywords brassinosteroids; heat shock factors; hormone; HSFA1; steroid
Subject Categories Chromatin, Transcription & Genomics; Plant Biology
DOI 10.15252/embj.2021108664 | Received 6 May 2021 | Revised 5 December 2021 | Accepted 8 December 2021 | Published online 4 January 2022
The EMBO Journal (2022) 41: e108664

Introduction

Plants strongly adapt to their temperature environment, and while warmth can accelerate growth (Quint *et al*, 2016; Vu *et al*, 2019), heat stress restricts vegetative and reproductive development (Desaint *et al*, 2021). This is a major threat to yield stability and, as a result of global warming, has increasing relevance for crop production (Zhao *et al*, 2017).

Whereas severe heat stress leads to cellular damage and cell death, sublethal doses induce the heat shock response, an ancient signaling pathway that organisms employ to protect their cells and allow resumption of normal cellular and physiological activities. The heat shock response induces the expression of heat shock-controlled genes through regulatory motifs called heat shock elements (HSEs), which are bound by the heat shock factors (HSFs), transcription factors (TFs) that are present in multiple copies in plants and vertebrates (Richter *et al*, 2010; Jacob *et al*, 2017). In addition, heat, like other abiotic stress types, represses plant growth, and while our understanding of these effects is still very limited, it is clear that the coordination of growth and stress responses in plants is governed by hormones including the brassinosteroids (BRs) (Bechtold & Field, 2018).

BRs are steroid hormones that stimulate cell elongation and participate in a multitude of physiological processes (Fridman & Savaldi-Goldstein, 2013; Nolan *et al*, 2020). Moreover, BRs control adaptive morphological changes, such as the warmth-induced promotion of growth (Quint *et al*, 2016), and can protect plants from abiotic stress including heat stress. When applied externally, or when hyperaccumulated in plants, BRs reduce heat-induced damage. This involves an impact on the heat-responsive transcriptome and a promotion of the synthesis of heat shock proteins (HSPs), chaperons with protective function during heat stress and other types of environmental insult (Dhaubhadel *et al*, 2002; Mazorra *et al*, 2011; Samakovli *et al*, 2014; Shigeta *et al*, 2015; Divi *et al*, 2016).

BRs are perceived by the receptor-like kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1) and signal in a phosphorylation-dependent mode to alter the phosphorylation state of TFs and thereby regulate their protein stability, nuclear localization, and DNA binding capacities (Kim & Wang, 2010; Belkhadir & Jaillais, 2015). BR-controlled TFs include basic helix-loop-helix (bHLH) proteins such as CESTA (CES), the BR ENHANCED EXPRESSION (BEE) 1–3 and BES1-INTERACTING MYC-LIKEs (BIM) proteins, PHYTOCHROME INTERACTING FACTOR 4 (PIF4) (Friedrichsen *et al*, 2002; Yin *et al*, 2005; Poppenberger *et al*, 2011; Bernardo-Garcia *et al*, 2014), and also a small subfamily of six proteins with bHLH-like features, the most-studied being BRI1 EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE RESISTANT 1 (BZR1). BR signaling controls the phosphorylation state of BES1 and BZR1 through activities of GSK3/shaggy-like kinases such as BR-INSENSITIVE 2 (BIN2) and phosphatases such as BRI1 SUPPRESSOR 1 (BSU1). An activation of BR signaling results in a hyper-accumulation of BES1 and BZR1 and enriches non-phosphorylated forms, which are active (He *et al*, 2002; Wang *et al*, 2002; Yin *et al*, 2002; Mora-Garcia *et al*, 2004).

BES1 and BZR1 functions are further modulated by heterodimerization with bHLH proteins, which can be antagonistic or synergistic

1 Biotechnology of Horticultural Crops, TUM School of Life Sciences, Technical University of Munich, Freising, Germany
2 Plant Growth Regulation, TUM School of Life Sciences, Technical University of Munich, Freising, Germany
*Corresponding author. Tel: +49 8161 712401; E-mail: brigitte.poppenberger@tum.de

© 2021 The Authors. Published under the terms of the CC BY NC ND 4.0 license

depending on the nature of the target promoter and the type of regulatory motifs present (Yin *et al*, 2002, 2005; Bai *et al*, 2012; Oh *et al*, 2012; Martinez *et al*, 2018). BES1/BZR1 bind the so-called BR response element (BRRE; 5′-CGTG(T/C)G-3′), but also have affinity for E-boxes (5′-CAnnTG-3′), G-boxes (5′-CACGTG-3′), and a certain non-E-box element (5′-AA(T/A)CAAnnnC(C/T)T) (He *et al*, 2005; Yin *et al*, 2005; Sun *et al*, 2010; Yu *et al*, 2011; Unterholzner *et al*, 2015). This enables these TFs to promote growth and facilitate developmental processes, but also repress BR biosynthetic genes for feedback adjustment of BR homeostasis (Wang *et al*, 2002; Yin *et al*, 2002).

Here, we present an alternative mode of BES1 activity, which allows BES1 to be recruited to the heat shock response pathway. We show that in *Arabidopsis thaliana* BES1 is rapidly de-phosphorylated and activated by heat stress and that this can occur even in the absence of canonical BR signaling and is mediated, at least in part, by abscisic acid (ABA)-repressed PP2C-type phosphatases. Evidence is provided that BES1 induces the expression of HSPs of the HSP70 and HSP90 type through direct binding to HSEs, an activity that is facilitated by HSFs of the HSFA1 subfamily. Based on these results, an expanded model for heat shock responses is proposed, where BES1 cooperates with HSFA1s for stress protection, facilitating plant survival when heat stress occurs.

Results

BES1 is de-phosphorylated and activated in response to heat

Using transcriptome analyses, BRs were previously shown to induce heat stress-responsive genes. Since BES1/BZR1-binding sites were enriched in the promoters of subclasses of these genes, it was proposed that they may contribute to the control of the heat-responsive transcriptome (Divi *et al*, 2016). To investigate whether heat impacts BES1/BZR1 activities, we assessed the phosphorylation state of BES1 following heat treatment. 14-day-old, tissue-cultured plants overexpressing BES1-CFP (*35S:BES1-CFP*) (Rozhon *et al*, 2010) were shifted from 21°C directly to different high temperatures and incubated for 60 min, before BES1-CFP was immuno-detected. At normal ambient temperatures of 21 or 25°C, the majority of BES1 was present in a phosphorylated (inactive) form. However, when plants were transferred to a temperature of 30°C or higher, BES1 levels strongly increased and non-phosphorylated (active) forms appeared. After an exposure to 45 or 50°C, almost all BES1 was non-phosphorylated (Fig 1A). BZR1 showed a similar response (Appendix Fig S1).

For further analyses, we focused on BES1 and continued with 45°C, as a temperature highly effective for BES1 de-phosphorylation. We found that BES1 was maintained in a non-phosphorylated state during prolonged heat stress (Fig 1B). However, when plants were shifted back to 21°C, the majority of BES1 reverted to phosphorylated forms after 180 min (Fig 1C). In accordance with the model that de-phosphorylation of BES1 induces its nuclear acquisition (Yin *et al*, 2002), the heat treatment resulted in a nuclear enrichment of BES1-CFP (Fig 1D).

To test whether heat stress induces BES1 transcriptional activity, expression levels of the BR biosynthesis genes *ROT3*, *DWF4*, and *BR6ox2*, which are repressed by BES1 (Yu *et al*, 2011; Martinez *et al*, 2018), were quantified. This showed that a 60-min treatment with 45°C significantly decreased the transcript abundance of all three genes. In line with BES1 returning to phosphorylated, inactive forms, this repression was released after 180 min of recovery at 21°C (Fig 1E). Interestingly, after stress recovery *DFW4* and *BR6ox2* expression levels were even higher than before the heat treatment, indicating that BR biosynthesis is stimulated when heat stress subsides.

BES1 de-phosphorylation by heat does not require BRs

Since there was evidence that BES1 is activated by heat, we tested if BES1 impacts the heat stress resistance of plants. For this purpose, we used the *bes1* T-DNA insertional mutants *bes1-1* and *bes1-2* (Lachowiec *et al*, 2013; Saito *et al*, 2018; Chen *et al*, 2019) and the dominant *bes1-D* mutant, in which BES1 is stabilized and constitutively active (Yin *et al*, 2002); all mutants are in the Columbia-0 (Col-0) background (Ibanes *et al*, 2009). To assess basal heat stress tolerance, 14-day-old, tissue-cultured wild-type and *bes1*-mutant plants were directly exposed to 45°C for the indicated periods of time using a light incubator and survival rates were assessed after 10 days of recovery at 21°C. It should be noted that this setup, in which the light environment is maintained, may yield some heat acclimation, since the temperature increase is slower than if a water bath is used for heat treatments (Yeh *et al*, 2012).

The results showed that the knock-outs *bes1-1* and *bes1-2* had a significantly decreased, whereas *bes1-D* had a significantly increased heat stress tolerance as compared to wild type (Fig 1F). In addition, also acquired heat stress tolerance was tested by priming plants with 30-35°C for 120 min and then exposing them to 45°C for 150 min, a condition where, without the priming treatments, 100% of wild type died. The results provided further support that heat stress tolerance of *bes1-1* and *bes1-2* is decreased and of *bes1-D* is increased. The degree of the phenotypes was correlated with the degree of BES1 activity changes. *bes1-1*, a weak knock-out allele (Chen *et al*, 2019) showed a weaker phenotype than *bes1-2*, which is a complete knock-out (Lachowiec *et al*, 2013). *bes1-D*, in which non-phosphorylated BES1 hyper-accumulates (Yin *et al*, 2002), showed the clearest changes to wild type, and we therefore used this line, for further analyses of BES1 modes of activity in heat stress resistance.

To investigate whether BR perception by BRI1 is required for BES1-conferred heat stress tolerance, we introduced the *bri1-1* mutant, a *bri1* null allele (Li & Chory, 1997), into the same phenotyping setup. However, *bri1-1* plants did not show an altered heat stress sensitivity (Fig 2A), indicating that BR perception may not be required for BES1 activity in heat stress responses. To verify this, we analyzed BES1 phosphorylation states following 45°C exposure in plants treated with 24-epi-brassinolide (epiBL), a bioactive BR analog, or the BR biosynthesis inhibitor Brassinazole (BRZ) (Asami *et al*, 2000). This showed that, as anticipated, epiBL application induced BES1 de-phosphorylation at 21°C and that this was further promoted by heat treatment. More importantly, even in the presence of 5 μM BRZ, which severely compromises BR biosynthesis (Asami *et al*, 2000), BES1 de-phosphorylation by heat was still effective (Fig 2B).

For genetic verification of this result, the *35S:BES1-CFP* reporter was introduced into *bri1-1* and the BR biosynthetic mutant *det2-1* (Chory *et al*, 1991) by crossing. In accordance with the expectation, BES1-CFP was resistant to epiBL treatment in the *bri1-1* and more responsive in the *det2-1* background, with all BES1-CFP being present in de-phosphorylated forms in the latter experimental setting (Fig 2C and D). Also as expected, BES1 phosphorylation was

 © 2021 The Authors

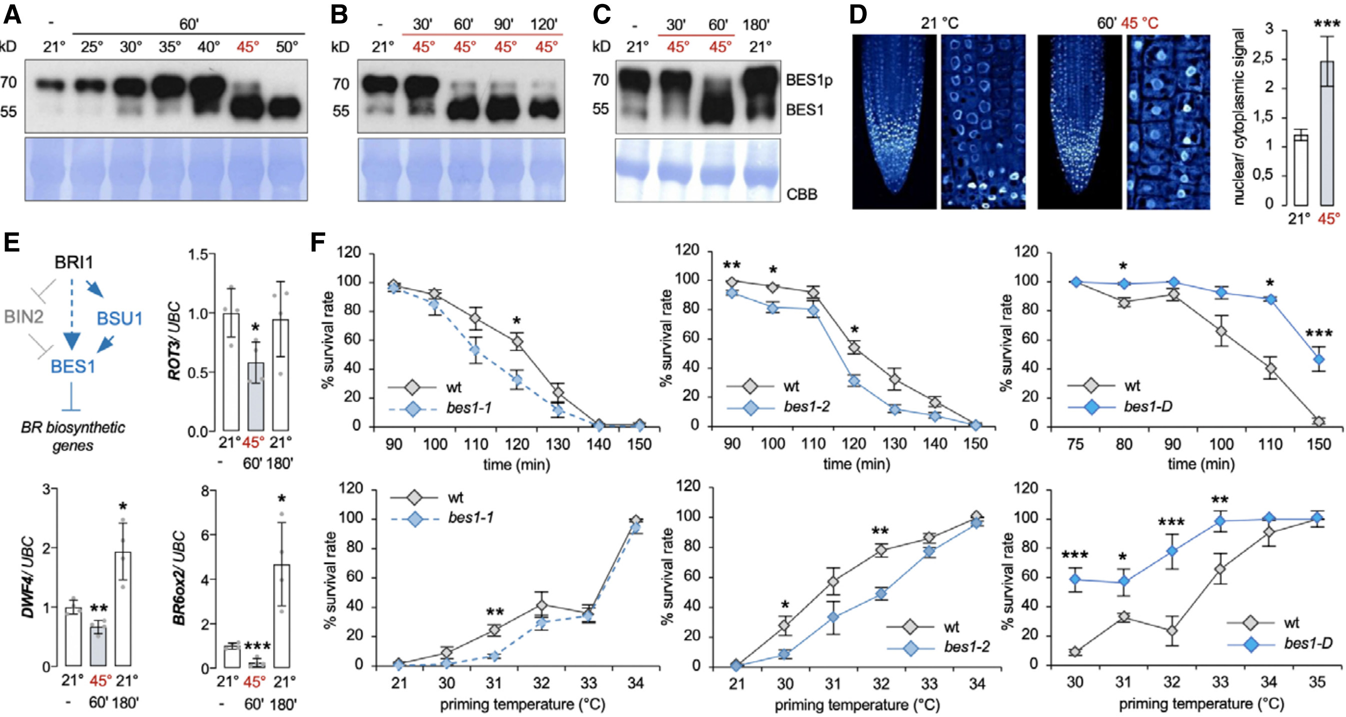

Figure 1. BES1 is activated by heat and contributes to heat stress resistance.

A–C Immunodetection of BES1-CFP from heat-treated plants. Plants pre-grown at 21°C were (A) exposed for 60 min to the indicated temperatures, (B) to 45°C for the indicated period of time, or (C) to 45°C and, following sampling after 30 min (lane 2) and 60 min (lane 3), returned to 21°C for 3 h (lane 4). CBB, Coomassie Brilliant Blue stained gel.

D Subcellular localization of BES1-CFP in roots of 7-day-old plants after heat shock. *35S:BES1-CFP* seedlings were grown at 21°C and either left untreated or exposed for 60 min to 45°C. Left: representative photos. Right: quantification of the nuclear/cytoplasmic signal. Data show the mean ± SD; $n = 25$. The asterisks indicate significant differences to the untreated plants by Student's *t*-test (***$P \leq 0.001$).

E Expression of the BES1-repressed BR biosynthetic genes *ROT3*, *DWF4*, and *BR6ox2* (the basic regulatory pathway is illustrated) after a heat shock of 45°C. Plants were treated as in C, samples were harvested, and qPCR analyses were performed. Data show the mean ± SD. $n = 4$ biological repeats, each measured in three technical replicates, normalized to *UBC*. The asterisks indicate significant differences compared to the control condition at 21°C by Student's *t*-test (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

F Heat stress resistance of *bes1-1*, *bes1-2*, and *bes1-D* as compared to wild type. For basal resistance assays (top charts), plants were directly exposed to 45°C for the indicated time. For acquired resistance (bottom charts), plants were primed with the respective temperatures for 120 min before transfer to 45°C for 150 min. Data show the mean ± SE. $n = 7$. The asterisks indicate significant differences compared to wild type by Student's *t*-test (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

Source data are available online for this figure.

not altered by BRZ in these mutants. Importantly, when BES1 was immuno-detected from 45°C-treated plants, it was seen that BES1 was efficiently de-phosphorylated by heat in both lines (Fig 2C and D), showing that BR perception is not required for this response.

To investigate whether BES1 de-phosphorylation may occur through BIN2 inhibition, the dominant *bin2-1* mutant, which hyper-accumulates phosphorylated, constitutively active BIN2 (Li *et al*, 2001; Peng *et al*, 2008) was analyzed for altered heat stress resistance, but no differences to wild type were seen (Fig 2E). Moreover, also in this mutant background, heat-induced de-phosphorylation of BES1 was not compromised (Fig 2F), indicating that it may not primarily be a repression of BIN2 that is responsible.

BES1 de-phosphorylation by heat is mediated by ABA-controlled PP2C phosphatases

Since there was evidence that heat stress de-phosphorylates BES1 by means independent of canonical BR signaling, we aimed to identify responsible factors and a candidate was the hormone abscisic acid (ABA). The ABA-repressed type 2C phosphatases (PP2Cs) ABI1 and ABI2 can interact with and de-phosphorylate BIN2, which promotes accumulation of non-phosphorylated BES1/BZR1 forms and allows for an activation of BR responses even in the absence of BRI1 (Zhang *et al*, 2009; Wang *et al*, 2018). To test whether ABA may be implicated, the 45°C treatment of the *35S:BES1-CFP* reporter line was performed in the presence of ABA or the ABA biosynthesis inhibitor Fluridone (Flu) (Chae *et al*, 2004). This showed that ABA strongly impaired BES1 de-phosphorylation, whereas Flu promoted it (Fig 3A), providing evidence for an inhibitory function of ABA in the process.

ABA represses multiple PP2C phosphatases, which act partially redundant in ABA-controlled physiological processes (Ma *et al*, 2009; Park *et al*, 2009; Fuchs *et al*, 2013). To test their implication and avoid redundancy problems, we analyzed a higher order *abi1* loss-of-function mutant, the triple *abi1-2 hab1-1 pp2ca-1* knock-out line, which combines defects in the two major PP2C A-type subbranches of ABA signaling, the ABI1 and PP2CA branch (Fuchs

© 2021 The Authors

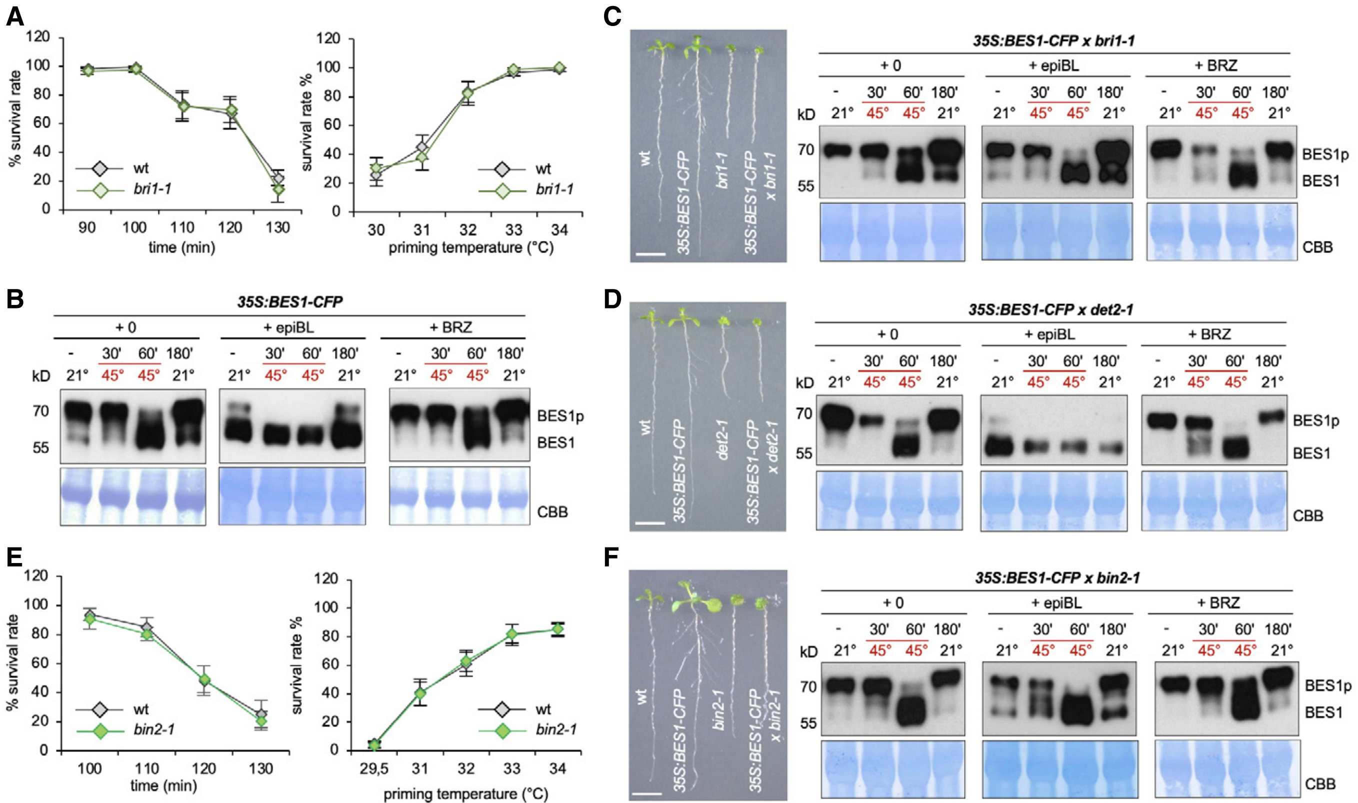

Figure 2. Canonical BR signaling is not required for heat-induced BES1 de-phosphorylation.

A Basal and acquired heat stress resistance of *bri1-1*. For basal resistance assays (left), plants were directly exposed to 45°C for the indicated time. For acquired resistance (right), plants were primed with the respective temperatures for 120 min before transfer to 45°C for 150 min. The data are shown as means ± SE; n = 8.

B Immunodetection of BES1-CFP from heat-stressed plants, treated with epiBL or BRZ. *35S:BES1-CFP* plants were treated for 24 h with DMSO (control), 1 μM 24-epiBL, or 5 μM BRZ and then heat-stressed as follows: lane 1: untreated control; lane 2: 45°C for 30 min; lane 3: 45°C for 60 min; lane 4: 45°C for 60 min and recovery at 21°C for 180 min.

C, D Immunodetection of BES1-CFP from heat-treated *bri1-1* or *det2-1* plants. Left: phenotypes of 10-day-old plants of all lines as compared to wild-type Col-0 (wt). Right: Immunoblots from the lines grown and treated as in B.

E Basal and acquired heat stress resistance of *bin2-1*. The experiment was performed as in A.

F Immunodetection of BES1-CFP from heat-treated *bin2-1* plants. The experiment was performed as in C and D.

Source data are available online for this figure.

et al, 2013), and exhibits extreme ABA hypersensitivity (Rubio *et al*, 2009). This *abi1* triple mutant (*abi1tM*) was introduced into the heat stress assays and showed a significantly reduced resistance as compared to wild type (Fig 3B). In addition, BES1 de-phosphorylation by heat, which was analyzed with a BES1 antibody, was clearly impaired in the *abi1tM* mutant background as compared to wild type (Fig 3C), albeit not completely abolished. Interestingly, BL treatment further promoted heat-induced BES1 de-phosphorylation in wild type, but not in *abi1tM* (Fig 3C). Therefore, there is evidence that plants utilize ABI1 and homologous ABA-controlled PP2C phosphatases for de-phosphorylation and activation of BES1 when heat stress occurs and that BRs, while not needed for this activity, do promote it.

BES1 can bind HSEs, an activity that is promoted by HSFA1a

BRs had previously been shown to induce heat-responsive genes (Divi *et al*, 2016) and an important subgroup are genes encoding HSPs of the HSP70 and HSP90 type (Akerfelt *et al*, 2010; Jacob *et al*, 2017). To test whether members of these HSP families are BES1-controlled, we quantified the expression of *HSP70.1, HSP70.2, HSP70.3, HSP70.4, HSP70.14,* and *HSP90.1* before and after heat stress in wild type and *bes1-D*. The genes where selected because they play roles in heat stress protection (Lee & Schoffl, 1996; Meiri & Breiman, 2009; Leng *et al*, 2017), and their promoters contain both HSEs (or a modified variant; mHSE) as well as BES1 binding sites (Fig 4A; Appendix Fig S2A). The analysis showed that in the *bes1-D* mutant the heat stress induction of all tested *HSPs*, except for *HSP70.2*, was significantly promoted (Fig 4B; Appendix Fig S2B) with *HSP70.3*, *HSP70.4,* and *HSP90.1* showing the clearest increases in this setting (Fig 4B).

To investigate whether BES1 can directly bind to the promoters of these genes, we conducted *in vivo* DNA binding studies. BES1-CFP was used as a bait for immuno-precipitation of BES1-bound DNA from plants exposed to heat stress and the presence of *HSP70.3*, *HSP70.4,* and *HSP90.1* promoter fragments (shown as purple bars in Fig 4A) was evaluated by qPCR. Whereas in normal ambient temperatures statistically significant, but low amounts of

 © 2021 The Authors

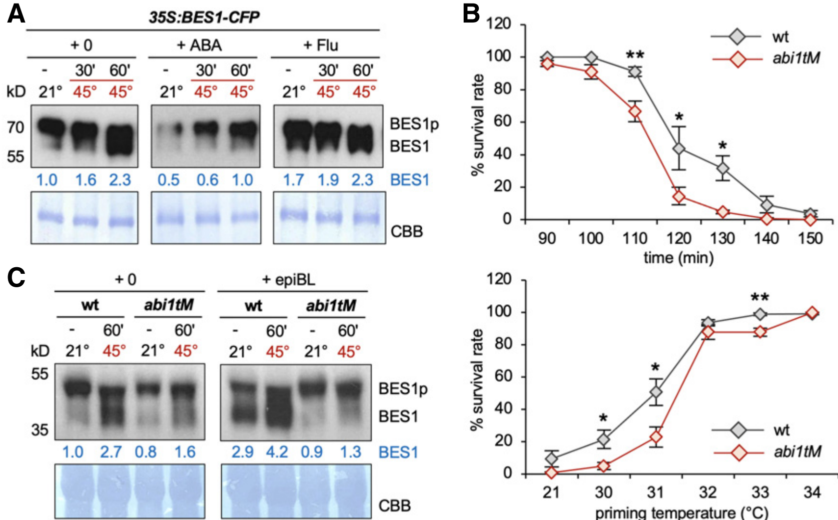

Figure 3. ABA represses BES1 de-phosphorylation by heat.

A Immunodetection of BES1-CFP from heat-stressed plants, treated with ABA or Flu. *35S:BES1-CFP* plants were treated for 24 h with DMSO (control), 5 μM ABA, or 10 μM Flu and heat-stressed as follows: lane 1: untreated control; lane 2: 45°C for 30 min; lane 3: 45°C for 60 min. The non-phosphorylated BES1 bands were quantified with ImageJ, and the obtained values are shown in blue.

B Basal and acquired heat stress resistance of *abi1tM*. For basal resistance assays (top), plants were directly exposed to 45°C for the indicate time. For acquired resistance (bottom), plants were primed with the respective temperatures for 120 min before transfer to 45°C for 150 min. Data show the mean ± SE; $n = 7$. The asterisks indicate significant differences compared to wild type by Student's *t*-test (*$P \leq 0.05$; **$P \leq 0.01$).

C Immunodetection of native BES1 from heat-stressed and epiBL-treated *abitM* plants. *abitM* and wild-type plants were grown on ½ MS medium either containing DMSO as a control or 1 μM epiBL and either left untreated or exposed to 45°C for 60 min. The non-phosphorylated BES1 bands were quantified with ImageJ, and the obtained values are shown in blue.

Source data are available online for this figure.

the promoter fragments were enriched, a strong enrichment of approximately 6- to 8-fold was seen after heat stress (Fig 4C), providing evidence that heat stress promotes BES1 binding to these promoters. Also, on the promoters of *HSP70.1*, *HSP70.2,* and *HSP70.14* a significant enrichment of BES1 was detectable after heat stress (Appendix Fig S2C).

The promoter regions on which BES1 was enriched contain HSEs, which are located in close proximity to established BES1 binding sites (Fig 4A; Appendix Fig S2A). Since heat induces *HSPs* via HSFs, which regulate gene expression through HSE binding, we investigated if BES1 activity may be impacted by HSFs. For this purpose, we chose HSFA1a, a central, positive regulator of heat stress tolerance (Liu *et al*, 2011; Yoshida *et al*, 2011). Recombinant MBP-tagged HSFA1a and GST-tagged BES1 were produced and tested for binding to the promoters of *HSP70.3* and *HSP90.1*, which both contain the perfect three-box HSE consensus sequence 5′-nGAAnnTTCnnGAAn-3′, *HSP70.3* in two copies and *HSP90.1* in one (Fig 4A). *In vitro* DNA binding studies were carried out using short promoter fragments that contain the HSEs only (shown as gray bars in Fig 4A) and analyzed by electrophoretic mobility shift assays (EMSAs). This showed that HSFA1a bound the HSE-containing fragments *in vitro* (Fig 5A), confirming previous results (Lohmann *et al*, 2004; Liu *et al*, 2013). Surprisingly, also BES1 alone was able to directly bind to fragments of the *HSP70.3* and *HSP90.1* promoter that contained only HSEs, albeit with low activity (Fig 5A, Appendix Fig S2D). Moreover, also BRREs present in both promoters (approximately 170 bp upstream of the ATG in the *HSP70.3* promoter and 1,790 bp upstream of the ATG in the *HSP90.1* promoter) were bound by BES1 (Appendix Fig S2E).

When both BES1 and HSFA1a were added to the DNA binding reaction, a strong shift of the DNA occurred, showing that the HSE-containing fragments of the *HSP70.3* and *HSP90.1* promoter were bound with very high efficiency (Fig 5A). Increasing BES1 amounts enhanced the shift, suggesting that the ratios of BES1 and HSFA1a are relevant for binding efficiencies. Also, the DNA–protein complexes shifted to different molecular weights, some with the same size like the HSFA1a–DNA complexes, some with lower and some with higher molecular mass producing a smeary ladder. When unlabeled competitor nucleotides with intact or mutated HSEs were included in the *in vitro* DNA-binding reactions, the non-mutated promoter fragments (C1 +HSE) efficiently competed for binding, whereas the HSE-mutant versions (C2 −HSE) did not, showing that it is the HSEs that are bound by BES1 and HSFA1a (Fig 5A).

BES1 can directly interact with HSFA1a

The formation of DNA–protein complexes with different molecular mass indicated that BES1 and HSFA1a may be able to physically interact. To verify this, we carried out *in vitro* pull-down assays using as baits GST-tagged BES1 or GST alone (as a control) and assessing the presence of MBP-tagged HSFA1a in the immunoprecipitates with an anti-MBP antibody. Some background bands were pulled with GST alone, but in addition a specific band was immuno-detected with anti-MBP in the BES1-GST precipitates (Appendix Fig S2F), an indication for an interaction.

© 2021 The Authors

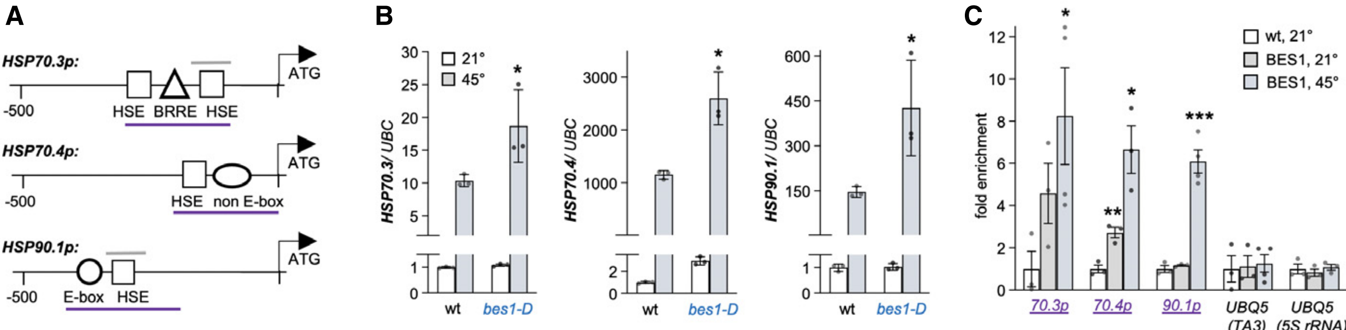

Figure 4. BES1 binds to *HSP* promoters.

A Illustration of the promoters of *HSP70.3*, *HSP70.4*, and *HSP90.1*. Relevant regulatory motifs and promoter regions that were assessed in ChIP experiments (C) or *in vitro* DNA-binding studies (Fig 5A) are shown with purple and gray lines, respectively.

B Relative expression changes of *HSP70.3*, *HSP70.4*, and *HSP90.1* in *bes1-D* as compared to wild type following heat shock. 14-day-old plants were exposed to 45°C for 60 min, expression was analyzed by qPCR, and the results were normalized to *UBC*. Data show the mean ± SD. $n = 3$ biological replicates measured in three technical repeats. Significant differences as compared to wild type at 45°C, calculated by Student's *t*-test, are shown (*$P \leq 0.05$).

C ChIP to determine BES1-CFP enrichment on the promoters of *HSP70.3*, *HSP70.4*, and *HSP90.1* following heat stress. *35S:BES1-CFP* and wild-type plants grown at 21°C were either left untreated or treated with 45°C for 60 min. BES1-CFP was immunoprecipitated with α-GFP beads. Fragments were quantified in the precipitates with qPCR, and the ratios of samples with antibody to without antibody were calculated. *TA3* (*70.3p* and *90.1p*) or *5s rRNA (70.4p)* was used for normalization. *UBQ5* served as an internal control. Data show the mean ± SE. $n = 3$ biological replicates measured in three technical repeats. Significant differences as compared to wild type at 21°C, calculated by Student's *t*-test, are shown (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

For an *in vivo* verification of this results, bimolecular fluorescence complementation (BiFC) assays were carried out. Tobacco leave mesophyll cells were transiently co-transformed with nYFP-BES1 and cYFP-HSFA1a, and in combinations with nYFP and cYFP as controls, and YFP activity was detected with fluorescence microscopy. Whereas no signal was seen in the controls (Appendix Fig S3), strong fluorescence was visible in nuclei of cells co-transformed with the two fusion proteins (Fig 5B), providing evidence for a direct interaction of BES1 and HSFA1a *in vivo*.

HSFA1s are required for BES1 activity in heat stress responses

HSFA1a acts redundantly with three additional HSFs of the HSFA1 type, namely, HSFA1b, HSFA1d, and HSFA1e, in heat stress protection and other biological processes (Liu *et al*, 2011; Yoshida *et al*, 2011). Since the *in vitro* analyses had indicated that HSFA1a promotes BES1 binding to the promoters of *HSP70.3*, *HSP70.4,* and *HSP90.1,* we used a *hsfA1a,b,d,e* quadruple mutant (*hsfA1qM*) (Liu *et al*, 2011) to verify this finding *in vivo*.

LUC reporter assays were carried out in protoplasts of wild type, *bes1-D,* and *hsfA1qM*, assessing constitutive changes in LUC expression between the lines, as well as analyzing effects on LUC activity when BES1 or HSFA1a were added as effectors (Fig 5C). This showed that without effectors the expression of *HSP70.3p-LUC* was constitutively increased in *bes1-D*, but not changed in the *hsfA1qM* as compared to wild type. Adding BES1 as an effector increased HSP70.3p-LUC activity in the *hsfA1qM* background, but not in wild type, whereas HSFA1a increased HSP70.3p-LUC activity in all genotypes (Fig 5C), showing that, while HSFA1s appear not to be required for *HSP70.3* expression, HSFA1a can activate this promoter.

The activity of the HSP70.4p-LUC and HSP90.1p-LUC reporters was constitutively increased in *bes1-D* and decreased in the *hsfA1qM*. Adding BES1 or HSFA1a as effectors to wild-type or *bes1-D* protoplasts increased HSP70.4p-LUC and HSP90.1p-LUC activity by similar means, showing that both TFs can promote their expression. In the *hsfA1qM* background, the basal expression of both reporters was significantly decreased as compared to wild type. Adding BES1 had mild, but significant effects, whereas HSFA1a strongly increased LUC activity of HSP70.4p-LUC and HSP90.1p-LUC. In summary, these results showed that HSFA1s are required for efficient BES1 activity in inducing *HSP* expression, albeit BES1 showed some transactivation capacity even in the absence of HSFA1s, which was in line with the results from the *in vitro* DNA binding studies.

For genetic verification of these results, we introduced *bes1-D* into *hsfA1qM* by crossing and isolated a *bes1-DxhsfA1qM* quintuple mutant (Appendix Fig S4A). An analysis of the BES1 phosphorylation state following BL or BRZ treatment showed that the cross had been successful, since BES1 levels were comparable between *bes1-D* and the quintuple mutant (Appendix Fig S4B). Interestingly, while in untreated conditions and following epiBL application similar amounts of non-phosphorylated BES1 were present in both wild-type and the *hsfA1qM* background, when exposed to BRZ, slightly less non-phosphorylated BES1 was seen in *bes1-DxhsfA1qM* plants (Appendix Fig S4B), suggesting that HSFA1s may contribute to the effects of BR depletion on BES1 phosphorylation.

The quintuple mutant was subjected to heat stress tolerance assays and compared to the *hsfA1qM* parent line. In line with previous results (Liu *et al*, 2011; Yoshida *et al*, 2011; Liu & Charng, 2012), the *hsfA1qM* was highly heat stress sensitive: It died off completely already after 60 min of 45°C (Fig 5D), a treatment that wild type fully survived (Fig 1F). The *bes1-DxhsfA1qM* quintuple mutant did not show any increases in heat stress tolerance as compared to *hsfA1qM* (Fig 5D), providing further support to the notion, that for the ability of BES1 to increase heat stress resistance HSFA1s are required. Thus, in summary, multiple lines of evidence support a model, in which BES1 induces expression of *HSPs* via

 © 2021 The Authors

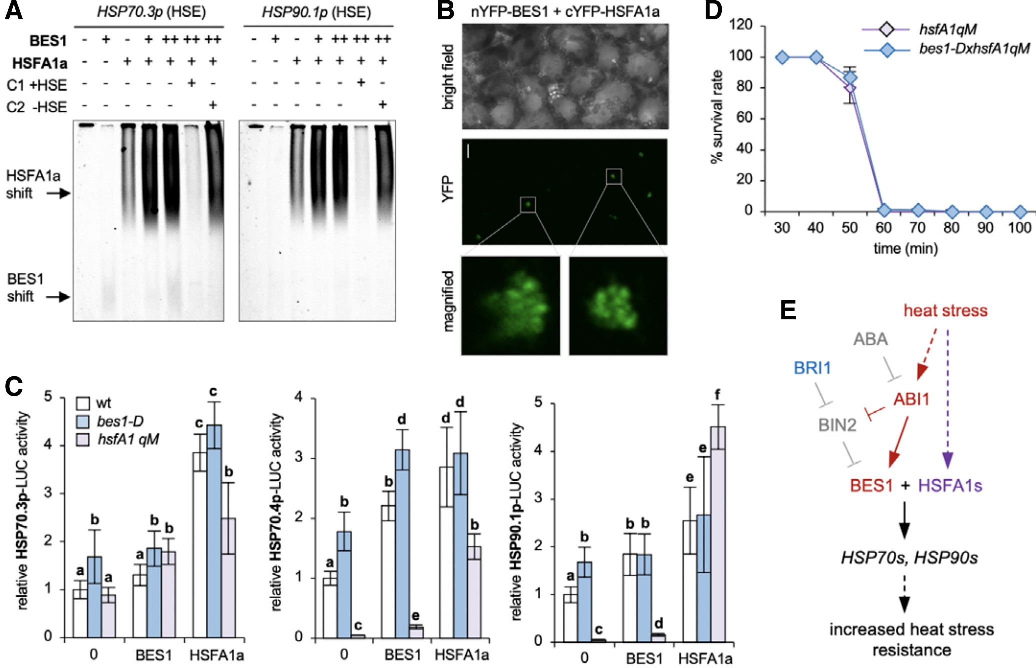

Figure 5. HSFA1s promote BES1 activity in *HSP* induction and heat stress resistance.

A EMSAs showing *in vitro* DNA interaction of recombinant BES1 and/or HSFA1 with HSE-containing fragments of the *HSP70.3* and *HSP90.1* promoters. Unlabeled competitor fragments with functional HSEs (C1 +HSE) or mutated versions (C2 −HSE) were used for competition studies in 100x molar access (lanes 6 and 7).

B BiFC assays showing cells co-transformed with nYFP-BES1 and cYFP-HSFA1a. The fusion proteins were co-expressed in *N. benthamiana* leaves, and fluorescence was visualized. Two nuclei are highlighted and shown in a 6-fold magnification. Scale bar = 20 μm.

C LUC assays in protoplasts of wild-type, *bes1-D*, or the *hsfA1qM* plants. LUC reporter constructs, driven by 2 kb of the *HSP70.3*, *HSP70.4*, and *HSP90.1* promoters, were tested either alone (0, with empty effector plasmid) or with BES1 or HSFA1a as effectors, and LUC transactivation was analyzed. Data show the mean ± SD; $n = 5$. Statistically significant difference at $P \leq 0.05$ of results is indicated with different letters and was determined by Student's *t*-test.

D Basal heat stress resistance of *hsfA1qM* and *bes1-DxhsfA1qM* plants. Plants were directly exposed to 45°C for the indicated time. Data show the mean ± SE; $n = 8$.

E Working model for the contribution of BES1 to heat stress signaling. BES1 is de-phosphorylated and activated by heat stress, utilizing ABI1 and redundant ABA-repressed PP2C phosphatases. It then interacts with HSFA1s, which are also activated by heat, for binding to HSEs in promoters of *HSP70s* and *HSP90s* genes, which induces them and increases heat stress resistance. BRs, while not needed for the activity, can promote it.

Source data are available online for this figure.

direct binding to HSE, an activity that is promoted by HSFs of the HSFA1 type, potentially through direct interaction (Fig 5E).

HSFA1s repress *bes1-D* effects on growth and BR-responsive genes

While the *hsfA1qM* background repressed *bes1-D* activity in heat stress resistance, we noted that it promoted *bes1-D* effects on growth. Both in the dark and in white light, the *bes1-DxhsfA1qM* quintuple mutant had significantly longer hypocotyls than *bes1-D* and also the BRZ hyper-responsiveness of *bes1-D* (Yin *et al*, 2002) was stimulated in the absence of HSFA1s in both dark and light conditions (Fig 6A and B). Moreover, in the light, the promotive effects of *bes1-D* on BL-induced hypocotyl elongation were strongly enhanced in *bes1-DxhsfA1qM* (Fig 6B), which was not due to increased BES1 accumulation or de-phosphorylation (Appendix Fig S4B).

To investigate whether the promotion of *bes1-D*-induced growth defects in the *hsfA1qM* background was correlated with enhanced expression changes of BR-responsive genes, mRNA levels of the BR-repressed biosynthetic genes *DFW4*, *ROT3*, and *BR6ox2*, and the BR-induced genes *GA3ox1*, *GA20ox1*, *IAA5*, *SAUR-AC1*, and *XTH17* (Bancos *et al*, 2002; Nakamura *et al*, 2003; Yin *et al*, 2005; Li *et al*, 2012; Unterholzner *et al*, 2015) were analyzed. This showed that, when compared to wild type, the expression of *DWF4* and *BR6ox2* was significantly more repressed in *bes1-DxhsfA1qM* than in *bes1-D*, whereas *ROT3* mRNA levels were not altered (Fig 6C). Moreover, the expression of the GA biosynthetic gene *GA3ox1*, and the auxin-responsive genes *IAA5* and *SAUR-AC1* were more increased in *bes1-DxhsfA1qM* than in *bes1-D* (Fig 6C), while *GA20ox1* and *XTH17* showed no significant differences between the two lines (Appendix Fig S4C). Therefore, in summary there is evidence that HSFs of the HSFA1 subfamily while promoting *bes1-D* activity in heat stress resistance repress *bes1-D* mutant effects on BR-responsive growth and the expression of a subset of BR-responsive genes.

Discussion

Heat stress occurs when the ambient temperature rises above a threshold level for a period of time that is sufficient to cause irreversible damage and species-specific abilities to cope with heat stress exist. *Arabidopsis thaliana*, a plant from temperate climates,

© 2021 The Authors

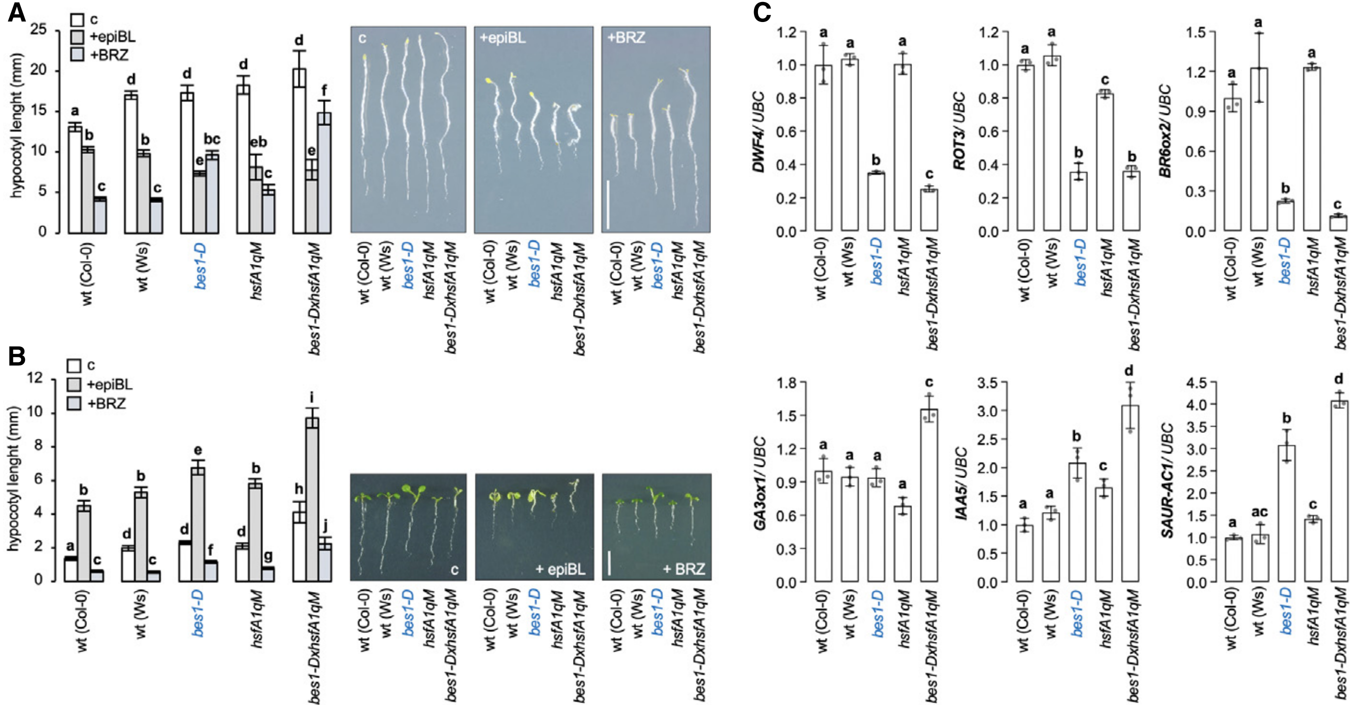

Figure 6. HSFA1s repress *bes1-D* activity in BR responses.

A, B Constitutive and BR-responsive growth of *bes1-DxhsfA1qM* seedlings. Hypocotyl elongation of 7-day-old dark-grown (A) and light-grown (B) seedlings on medium containing either 1 μM epiBL, 1 μM BRZ, or DMSO as a control (C). Left: Mean and SD of 30 plants. Significant difference at $P \leq 0.05$ of results is indicated with different letters and was determined with a Student's *t*-test. Right: photos of representative plants.

C Expression of BR-regulated genes in *bes1-DxhsfA1qM*, as compared to its parents and respective wild types analyzed by qPCRs. Data show the mean ± SD. $n = 3$ biological repeats, each measured in three technical replicates, normalized to *UBC*. Statistically significant difference at $P \leq 0.05$ of results is indicated with different letters and was determined by Student's *t*-test.

Source data are available online for this figure.

has an optimum of 20–25°C for vegetative growth, and while a rise of 5–10°C can accelerate growth and development, temperatures above that range impair cellular processes, with negative effects on development and reproductive success (Yeh *et al*, 2012; Quint *et al*, 2016; Casal & Balasubramanian, 2019; Desaint *et al*, 2021).

BRs had previously been shown to increase heat stress tolerance via effects on the heat-responsive transcriptome (Divi *et al*, 2016), and here, we provide evidence that the BR-regulated TF BES1 can directly activate heat stress-responsive genes via HSE binding. *In vivo* DNA binding studies, complemented with *in vitro* DNA binding experiments and LUC reporter analyses in protoplasts, showed that BES1 had a weak affinity for HSEs on its own. However, when HSFs of the HSFA1 type were added, BES1 activity was strongly increased and in a *hsfA1qM* background *bes1-D* was unable to increase heat stress tolerance. Moreover, BES1 as an effector was unable to efficiently increase *HSP-LUC* expression, evidence for an interaction with HSFA1s in the direct regulation of heat stress-responsive genes.

HSFA1s are essential for both basal and acquired heat stress resistance (Liu *et al*, 2011; Yoshida *et al*, 2011). Their activity is controlled by HSP90s, which bind HSFA1s at ambient temperatures and release them for nuclear transfer when heat stress occurs (Jacob *et al*, 2017; Ohama *et al*, 2017). It is interesting that also BES1 and BIN2 are HSP90 clients (Samakovli *et al*, 2014; Shigeta *et al*, 2015; Lachowiec *et al*, 2018). Chemical inhibitor studies paired with protein–protein interaction analyses indicated that HSP90 removes BIN2 from the nucleus when BR signaling is activated (Lachowiec *et al*, 2013). Since heat triggers HSP90 accumulation, it is conceivable that HSP90 restrains BIN2 activity when heat stress develops, yielding a stabilization of BES1 and enrichments of its non-phosphorylated, active forms. A result, which may speak against this idea, is that in the *hsfA1qM* background, which is defective in HSP90 synthesis (Liu *et al*, 2011; Yoshida *et al*, 2011), BES1 abundance is not significantly altered, albeit a slight reduction of non-phosphorylated forms, at least in BRZ-treated plants, was seen.

Another, not mutually exclusive way of BES1 activation by heat is the recruitment of phosphatases that can act by BRI1-independant means. Candidates are proteins of the PP2C-type; in particular, ABI1 and ABI2, which are repressed by ABA, are required for heat stress resistance (Larkindale *et al*, 2005; Suzuki *et al*, 2016) and can induce BES1/BZR1 de-phosphorylation even in the absence of BRs. This has been shown to be relevant for an ABA impact on BR-responsive growth (Zhang *et al*, 2009; Wang *et al*, 2018), and here, we find that it also contributes to heat-induced BES1 de-phosphorylation, which was impaired in an *abi1* higher order mutant and in ABA-treated plants. Since ABA repressed BES1 de-phosphorylation by heat, it appears that ABA acts as a negative regulator of this process and it will be interesting to explore this further.

 © 2021 The Authors

ABI1 and ABI2 can directly interact with and de-phosphorylate BIN2, and it was therefore possible that BES1 de-phosphorylation by heat stress occurs indirectly via BIN2 inactivation. To investigate this, we analyzed the dominant *bin2-1* mutant, which hyper-accumulates a constitutively active BIN2 version. However, heat-induced BES1 de-phosphorylation was not compromised in this line. This indicates that BES1 may be de-phosphorylated by additional modes, for example, directly, and this will require testing. Since BZR1 is also de-phosphorylated by heat, it could act redundantly in heat stress responses, and although we here confirmed previous results (Setsungnern *et al*, 2020) that a loss of BES1 function only is sufficient to increase heat stress sensitivity in *A. thaliana*, higher order mutation of BES1 with BZR1 may further increase heat stress hypersensitivity.

BES1 de-phosphorylation was most efficient at temperatures of 40°C or higher. However, also at around 30°C de-phosphorylation took place, which is in line with previous results that BES1 de-phosphorylation is promoted by high ambient temperatures. BES1 activation by warmth initiates a cooperation with PIF4 for a synergistic induction of elongation-related genes (Martinez *et al*, 2018), and interestingly, PIF4 activity contributes to the priming of plants for improved heat stress resistance (Zhu *et al*, 2016). In addition, BZR1, which is also de-phosphorylated by warmth, directly induces PIF4 expression (Ibanez *et al*, 2018), which may enable a feed-forward amplification of the growth response. Since PIF4 and other bHLH proteins cooperate with BES1/BZR1 in different physiological processes (Yin *et al*, 2005; Oh *et al*, 2012; Ibanez *et al*, 2018; Martinez *et al*, 2018), an investigation of a potential contribution of additional bHLH TFs to heat shock responses is warranted.

BES1 acts in both warmth-induced growth and heat stress responses, which at extreme levels can impair development, and it is thus important to ask how these dual roles are realized. Specificity could be conferred by interactions with PIF4 and other bHLH TFs on the one hand and HSFA1s on the other. Whereas BES1 homodimers bind BRREs and BES1+PIF4 heterodimers bind E-box motifs (Martinez *et al*, 2018), a cooperation with HSFA1s promotes BES1 binding to HSEs. Consequently, alternating interactions enable diversification of BES1 activities, and also, HSFs are known to heterodimerize. In mammals, human HSF1 interacts with STAT-1 to activate *HSP70* and *HSP90* promoters (Stephanou *et al*, 1999) and chicken HSF3 interacts with the TF c-Myb to facilitate HSF3 binding to HSEs (Kanei-Ishii *et al*, 1997). In *A. thaliana*, NONEXPRESSOR OF PR GENES 1, a component of immune signaling, interacts with HSFA1s to directly activate cold-induced, heat stress-responsive genes (Olate *et al*, 2018). While our results show that HSFA1a can heterodimerize with BES1, the nature of the complexes and under which conditions they are formed *in vivo* is not clear yet.

Among environmental factors, heat stress is a clear candidate for impacting BES1+HSFA1 interaction. However, in addition also developmental context may be relevant, since there are first indications that HSFA1s, while stimulating BES1 activity in heat stress responses, may repress BES1 function in BR-controlled growth. This is indicated by the fact that in the *hsfA1qM* background *bes1-D* growth-promoting effects and BL and BRZ hypersensitivity are enhanced. Moreover, the BR biosynthetic genes *DWF4* and *BR6ox2* are more strongly repressed and the GA biosynthetic gene *GA3ox1*, as well as *IAA5* and *SAUR-AC1*, are more strongly induced by *bes1-D*, when HSFA1 activity is lost. Whether this is of biological relevance and contributes to BR response remains to be investigated though, since the *hsfA1qM* did not show any clear defects in BR responses, at least in the specific developmental context and environmental setting analyzed.

In summary, based on the results of this and other studies, an expanded working model for heat stress responses in plants can be proposed, which employs BES1 (Fig 5E). In this model, heat stress activates BES1 through de-phosphorylation mediated by PP2Cs including ABI1 and, while heat achieves this even in the absence of BRs, the hormones promote the effect. In addition, heat stress activates HSFA1s, which cooperate with BES1 for binding to HSEs. This induces the expression of heat shock-controlled genes, such as those encoding HSPs of the HSP70- and HSP90-type, and facilitates heat stress protection.

In future work, it will be important to determine the relative contribution of BES1 and potential functional homologous to the control of the heat-responsive transcriptome. Moreover, it will be interesting to investigate whether the cooperation of BES1 with HSFA1s also contributes to other stress response reactions, since these HSFs are not only required for resistance against heat, but also other abiotic stress types, including drought, soil salinity, and excess light (Larkindale *et al*, 2005; Liu *et al*, 2011; Jung *et al*, 2013).

Materials and Methods

Plant material and growth conditions

The *bes1-D* mutant utilized is a version that has been introgressed into Col-0 (Ibanes *et al*, 2009). *bes1-1* (Saito *et al*, 2018; Chen *et al*, 2019), *bes1-2* (Lachowiec *et al*, 2013), and *abi1-2 hab1-1 pp2ca-1* (Rubio *et al*, 2009) are also in the Col-0 background. The *hsfA1qM* combines the Col-0 and Wassilevskia (Ws) genetic backgrounds (Liu *et al*, 2011), which is why both Col-0 and Ws were used as controls in experiments with this line. The *hsfA1qMxbes1-D* quintuple mutant was generated by crossing *hsfA1qM* with *bes1-D* and preselecting lines based on heat stress sensitivity and *bes1-D* morphologies in the F2 generation. The F3 progeny was then screened for plants homozygous for the T-DNA insertions in HSFA1a, HSFA1b, HSFA1d, and HSFA1e by genotyping and for the *bes1-D* mutation by sequencing (for all primer sequences, see Appendix Table S1).

For phenotyping in standard conditions, the plants were grown on ½ MS plates in growth cabinets (Bright Boys, CLF Plant Climatics GmbH) at 21°C ± 2° and in long days of 16 h white light (80 $\mu mol/m^2/s$)/ 8 h dark. Treatments with BL, BRZ, ABA, and Flu were performed by growing plants for 10 days on ½ MS plates and transferring the plants to new ½ MS plates containing 24-epiBL, BRZ, ABA, Flu, and DMSO, the solvent used for compound solution, in equivalent concentrations as control.

Heat stress assays

Heat stress resistance assays were performed with tissue-cultured, 14-day-old plants, pre-grown at 21°C in long days, as described previously (Larkindale *et al*, 2005). The mutants were always analyzed together with wild type on one ½ MS plate, to ensure that exactly the same conditions were applied and minimize positional effects. For basal resistance assays, plants were directly exposed to 45°C. For acquired resistance, plants were primed with temperatures between 30 and 35°C for 120 min and immediately transferred back to 21°C

© 2021 The Authors

for 120 min before a heat shock of 45°C was applied for 150 min. Survival rates were assessed after 10 days of recovery at 21°C and were defined as the ability of the plants to form new leaves.

Protein immunoblotting

Immunoblot analysis was done as described previously (Albertos *et al*, 2015) using HRP-conjugated anti-GFP antibody (Miltenyi Biotec), rabbit anti-BES1 (Yin *et al*, 2002), and mouse anti-Actin (Sigma) as primary antibodies and alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG (Sigma-Aldrich) as secondary antibodies. Detection was performed with enhanced chemiluminescence using the Amersham ECL Select Western Blotting Detection reagent (GE Healthcare) or PhosphaGLO Reserve AP Substrat (Medac Diagnostic). For loading control, the membranes were stained with the Coomassie Brilliant Blue dye.

Fluorescence microscopy

For fluorescence microscopic imaging of BES1 re-localization in response to heat stress, 7-day-old *35S:BES1-CFP* plants were kept either at 21°C or treated for 60 min with 45°C. The fluorescent photographs were taken using an Olympus Bx61 fluorescence microscope with a FV1000 confocal laser scanning unit (Olympus). CFP-tagged fusion proteins were excited using the Ar laser line at 435 nm and detected at 470–500 nm (blue channel). The nuclear/cytoplasmic rate was measured from 34 cells using ImageJ (Schneider *et al*, 2012).

Quantitative PCRs

For qPCRs, 14-day-old plants, grown on ½ MS medium, were used to extract total mRNA with the E.Z.N.A. Plant RNA (OMEGA Biotek). Following DNaseI treatment (Thermo Scientific), first-strand cDNA was synthesized from 1 µg of RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham). qPCRs were performed with the Mastercycler Realplex (Eppendorf) using qPCRBIO SyGreen Mix No-ROX (2000) (Nippon Genetics) and primers specific for the relevant genes. *UBC (Ubiquitin-conjugating enzyme 21)* (Eremina *et al*, 2016) was used for normalization. Analyses were done in at least three independent biological samples, each measured in at least three technical repeats. Mean and SDs were calculated and statistical significance of differences was evaluated with two-tailed Student's *t*-test.

In vivo DNA binding studies: LUC and ChIP assays

For LUC transactivation assays, *35S:BES1* and *35S:HSFA1a* constructs were used for effector expression. The LUC reporters were generated by PCR-amplifying 2.0 kb promoter fragments of *HSP70.3p, HSP70.4p,* and *HSP90.1p* and cloning them into the pGreenII-0800-LUC vector to drive LUC expression (Hellens *et al*, 2005). Protoplasts isolated from 21-day-old wild-type, *bes1-D, or hsfA1qM* plants were transiently transformed using a PEG-mediated transformation protocol (Yoo *et al*, 2007), and LUC assays were performed using a Dual-Luciferase® Reporter Assay System (Promega) and a Lumat LB9501 luminometer (Berthold) as described previously (Khan *et al*, 2014).

For ChIPs, 3-week-old *35S:BES1-CFP* plants and wild type were treated for 60 min at 45°C or left at 21°C, 1.5 g of plant material was harvested for each biological replicate, and ChIP was performed in three biological repeats as described previously (Unterholzner *et al*, 2015). Statistical significance was evaluated with the Student's *t*-test.

Recombinant protein production, *in vitro* DNA binding studies and EMSAs

Recombinant proteins of BES1-GST, BES1-MBP, and HSFA1a-MBP were expressed in *Escherichia coli* BL21 (New England Biolabs) using the expression vectors pGEX-BES1 and pMAL-BES1, pMAL-HSFA1a, and subsequently purified using GSH beads (GE Healthcare) or Amylose resin (New England Biolabs). Protein elution from GSH beads was performed with elution buffer (150 mM NaCl, 5 mM DTT, 20 mM GSH reduced form, and 50 mM Tris–HCl, pH 8.0) and from Amylose resin (150 mM NaCl, 5 mM DTT, 10 mM Maltose, and 50 mM Tris–HCl, pH 8.0) concentrated using Roti-spin MINI-3 columns (Roth). HEX-labeled probes for EMSAs were prepared by PCR and purified using a Phenol:Chloroform protocol (Unterholzner *et al*, 2017). The probes (0.5 pmol per reaction) were incubated with purified BES1-MBP, HSFA1A-MBP, or combinations of both proteins and separated on 3–6% PAGE gels. The bands were detected using a Molecular Imager FX Pro (Hercules, Bio-Rad) equipped with a 532 nm laser for excitation and a 555 nm long pass emission filter as described previously (Unterholzner *et al*, 2017).

In vitro and *in vivo* protein pull-down and BiFC assays

For *in vitro* pull-down experiments, recombinant BES1-GST or GST alone (as a control) was incubated with GST beads for 1 h in the reaction buffer (150 mM NaCl and 50 mM Tris–HCl, pH 8.0). After BES1-GST or GST was immunoprecipitated and washed, recombinant HSFA1a-MBP was added and incubated for 1 h more (input sample). Then, five washing steps were performed before the proteins were eluted with 4x SDS buffer (pull-down sample). Input and pull-down samples were separated by PAGE and transferred to membranes. The presence of BES1-GST or GST was verified with a mouse anti-GST (Sigma-Aldrich) and co-immunoprecipitated HSFA1A-MBP was detected with a mouse anti-MBP (New England Biolabs) and alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma-Aldrich) as the secondary antibody.

For BiFC assays (Walter *et al*, 2004), BES1 was fused to the N-terminal and HSFA1a to the C-terminal part of YFP as described previously (Khan *et al*, 2013). The constructs were transiently co-introduced into *Nicotiana benthamiana* leaves via Agrobacterium-mediated transformation, and after 2 days of incubation, fluorescence was visualized using confocal microscopy.

Quantification and statistical analysis

Information about statistical tests used and technical and biological replicate experiments for each experiment are detailed in the relevant methods sections. Values are represented as mean ± SD or ± SE. The statistical analysis was performed by a two-tailed Student's *t*-test where $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$ are considered significant and expressed as *, **, and *** respectively.

© 2021 The Authors

Data availability

This study includes no data deposited in external repositories.

Expanded View for this article is available online.

Acknowledgements
We thank Erwin Grill for very interesting and helpful discussions, Yanhai Yin for the BES1 antibody, Ana Cano for the *bes1-D* mutant in the Col-0 background, and Jennifer Lachowiec for the *hsfA1qM* line and the *bes1-2* allele. Also, we thank the Nottingham *Arabidopsis* Stock Center for T-DNA insertion lines and Irene Ziegler for technical assistance. This work was supported by the TUM University Foundation and Alexander von Humboldt Foundation (postdoctoral fellowships to P.A.), as well as the Deutsche Forschungsgemeinschaft (DFG project PO1640/6-1 to B.P.). Open Access funding enabled and organized by Projekt DEAL.

Author contributions
PA and BP designed the research. PA, GD, TS, and BP analyzed the data. PA and GD performed the research with PS, PC, and SC contributing preliminary experiments and providing technical assistance. BP wrote the article with input from PA, GD, and TS. All authors reviewed and approved the final version.

Conflict of interest
The authors declare that they have no conflict of interest.

References

Akerfelt M, Morimoto RI, Sistonen L (2010) Heat shock factors: integrators of cell stress, development and lifespan. *Nat Rev Mol Cell Biol* 11: 545 – 555

Albertos P, Romero-Puertas MC, Tatematsu K, Mateos I, Sanchez-Vicente I, Nambara E, Lorenzo O (2015) S-nitrosylation triggers ABI5 degradation to promote seed germination and seedling growth. *Nat Commun* 6: 8669

Asami T, Min YK, Nagata N, Yamagishi K, Takatsuto S, Fujioka S, Murofushi N, Yamaguchi I, Yoshida S (2000) Characterization of brassinazole, a triazole-type brassinosteroid biosynthesis inhibitor. *Plant Physiol* 123: 93 – 100

Bai MY, Shang JX, Oh E, Fan M, Bai Y, Zentella R, Sun TP, Wang ZY (2012) Brassinosteroid, gibberellin and phytochrome impinge on a common transcription module in *Arabidopsis*. *Nat Cell Biol* 14: 810 – 817

Bancos S, Nomura T, Sato T, Molnar G, Bishop GJ, Koncz C, Yokota T, Nagy F, Szekeres M (2002) Regulation of transcript levels of the *Arabidopsis* cytochrome p450 genes involved in brassinosteroid biosynthesis. *Plant Physiol* 130: 504 – 513

Bechtold U, Field B (2018) Molecular mechanisms controlling plant growth during abiotic stress. *J Exp Bot* 69: 2753 – 2758

Belkhadir Y, Jaillais Y (2015) The molecular circuitry of brassinosteroid signaling. *New Phytol* 206: 522 – 540

Bernardo-Garcia S, de Lucas M, Martinez C, Espinosa-Ruiz A, Daviere JM, Prat S (2014) BR-dependent phosphorylation modulates PIF4 transcriptional activity and shapes diurnal hypocotyl growth. *Genes Dev* 28: 1681 – 1694

Casal JJ, Balasubramanian S (2019) Thermomorphogenesis. *Annu Rev Plant Biol* 70: 321 – 346

Chae SH, Yoneyama K, Takeuchi Y, Joel DM (2004) Fluridone and norflurazon, carotenoid-biosynthesis inhibitors, promote seed conditioning and germination of the holoparasite Orobanche minor. *Physiol Plant* 120: 328 – 337

Chen W, Lv M, Wang Y, Wang PA, Cui Y, Li M, Wang R, Gou X, Li J (2019) BES1 is activated by EMS1-TPD1-SERK1/2-mediated signaling to control tapetum development in *Arabidopsis thaliana*. *Nat Commun* 10: 4164

Chory J, Nagpal P, Peto CA (1991) Phenotypic and genetic analysis of det2, a new mutant that affects light-regulated seedling development in *Arabidopsis*. *Plant Cell* 3: 445 – 459

Desaint H, Aoun N, Deslandes L, Vailleau F, Roux F, Berthome R (2021) Fight hard or die trying: when plants face pathogens under heat stress. *New Phytol* 229: 712 – 734

Dhaubhadel S, Browning KS, Gallie DR, Krishna P (2002) Brassinosteroid functions to protect the translational machinery and heat-shock protein synthesis following thermal stress. *Plant J* 29: 681 – 691

Divi UK, Rahman T, Krishna P (2016) Gene expression and functional analyses in brassinosteroid-mediated stress tolerance. *Plant Biotechnol J* 14: 419 – 432

Eremina M, Unterholzner SJ, Rathnayake AI, Castellanos M, Khan M, Kugler KG, May ST, Mayer KF, Rozhon W, Poppenberger B (2016) Brassinosteroids participate in the control of basal and acquired freezing tolerance of plants. *Proc Natl Acad Sci USA* 113: E5982 – E5991

Fridman Y, Savaldi-Goldstein S (2013) Brassinosteroids in growth control: how, when and where. *Plant Sci* 209: 24 – 31

Friedrichsen DM, Nemhauser J, Muramitsu T, Maloof JN, Alonso J, Ecker JR, Furuya M, Chory J (2002) Three redundant brassinosteroid early response genes encode putative bHLH transcription factors required for normal growth. *Genetics* 162: 1445 – 1456

Fuchs S, Grill E, Meskiene I, Schweighofer A (2013) Type 2C protein phosphatases in plants. *FEBS J* 280: 681 – 693

He JX, Gendron JM, Yang Y, Li J, Wang ZY (2002) The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in *Arabidopsis*. *Proc Natl Acad Sci USA* 99: 10185 – 10190

He JX, Gendron JM, Sun Y, Gampala SS, Gendron N, Sun CQ, Wang ZY (2005) BZR1 is a transcriptional repressor with dual roles in brassinosteroid homeostasis and growth responses. *Science* 307: 1634 – 1638

Hellens RP, Allan AC, Friel EN, Bolitho K, Grafton K, Templeton MD, Karunairetnam S, Gleave AP, Laing WA (2005) Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. *Plant Methods* 1: 13

Ibanes M, Fabregas N, Chory J, Cano-Delgado AI (2009) Brassinosteroid signaling and auxin transport are required to establish the periodic pattern of *Arabidopsis* shoot vascular bundles. *Proc Natl Acad Sci USA* 106: 13630 – 13635

Ibanez C, Delker C, Martinez C, Burstenbinder K, Janitza P, Lippmann R, Ludwig W, Sun H, James GV, Klecker M *et al* (2018) Brassinosteroids dominate hormonal regulation of plant thermomorphogenesis via BZR1. *Curr Biol* 28: 303 – 310 e303

Jacob P, Hirt H, Bendahmane A (2017) The heat-shock protein/chaperone network and multiple stress resistance. *Plant Biotechnol J* 15: 405 – 414

Jung HS, Crisp PA, Estavillo GM, Cole B, Hong F, Mockler TC, Pogson BJ, Chory J (2013) Subset of heat-shock transcription factors required for the early response of *Arabidopsis* to excess light. *Proc Natl Acad Sci USA* 110: 14474 – 14479

Kanei-Ishii C, Tanikawa J, Nakai A, Morimoto RI, Ishii S (1997) Activation of heat shock transcription factor 3 by c-Myb in the absence of cellular stress. *Science* 277: 246 – 248

Khan M, Rozhon W, Bigeard J, Pflieger D, Husar S, Pitzschke A, Teige M, Jonak C, Hirt H, Poppenberger B (2013) Brassinosteroid-regulated GSK3/Shaggy-like kinases phosphorylate mitogen-activated protein (MAP) kinase kinases,

© 2021 The Authors

which control stomata development in *Arabidopsis thaliana*. *J Biol Chem* 288: 7519 – 7527

Khan M, Rozhon W, Unterholzner SJ, Chen T, Eremina M, Wurzinger B, Bachmair A, Teige M, Sieberer T, Isono E *et al* (2014) Interplay between phosphorylation and SUMOylation events determines CESTA protein fate in brassinosteroid signalling. *Nat Commun* 5: 4687

Kim TW, Wang ZY (2010) Brassinosteroid signal transduction from receptor kinases to transcription factors. *Annu Rev Plant Biol* 61: 681 – 704

Lachowiec J, Lemus T, Thomas JH, Murphy PJ, Nemhauser JL, Queitsch C (2013) The protein chaperone HSP90 can facilitate the divergence of gene duplicates. *Genetics* 193: 1269 – 1277

Lachowiec J, Mason GA, Schultz K, Queitsch C (2018) Redundancy, feedback, and robustness in the *Arabidopsis thaliana* BZR/BEH gene family. *Front Genet* 9: 523

Larkindale J, Hall JD, Knight MR, Vierling E (2005) Heat stress phenotypes of *Arabidopsis* mutants implicate multiple signaling pathways in the acquisition of thermotolerance. *Plant Physiol* 138: 882 – 897

Lee JH, Schoffl F (1996) An Hsp70 antisense gene affects the expression of HSP70/HSC70, the regulation of HSF, and the acquisition of thermotolerance in transgenic *Arabidopsis thaliana*. *Mol Gen Genet* 252: 11 – 19

Leng L, Liang Q, Jiang J, Zhang C, Hao Y, Wang X, Su W (2017) A subclass of HSP70s regulate development and abiotic stress responses in *Arabidopsis thaliana*. *J Plant Res* 130: 349 – 363

Li J, Chory J (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* 90: 929 – 938

Li J, Nam KH, Vafeados D, Chory J (2001) BIN2, a new brassinosteroid-insensitive locus in *Arabidopsis*. *Plant Physiol* 127: 14 – 22

Li QF, Wang C, Jiang L, Li S, Sun SS, He JX (2012) An interaction between BZR1 and DELLAs mediates direct signaling crosstalk between brassinosteroids and gibberellins in *Arabidopsis*. *Sci Signal* 5: ra72

Liu HC, Charng YY (2012) Acquired thermotolerance independent of heat shock factor A1 (HsfA1), the master regulator of the heat stress response. *Plant Signal Behav* 7: 547 – 550

Liu HC, Liao HT, Charng YY (2011) The role of class A1 heat shock factors (HSFA1s) in response to heat and other stresses in *Arabidopsis*. *Plant Cell Environ* 34: 738 – 751

Liu Y, Zhang C, Chen J, Guo L, Li X, Li W, Yu Z, Deng J, Zhang P, Zhang K *et al* (2013) Arabidopsis heat shock factor HsfA1a directly senses heat stress, pH changes, and hydrogen peroxide via the engagement of redox state. *Plant Physiol Biochem* 64: 92 – 98

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

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* 324: 1064 – 1068

Martinez C, Espinosa-Ruiz A, de Lucas M, Bernardo-Garcia S, Franco-Zorrilla JM, Prat S (2018) PIF4-induced BR synthesis is critical to diurnal and thermomorphogenic growth. *EMBO J* 37: e99552

Mazorra LM, Holton N, Bishop GJ, Nunez M (2011) Heat shock response in tomato brassinosteroid mutants indicates that thermotolerance is independent of brassinosteroid homeostasis. *Plant Physiol Biochem* 49: 1420 – 1428

Meiri D, Breiman A (2009) *Arabidopsis* ROF1 (FKBP62) modulates thermotolerance by interacting with HSP90.1 and affecting the accumulation of HsfA2-regulated sHSPs. *Plant J* 59: 387 – 399

Mora-Garcia S, Vert G, Yin Y, Cano-Delgado A, Cheong H, Chory J (2004) Nuclear protein phosphatases with Kelch-repeat domains modulate the response to brassinosteroids in *Arabidopsis*. *Genes Dev* 18: 448 – 460

Nakamura A, Higuchi K, Goda H, Fujiwara MT, Sawa S, Koshiba T, Shimada Y, Yoshida S (2003) Brassinolide induces IAA5, IAA19, and DR5, a synthetic auxin response element in *Arabidopsis*, implying a cross talk point of brassinosteroid and auxin signaling. *Plant Physiol* 133: 1843 – 1853

Nolan TM, Vukasinovic N, Liu D, Russinova E, Yin Y (2020) Brassinosteroids: multidimensional regulators of plant growth, development, and stress responses. *Plant Cell* 32: 295 – 318

Oh E, Zhu JY, Wang ZY (2012) Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. *Nat Cell Biol* 14: 802 – 809

Ohama N, Sato H, Shinozaki K, Yamaguchi-Shinozaki K (2017) Transcriptional regulatory network of plant heat stress response. *Trends Plant Sci* 22: 53 – 65

Olate E, Jimenez-Gomez JM, Holuigue L, Salinas J (2018) NPR1 mediates a novel regulatory pathway in cold acclimation by interacting with HSFA1 factors. *Nat Plants* 4: 811 – 823

Park S-Y, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow T-F *et al* (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* 324: 1068 – 1071

Peng P, Yan Z, Zhu Y, Li J (2008) Regulation of the Arabidopsis GSK3-like kinase BRASSINOSTEROID-INSENSITIVE 2 through proteasome-mediated protein degradation. *Mol Plant* 1: 338 – 346

Poppenberger B, Rozhon W, Khan M, Husar S, Adam G, Luschnig C, Fujioka S, Sieberer T (2011) CESTA, a positive regulator of brassinosteroid biosynthesis. *EMBO J* 30: 1149 – 1161

Quint M, Delker C, Franklin KA, Wigge PA, Halliday KJ, van Zanten M (2016) Molecular and genetic control of plant thermomorphogenesis. *Nat Plants* 2: 15190

Richter K, Haslbeck M, Buchner J (2010) The heat shock response: life on the verge of death. *Mol Cell* 40: 253 – 266

Rozhon W, Mayerhofer J, Petutschnig E, Fujioka S, Jonak C (2010) ASKtheta, a group-III *Arabidopsis* GSK3, functions in the brassinosteroid signalling pathway. *Plant J* 62: 215 – 223

Rubio S, Rodrigues A, Saez A, Dizon MB, Galle A, Kim TH, Santiago J, Flexas J, Schroeder JI, Rodriguez PL (2009) Triple loss of function of protein phosphatases type 2C leads to partial constitutive response to endogenous abscisic acid. *Plant Physiol* 150: 1345 – 1355

Saito M, Kondo Y, Fukuda H (2018) BES1 and BZR1 redundantly promote phloem and xylem differentiation. *Plant Cell Physiol* 59: 590 – 600

Samakovli D, Margaritopoulou T, Prassinos C, Milioni D, Hatzopoulos P (2014) Brassinosteroid nuclear signaling recruits HSP90 activity. *New Phytol* 203: 743 – 757

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9: 671 – 675

Setsungnern A, Munoz P, Perez-Llorca M, Muller M, Thiravetyan P, Munne-Bosch S (2020) A defect in BRI1-EMS-SUPPRESSOR 1 (bes1)-mediated brassinosteroid signaling increases photoinhibition and photo-oxidative stress during heat stress in *Arabidopsis*. *Plant Sci* 296. 110470

Shigeta T, Zaizen Y, Sugimoto Y, Nakamura Y, Matsuo T, Okamoto S (2015) Heat shock protein 90 acts in brassinosteroid signaling through interaction with BES1/BZR1 transcription factor. *J Plant Physiol* 178: 69 – 73

Stephanou A, Isenberg DA, Nakajima K, Latchman DS (1999) Signal transducer and activator of transcription-1 and heat shock factor-1 interact and activate the transcription of the Hsp-70 and Hsp-90beta gene promoters. *J Biol Chem* 274: 1723 – 1728

Sun YU, Fan X-Y, Cao D-M, Tang W, He K, Zhu J-Y, He J-X, Bai M-Y, Zhu S, Oh E *et al* (2010) Integration of brassinosteroid signal transduction with the

 © 2021 The Authors

transcription network for plant growth regulation in *Arabidopsis*. *Dev Cell* 19: 765 – 777

Suzuki N, Bassil E, Hamilton JS, Inupakutika MA, Zandalinas SI, Tripathy D, Luo Y, Dion E, Fukui G, Kumazaki A *et al* (2016) ABA is required for plant acclimation to a combination of salt and heat stress. *PLoS One* 11: e0147625

Unterholzner SJ, Rozhon W, Papacek M, Ciomas J, Lange T, Kugler KG, Mayer KF, Sieberer T, Poppenberger B (2015) Brassinosteroids are master regulators of gibberellin biosynthesis in *Arabidopsis*. *Plant Cell* 27: 2261 – 2272

Unterholzner SJ, Rozhon W, Poppenberger B (2017) Analysis of *in vitro* DNA interactions of brassinosteroid-controlled transcription factors using electrophoretic mobility shift assay. *Methods Mol Biol* 1564: 133 – 144

Vu LD, Xu X, Gevaert K, De Smet I (2019) Developmental plasticity at high temperature. *Plant Physiol* 181: 399 – 411

Walter M, Chaban C, Schütze K, Batistic O, Weckermann K, Näke C, Blazevic D, Grefen C, Schumacher K, Oecking C *et al* (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J* 40: 428 – 438

Wang H, Tang J, Liu J, Hu J, Liu J, Chen Y, Cai Z, Wang X (2018) Abscisic acid signaling inhibits brassinosteroid signaling through dampening the dephosphorylation of BIN2 by ABI1 and ABI2. *Mol Plant* 11: 315 – 325

Wang Z-Y, Nakano T, Gendron J, He J, Chen M, Vafeados D, Yang Y, Fujioka S, Yoshida S, Asami T *et al* (2002) Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. *Dev Cell* 2: 505 – 513

Yeh CH, Kaplinsky NJ, Hu C, Charng YY (2012) Some like it hot, some like it warm: phenotyping to explore thermotolerance diversity. *Plant Sci* 195: 10 – 23

Yin Y, Vafeados D, Tao Y, Yoshida S, Asami T, Chory J (2005) A new class of transcription factors mediates brassinosteroid-regulated gene expression in *Arabidopsis*. *Cell* 120: 249 – 259

Yin Y, Wang ZY, Mora-Garcia S, Li J, Yoshida S, Asami T, Chory J (2002) BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. *Cell* 109: 181 – 191

Yoo SD, Cho YH, Sheen J (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc* 2: 1565 – 1572

Yoshida T, Ohama N, Nakajima J, Kidokoro S, Mizoi J, Nakashima K, Maruyama K, Kim J-M, Seki M, Todaka D *et al* (2011) *Arabidopsis* HsfA1 transcription factors function as the main positive regulators in heat shock-responsive gene expression. *Mol Genet Genomics* 286: 321 – 332

Yu X, Li L, Zola J, Aluru M, Ye H, Foudree A, Guo H, Anderson S, Aluru S, Liu P *et al* (2011) A brassinosteroid transcriptional network revealed by genome-wide identification of BESI target genes in *Arabidopsis* thaliana. *Plant J* 65: 634 – 646

Zhang S, Cai Z, Wang X (2009) The primary signaling outputs of brassinosteroids are regulated by abscisic acid signaling. *Proc Natl Acad Sci USA* 106: 4543 – 4548

Zhao C, Liu B, Piao S, Wang X, Lobell DB, Huang Y, Huang M, Yao Y, Bassu S, Ciais P *et al* (2017) Temperature increase reduces global yields of major crops in four independent estimates. *Proc Natl Acad Sci USA* 114: 9326 – 9331

Zhu JY, Oh E, Wang T, Wang ZY (2016) TOC1-PIF4 interaction mediates the circadian gating of thermoresponsive growth in *Arabidopsis*. *Nat Commun* 7: 13692

License: This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.