HsfB2b-mediated repression of *PRR7* directs abiotic stress responses of the circadian clock

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The circadian clock perceives environmental signals to reset to local time, but the underlying molecular mechanisms are not well understood. Here we present data revealing that a member of the heat shock factor (Hsf) family is involved in the input pathway to the plant circadian clock. Using the yeast one-hybrid approach, we isolated several Hsfs, including HEAT SHOCK FACTOR B2b (HsfB2b), a transcriptional repressor that binds the promoter of *PSEUDO RESPONSE REGULATOR 7* (*PRR7*) at a conserved binding site. The constitutive expression of HsfB2b leads to severely reduced levels of the *PRR7* transcript and late flowering and elongated hypocotyls. HsfB2b function is important during heat and salt stress because *HsfB2b* overexpression sustains circadian rhythms, and the *hsfB2b* mutant has a short circadian period under these conditions. *HsfB2b* is also involved in the regulation of hypocotyl growth under warm, short days. Our findings highlight the role of the circadian clock as an integrator of ambient abiotic stress signals important for the growth and fitness of plants.

circadian clock | Hsf | heat compensation | salt tolerance

The circadian clock is an endogenous timing mechanism that ensures that daily rhythmic processes are synchronized with the environment. The circadian oscillator runs with a period of ∼24 h. It entrains to environmental signals, such as light and temperature, and in this way is able to anticipate daily environmental changes (1). Molecular genetics and modeling have revealed that the circadian system comprises several interconnected feedback loops involving multiple phase-specific gene products (2).

In the model plant *Arabidopsis*, the morning feedback loops are composed of the major oscillator proteins CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which peak in abundance at dawn and activate the expression of early-late morning genes *PSEUDO RESPONSE REGULATOR 9* (*PRR9*) and *PRR7* (3). Pseudo-response regulator (PRR) proteins are transcriptional repressors, and PRR9 and PRR7 close the morning loops by binding to the *CCA1* and *LHY* promoters (4). The transcription of *PRR9* is acutely activated by light, a feature that is not shared by *PRR7* (3, 5). Disruption of either locus results in a mild clock phenotype, including an increased clock period of ∼1 h and an elongated hypocotyl (6–8). The double loss-of-function mutant exhibits a strong synergistic phenotype with free-running period lengths of up to 35 h and loss of temperature entrainment and compensation, indicating overlapping roles for *PRR9* and *PRR7* (3, 9, 10).

The ambient environment influences the circadian clock in a number of different ways. Although the most well-studied input cue is light, temperature is a major zeitgeber as well (11). Brief pulses (from minutes to hours, depending on the intensity) result in acute responses from the clock. Importantly, the severity of such responses is time-dependent and gated by the oscillator (12–14). Longer durations of zeitgeber change can function as an entrainment signal and generally follow the diurnal pattern of the light–dark cycle. In this way, clock phase is adjusted to match seasonal changes. In addition, the clock is temperature-compensated so that a ∼24-h period is maintained despite fluctuations in temperature.

The heat shock factor (Hsf) family of transcription factors is large in plants compared with fungi and animals, and the *Arabidopsis* genome encodes 21 Hsfs (15, 16). Many are activated on heat stress, but specific Hsfs respond to other abiotic stresses, including oxidative and drought stress (17). The function of many Hsfs remains to be characterized.

Hsfs bind to the heat shock element (HSE) repeat, which is an inverted repeat of the nucleotides nGAAn (17, 18). HsfB1 and HsfB2b have been confirmed to function in transcriptional repression, and their overexpression in the nucleus results in cell death (19, 20). *HsfB2b* is induced after heat stress (21) and has redundant functions with HsfB1 in the repression of *HsfA2* and *HEAT SHOCK PROTEIN* (*Hsp*) genes (22).

Here we have identified HsfB2b as a novel repressor of the morning clock gene *PRR7*. HsfB2b binds a conserved HSE site in the *PRR7* promoter and *HsfB2b-ox* plants have reduced *PRR7* expression, have elongated hypocotyls, and are late-flowering. We characterize *HsfB2b* as a circadian gene, and the loss-of-function mutant exhibits compromised temperature compensation under high temperature. *HsfB2b* also has a role in growth under warm conditions, along with the compensation of circadian rhythms under salt stress. Finally, the expression of *PRR7* target genes is altered by the *hsfB2b-1* mutation. We propose that *HsfB2b*-mediated repression is important for the appropriate expression pattern of *PRR7* and its target gene network involved in plant growth and stress responses.

Results

Identification of HsfB2b Binding to the *PRR7* Promoter. We performed a yeast one-hybrid screen with an arrayed *Arabidopsis* transcription factor library (23) using the *PRR7* promoter (*proPRR7*) as bait. We screened the 1-kb region upstream of

Significance

The daily environmental changes in light and dark set the pace of the circadian clock, which at the molecular level is composed of transcription-based feedback loops. It is well known that temperature fluctuations affect the circadian clock, but the molecules involved remain largely undefined. We found that the heat shock transcription factor HsfB2b works in the repression of the morning clock gene *PRR7*. We tested the properties of the circadian clock, and growth behavior, in the *hsfB2b* mutant and in transgenics with altered *HsfB2b* expression. Our results reveal that *HsfB2b* expression is important for accurate circadian rhythms following elevated temperature and/or salt treatment. Our work provides evidence of a molecular entry point for temperature signaling to the plant circadian clock.

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ATG and observed binding by several Hsf factors, including HsfA1b, HsfA3, HsfA6a, and HsfB2b, to a specific fragment of the promoter (−673/−328 bp; Fig. 1 and Fig. S1). On mutation of the critical G nucleotides in either of the two HSEs in *proPRR7* (termed HSE1mut or HSE2mut; *Materials and Methods*), Hsf binding was lost (Fig. 1*B*), supporting that Hsfs bind *proPRR7* in an HSE-dependent manner. In this validation of the Hsf candidates, we note that HSE1mut and HSE2mut most dramatically affected the fold change of HsfB2b binding to *proPRR7*. Thus, we focused follow-up studies on this Hsf isoform.

HsfB2b Is a Repressor of *PRR7*, and Its Basal Expression Is Circadian. To analyze the role of *HsfB2b* in regulating *PRR7* expression, we generated plants with altered *Hsf* expression and screened for phenotypes of the transgenic plants (*Hsf-ox*) in the Col-0 *PRR7:LUCIFERASE* (*LUC*) background. We used a construct encoding a GFP C-terminal tag under the control of the *UBIQUITIN10* (*UBQ10*) promoter (pro*UBQ10*:Hsf-*GFP*), which has intermediate strength compared with the popular 35S constitutive promoter. The 35S sequence was reported to contain potential HSE elements, and the overexpression of *HsfB2b* can lead to cell death (20, 24).

Interestingly, we found that independent *HsfB2b-ox* lines resulted in late flowering and loss of *PRR7:LUC* luminescence (Fig. 2*A* and Fig. S2). Under long days, *HsfB2b-ox* flowered with approximately double the number of leaves of WT Col-0. The *prr7-3* mutant flowered slightly late, at the ∼20-leaf stage, whereas the *hsfB2b-1* mutant had WT flowering time (Fig. 2*A*). The delay in flowering time for *HsfB2b-ox* was more extreme than the delayed flowering of the *prr7-3* null mutant under long days (8). This stronger phenotype of *HsfB2b-ox* suggests that additional flowering time regulators besides *PRR7* are targeted (22).

For the experiments reported herein, we chose *HsfB2b-ox* #23 as the representative line. The late flowering of *HsfB2b-ox* was also observed under short days (Fig. S3). Similar to the *prr7-3* mutant, *HsfB2b-ox* seedlings maintained under short photoperiods exhibited elongated hypocotyls (Fig. 2*B*). Interestingly, hypocotyl growth of *HsfB2b-ox* was increased on transfer to warm temperature in a similar manner to *prr7-3*, and, notably, the relative increase of *HsfB2b-ox* was less than that of Col-0, suggesting heat insensitivity (Fig. 2 *B* and *C*). In addition, the *hsfB2b-1* mutant had a heat-dependent, long hypocotyl phenotype (Fig. 2*C*). Taken together, our results suggest that *HsfB2b* has a role in regulating growth.

We next investigated for a circadian aspect of *HsfB2b*. Interestingly, although *HsfB2b* is a heat-induced gene (21), in the DIURNAL database *HsfB2b* is a rhythmic gene under most regimes, with a peak during the daytime (25). We fused the *HsfB2b* promoter to *LUC* and confirmed the circadian activity of the *HsfB2b* promoter under constant light (LL) conditions (Fig. 2*D*). In addition, we performed a circadian time course with samples obtained every 3 h and determined relative mRNA expression levels using quantitative RT-PCR (qPCR). Indeed, expression of *HsfB2b* was rhythmic under LL (Fig. 2*E*). *HsfB2b-ox* had similar amplitude of *HsfB2b* expression as the WT, but with a shifted phase (Fig. 2*E*). Specifically, the *HsfB2b-GFP* transgene profile, as measured by quantification of the *GFP* amplicon, indicated loss of rhythmicity of the *HsfB2b-GFP* transcript (Fig. 2*F*). Strikingly, and in accordance with the loss of *PRR7:LUC* bioluminescence (Fig. S2), we found very low levels of the *PRR7* transcript in *HsfB2b-ox*, revealing a strong repressor activity of HsfB2b (Fig. 2*G*).

We also analyzed the *hsfB2b-1* mutant, which has a T-DNA insertion in the first exon and confers a loss-of-function mutation (26). We detected subtle increases and phase advances of *PRR7* transcript levels (full-length and alternatively spliced *PRR7* transcripts, AS1 and AS3) in this background compared with WT, specifically an increase in *PRR7* expression during the first

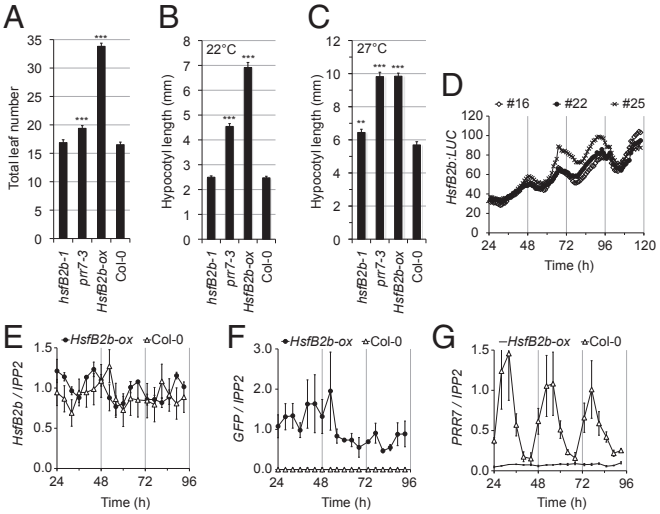

Fig. 2. *HsfB2b* alters growth and is a circadian gene. (*A*) Delayed flowering time of *HsfB2b-ox*. Plants were grown in soil under long-day conditions (16L:8D), and the total leaf number was determined at the time of bolting. The experiment was repeated twice, with similar results; $n > 18$. Error bars represent SD. (*B*) Hypocotyl length of 6-d-old *hsfB2b-1*, *prr7-3*, *HsfB2b-ox*, and Col-0 seedlings grown under short-day photoperiods at 22 °C. Error bars represent SEM of technical replicates; $n > 18$. (*C*) Hypocotyl length of 6-d-old seedlings after growth under constantly warm short days. Seedlings were grown at 22 °C for the first 3 d and then transferred to warm short days (27 °C). Error bars represent the SEM of technical replicates; $n > 23$. $^{**}P < 0.01$; $^{***}P < 0.001$ (Student *t* test). (*D*) *HsfB2b:LUC* activity under LL. Seedlings were monitored for 5 d under LL after entrainment in 12L:12D for 1 wk. Three independent T2 lines are shown. Error bars represent SEM of technical replicates; $n = 12$. (*E*–*G*) Gene expression in *HsfB2b-ox* (*proUBQ10:HsfB2b-GFP*). The values are relative to *IPP2* and measured by qPCR. (*E*) Endogenous *HsfB2b*, amplicon spanning the 3′ UTR. (*F*) *GFP*. Note that this line is in Col-0 WT background and thus has two *HsfB2b* transcripts (with and without *GFP*). (*G*) *PRR7*. Samples were obtained every 3 h on days 2–4 in LL after 12L:12D entrainment for 1 wk. The experiments were repeated at least twice. Error bars represent the SEM of biological replicates.

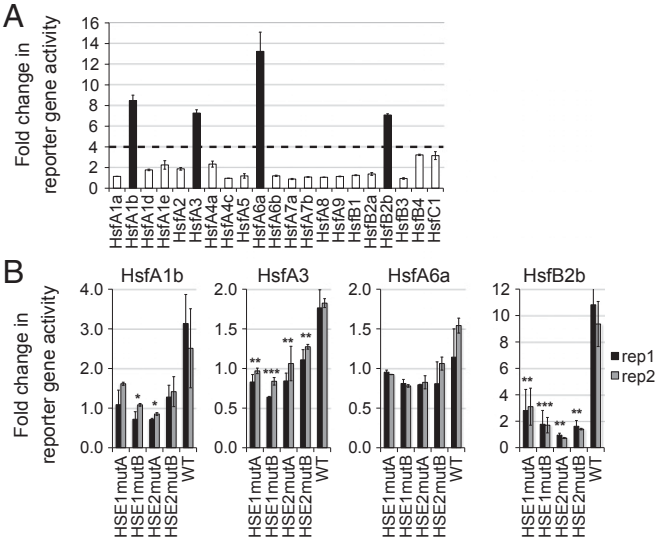

Fig. 1. Hsfs bind the *PRR7* promoter. (*A*) Hsf binding to the *PRR7* promoter in yeast one-hybrid assay. Twenty-one Hsf family members were tested. Data are the average of three technical replicates. Error bars represent SEM. The fold change in reporter activity is relative to the background in the pEXP502 negative control strain. (*B*) Mutation of HSE1 or HSE2 abolishes Hsf binding to *proPRR7* in yeast. The fold change of β-galactosidase activity of each HSE-mutated strain was obtained by normalizing to the negative control. Black and gray bars denote biological replicates, and error bars represent the SEM of the technical replicates. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student *t* test).

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subjective day, followed by a gradual decrease on days 2 and 3, indicative of a *PRR7* negative feedback (Fig. S4). Collectively, these phenotypes are in agreement with HsfB2b repressor function and the redundancy among multiple factors in heat-signaling pathways (22, 27).

HsfB2b Affects Temperature Compensation and Clock Resetting. The morning genes of the circadian clock are involved in temperature compensation of the circadian system (10). To investigate a role for HsfB2b in compensation of the circadian clock after heat exposure, we determined the free-running period length of the clock at 22 °C compared with 27 °C. To this end, we crossed the *PRR7:LUC* reporter into the *hsfB2b-1* mutant, and because the luminescence of *PRR7:LUC* was absent in *HsfB2b-ox*, we crossed the *CCA1:LUC* reporter into the *HsfB2b-ox* background. We found that *hsfB2b-1* failed to sufficiently compensate period length and displayed a 1- to 2-h shorter period compared with WT, and that this temperature defect was absent in *HsfB2b-ox* (Fig. 3*A*).

Temperature serves as an entrainment cue for the circadian clock. To test the effect of heat as an entrainment signal, we performed a temperature phase response curve (tPRC). We note that the shape of the warm tPRC (transfer from 18 °C to 22 °C) is similar to the cold tPRC (transfer from 22 °C to 12 °C); however, the break point (switch from phase delays to advances) is ∼CT6 (circadian time, CT) for the cold tPRC and ∼CT18 for warm tPRC (28, 29). We tested the resetting behavior of *CCA1:LUC* to heat pulses (38 °C) applied in LL after entrainment to 12-h light:12-h dark (12L:12D) cycles and constant 22 °C. In agreement with an earlier report (29), Col-0 seedlings displayed phase delays from subjective late day to middle of the night, and advances from late night to late morning (i.e., exhibiting little resetting during the day). Under these conditions, *HsfB2b-ox* displayed no phase advances compared with Col-0, along with a later breakpoint (Fig. 3*C*). Interestingly, we found a similar phenotype for the *prr7-3* mutant (Fig. 3*C*), in agreement with a role for HsfB2b-mediated control of *PRR7* expression in heat-responsive resetting.

HsfB2b Buffers the Clock Period After Salt Treatment. *OsHsfB2b*, the *HsfB2b* ortholog in rice, was recently reported to be involved in salt and drought tolerance (30). Thus, we tested the effect of salt on circadian phenotypes. To this end, we transferred seedlings to salt-containing medium and monitored the bioluminescence rhythms under LL at 22 °C. WT seedlings maintained robust circadian oscillations except for an ∼1-h phase delay compared with control medium (Fig. S5). In contrast, the *hsfB2b-1* mutant displayed short-period rhythms after transfer to salt (0.7 h shorter than Col-0 on salt and 0.7 h shorter than the *hsfB2b-1* control on no salt). *HsfB2b-ox* maintained WT period length on average; however, we observed a greater variation in period length and less robust rhythms, as indicated by higher relative amplitude error (RAE) values (Figs. 3 *A* and *B* and 4 *A* and *B*).

In nature, several stress conditions often occur simultaneously (31). We tested the circadian rhythms of *hsfB2b-1* and *HsfB2b-ox* seedlings under the combination of salt and higher temperature (27 °C). Interestingly, the heat-dependent short period of the *hsfB2b-1* mutant was enhanced by salt (Figs. 3*A* and 4*A*). *HsfB2b-ox* exhibited no major change in period, but had less robust rhythms compared with the control plants (Fig. 4*B*). Collectively, *HsfB2b* expression is required to buffer the detrimental effects of drought and heat on the clock system.

The circadian clock gates acute effects on stress. To assess the acute salt response, we used the *HsfB2b:LUC* and *PRR7:LUC* reporter lines and measured their activity after salt application at different times of the day. We found that both reporters had a gated response to salt uptake and, interestingly, were inversely correlated, in agreement with a repressor function for HsfB2b on *PRR7* expression (Fig. 4*C*). We note that the acute response to salt for *PRR7* is similar to the response of the desiccation-responsive gene *RD29A* (32).

An Upstream Role for *HsfB2b* in Hypocotyl Elongation Growth. To investigate the effect of the *hsfB2b-1* mutation in a *PRR7*-sensitized background, we crossed the *hsfB2b-1* mutation into *proPRR7:HA-PRR7* (*PRR7* minigene, *7MG*), which features elevated PRR7 levels and a shortened circadian period (33). Because *HsfB2b* is involved in growth (Fig. 2), we compared hypocotyls of seedlings grown under short days at 22 °C and seedlings grown at 27 °C. Both lines displayed elongated hypocotyls after heat exposure, but the *hsfB2b-1 7MG* seedlings grew more than the single *7MG* line (Fig. S6), indicating a role for HsfB2b-mediated repression of *PRR7* after heat exposure.

PRR7 is a transcriptional repressor and is associated with several genomic loci at ZT12 (34). To identify PRR7-regulated genes involved in the thermosensory regulation of growth, we looked at the diurnal expression of PRR7 target genes (PRR7-ChIP dataset) using available microarray data (DIURNAL database) (25, 34). Specifically, we reasoned that genes with increased expression in short-day photoperiods compared with long-day photoperiods were important, because the clock controls the phase of growth (35, 36). We identified 47 genes that were phase-advanced (1–9 h; Table S1). Apart from core clock genes (*CCA1*, *LUX*, and *PRR7*), many genes included in this list are known to be involved in hypocotyl growth, including *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*), *PIF5*, *REVEILLE2* (*RVE2*), *RVE7*, *ELONGATED HYPOCOTYL5* (*HY5*),

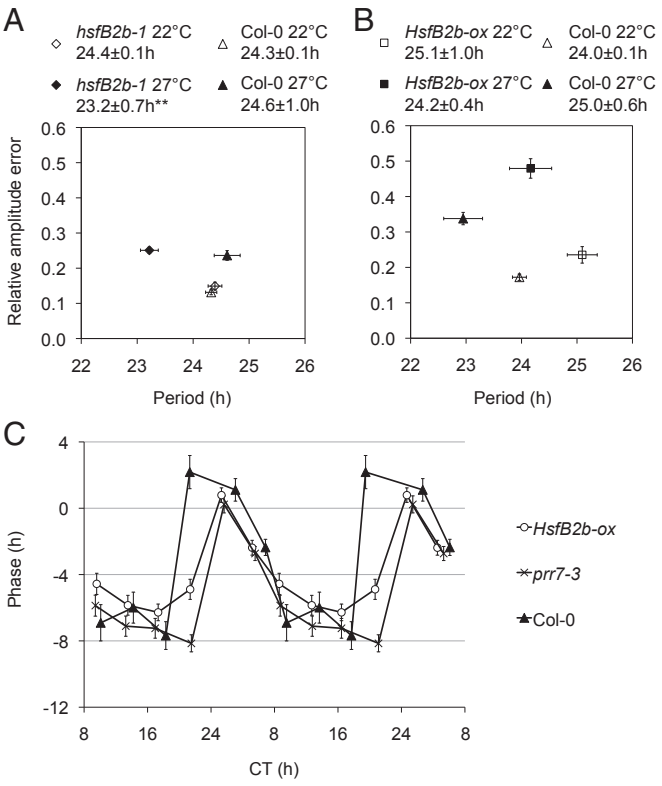

Fig. 3. HsfB2b is important for sustenance of period under high temperature. (*A* and *B*) Temperature compensation of *hsfB2b-1 PRR7:LUC* and *HsfB2b-ox CCA1:LUC* at 22 °C and 27 °C. Seedlings were entrained under 12L:12D at 22 °C for 1 wk and transferred to constant conditions at ZT0. The graphs show the mean period estimate of single seedlings vs. the mean RAE of the estimates. Low RAE indicates robust rhythmicity. Shown are RAE-weighted period means and SEM at 22 °C and 27 °C for *hsfB2b-1 PRR7:LUC* (*A*) and *HsfB2b-ox CCA1:LUC* (*B*). $^{**}P < 0.01$ (Student *t* test) for 22 °C compared with 27 °C; $n = 10–20$. (*C*) tPRCs of *HsfB2b-ox*, *prr7-3*, and Col-0. Seedlings harbored the *CCA1:LUC* reporter and were entrained for one week (under 12L:12D at 22 °C) and then released in LL at 22 °C. Phase resetting pulses of 38 °C were applied for 3h at different times during the circadian cycle (circadian time, CT; corrected for period length). The circadian phase changes were measured based on the circadian phase of nonpulsed plants. Error bars represent SEM of technical replicates; $n = 15–26$.

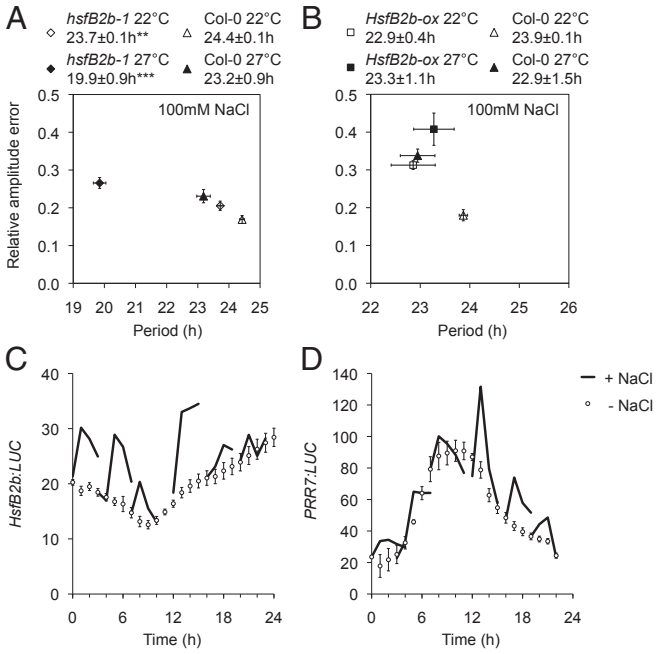

Fig. 4. Circadian and *HsfB2b*-mediated responses on salt exposure. (*A* and *B*) Compensation response of circadian rhythms to salt. Shown are RAE-weighted period means and SEM for *hsfB2b-1 PRR7:LUC* (*A*) and *HsfB2b-ox CCA1:LUC* (*B*) on 100 mM NaCl at 22 °C and 27 °C. $^{**}P < 0.01$; $^{***}P < 0.001$ (Student *t* test) for 22 °C compared with 27 °C; $n = 11$–19. (*C* and *D*) The *HsfB2b* and *PRR7* promoters have a gated response to salt exposure. One-week-old seedlings were sprayed with 5 M NaCl at 4-h intervals under LL, and luminescence was monitored for ∼6 h and compared with control seedlings. The luminescence at each time point was normalized to the luminescence before treatment. Error bars represent SEM of three biological replicates for *HsfB2b:LUC* and two biological replicates for *PRR7:LUC*; $n = 9$–18.

HY5 HOMOLOG (*HYH*), and members of the *B-BOX* (*BBX*) family (*BBX24*/*STO*, *BBX25*/*STH*, and *BBX29*) (35–41).

A subset of these genes (*RVE7*, *PIF4*, *BBX25*, and *BBX29*) exhibited elevated expression at certain time points at 27 °C compared with 22 °C in *hsfB2b-1 7MG*, a temperature phenotype that was not observed in the *7MG* line (Fig. 5 and Fig. S7). We note that the overexpression of *PIF4*, *BBX25*, or *BBX29* leads to elongated hypocotyls (35, 42) (Fig. S8), whereas *RVE7* may be a negative regulator of hypocotyl growth (37, 43).

We propose two possible scenarios for HsfB2b regulation of growth. First, HsfB2b may act through the repression of *PRR7* after a temperature increase. Second, HsfB2b may function independent of *PRR7* to regulate common target genes. Supporting the latter scenario, *RVE7* is derepressed at 27 °C in the *hsfB2b-1* single mutant, and *PRR7* levels are unchanged in *hsfB2b-1 7MG* and *7MG* (Fig. 5 and Fig. S7). Collectively, the output genes *PIF4*, *BBX25*, *BBX29*, and *RVE7* are candidate members of the HsfB2b- and PRR7-mediated pathway(s) controlling thermosensory hypocotyl growth.

Discussion

In this work, we positioned HsfB2b in the temperature input pathway to the circadian clock by transcriptional regulation of *PRR7*. A study of Hsf1's action on the circadian clock in mammalian cells was reported recently; however, the molecular mechanism remains unknown (44, 45). In general, Hsfs have been described to target *HSP* genes via multiple HSE repeats in their promoters, and few studies exist where Hsfs regulate non-*HSP* genes (46–48). Importantly, mammalian Hsfs are known to control the expression of various developmental genes in addition to *HSP*s, and it recently has become clear that the stress-independent expression of HSF1 is crucial in oncogenesis (49, 50). Thus, it appears that the low basal circadian expression of *HsfB2b* (DIURNAL; Fig. 2*E*) may have important functions, specifically in maintaining the transcriptional state of the circadian clock. In future studies, it would be interesting to determine the degree to which Hsf-mediated input to the circadian system is evolutionarily conserved.

Temperature compensation is a key property of the circadian clock. It has been shown that the morning genes of the circadian system, including *PRR7*, were important for maintenance of period length under divergent temperatures (10). We found that the *hsfB2b-1* mutant was uncompensated and had a shorter period than WT specifically at the higher temperature (Fig. 3 *A* and *B*). Repression of *PRR7* by HsfB2b may maintain *PRR7* levels, and this correlates with period length; that is, it is known that

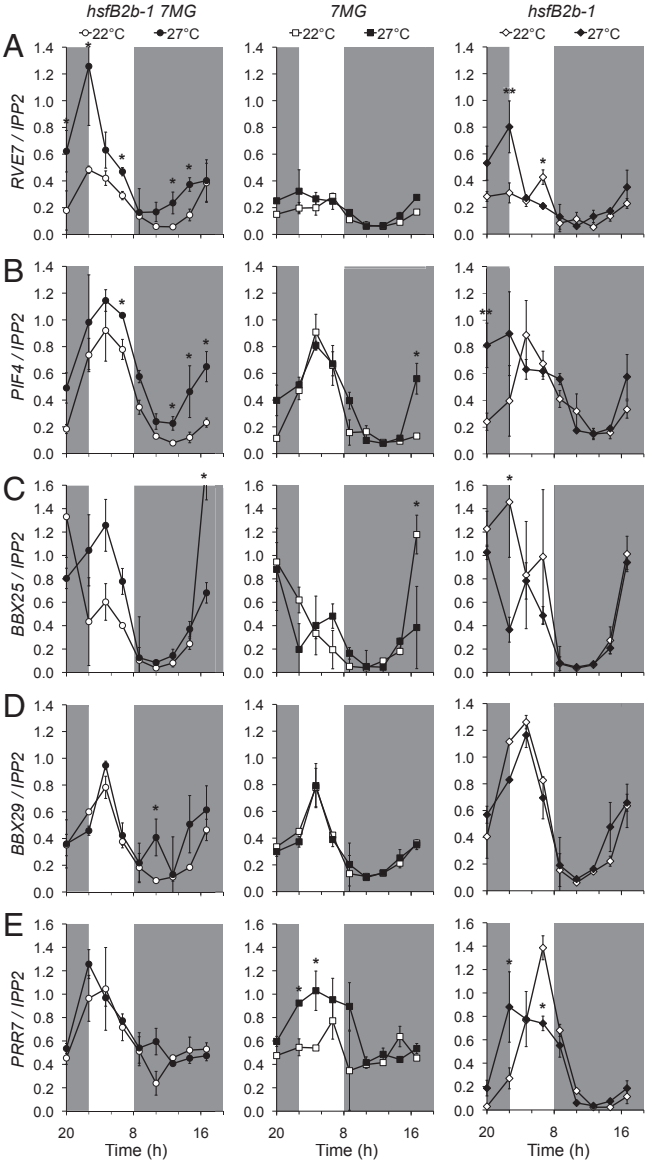

Fig. 5. Loss of *HsfB2b* in the *proPRR7:HA-PRR7* (*7MG*) background derepresses gene expression related to growth under warm short days. Shown are *RVE7* (*A*), *PIF4* (*B*), *BBX25* (*C*), *BBX29* (*D*), and *PRR7* (*E*) expression in *hsfB2b-1 7MG*, *7MG*, and *hsfB2b-1* under 22 °C and 27 °C short days. The seedlings were grown under short-day photoperiods at room temperature (22 °C) or under warm short days at 27 °C. For the latter, seedlings were grown at 22 °C for the first 3 d and then transferred to 27 °C. The seedlings were harvested on day 7. Error bars represent the SD of two biological replicates. $^{*}P < 0.05$ (Student *t* test) for 22 °C compared with 27 °C.

7MG lines can have a short period and the *prr7-3* mutant can have a long period under LL (3, 33). Gating of acute inputs to the circadian system is another important feature of the circadian clock. We found that the *HsfB2b* and *PRR7* promoters responded acutely to the application of salt, and that the salt-induced patterns had a circadian profile. Possibly, the gated profile of *HsfB2b* expression is directly connected to its circadian expression (Figs. 2 *D* and *E* and 4 *C* and *D*). It was previously shown that *RD29A* had a gated induction by salt, and that this likely is connected to GI, a core circadian protein involved in flowering time (32). Future studies will help determine whether there is a direct connection between PRR7 and GI activities regarding circadian compensation of salt and drought responses.

In the mammalian clock system, Hsf1 has a key role in clock resetting to temperature (44, 45). We found that *HsfB2b-ox* altered the resetting to warm temperature in *Arabidopsis* (Fig. 3*C*). The tPRC for *HsfB2b-ox* was not identical to the tPRC for the *prr7-3* mutant, possibly because HsfB2b also represses other genes involved in temperature perception (e.g., *Hsp*), which may provide feedback for the regulation of circadian rhythms (22, 51). Of note, misexpression due to the ubiquitous tissue expression of *proUBQ10:HsfB2b-GFP* could explain the strong growth phenotypes observed in *HsfB2b-ox*, given that endogenous *HsfB2b* expression is strongest in roots and floral organs and overlaps with *PRR7* expression only in these tissues (52). *PRR7* is expressed in all organs except siliques (52). We also note here that under short days, *HsfB2b-ox* flowered later than *prr7-3*, and that the heat-dependent acceleration of flowering at 27 °C compared with at 22 °C was diminished for *HsfB2b-ox* (Fig. S2). Hsp90 is involved in the proteostasis of clock proteins, and down-regulation of Hsp90 using RNAi resulted in late-flowering plants, a phenotype similar to *HsfB2b-ox* (53, 54).

Hsf genes are abundant in plants compared with other organisms, and the activity of certain Hsfs are specific to different biotic or abiotic stresses (16). *Thellungiella salsuginea*, an extremophile closely related to *Arabidopsis*, has 28 Hsfs, supporting the importance of this transcription factor family in abiotic stress responses (55). A previous transcriptomic study revealed the involvement of *HsfB2b* in the necrotrophic pathogen response, but the direct molecular target of HsfB2b is in the corresponding signaling pathway(s) remains unclear (26). Another microarray study revealed that *HsfB2b* has overlapping functions with *HsfB1* in thermotolerance, and that the *Hsp* genes are misregulated in the double-mutant background (22). In rice, *OsHsfB2b* is a negative regulator of drought and salt tolerance (30). We found that HsfB2b has a positive role in maintaining the circadian period under salt stress (Fig. 4). Future experiments will reveal to what degree the function of HsfB2b is conserved between monocotyledonous and dicotyledonous plants.

The circadian clock controls rhythmic hypocotyl growth, which occurs at the end of the night. With regard to PRR7 target genes known to be involved in growth, *PIF4* has been characterized extensively (35, 36, 56, 57). In addition to *PIF4*, we found increased levels of *RVE7*, *BBX25*, and *BBX29* at 27 °C compared with 22 °C in the *hsfB2b-1 7MG* double transgenic, an increase not seen in the single *7MG* line (Fig. 5). Our finding of several genes with altered expression in this analysis is hardly a surprise; for example, PRR5 also has *RVE7* and *BBX29* as direct targets (58), and *BBX25* is working with *HY5* in a signaling pathway specific to UV-B responses, whereas *PIF4* is involved in growth regarding red light, temperature, and GA signaling (56, 59). There is cross-talk between these pathways; for example, it was recently shown that the PIFs and HY5 share binding sites in their target promoters (60), and there are feedback loops within the circadian clock involving PRR7 and PRR5. *RVE7* is an interesting candidate because its promoter contains HSE repeats, and no HSE repeats are found in the promoters of the other PRR7 targets that we investigated here (www.arabidopsis.org). Future studies will help determine the molecular mode of action of the transcription factor complexes controlling hypocotyl growth, and identify whether the PRR7-mediated repression of the four candidate target loci is temperature-regulated.

Here we have provided further proof of concept that the *Arabidopsis* TF ORF clone collection is a sensitized genomic tool for identifying key transcriptional regulators in the expanding network of the circadian system (23). Our discovery that *HsfB2b* is involved in heat and salt input signaling to the circadian clock provides a molecular target for improving stress tolerance in plants.

Materials and Methods

Promoter Analysis. Details are provided in *SI Materials and Methods*.

Plant Materials and Growth Conditions. All *Arabidopsis* lines used were in the Col-0 background (reporter lines *PRR7:LUC* for *Hsf-ox*; *CCR2:LUC* for 7MG lines). Mutant and transgenic lines were *prr7-3* (3), *hsfB2b-1* (SALK-047291) (26), *proPRR7:HA-PRR7 prr7-3 CCR2:LUC #151* (7MG) (33), and *CCA1:LUC* (61). *Hsf-ox* transgenic lines were generated in the *PRR7:LUC* background using *A. tumefaciens*-mediated transformation (floral dip) (62). *HsfB2b-ox CCA1:LUC* was generated by crossing *HsfB2b-ox* #23 to *CCA1:LUC*. For flowering time measurements, seeds were sown in soil (Sunshine Mix #3), fertilized once monthly (15–16-17 Peat Lite Special; Everris) and grown under a controlled environment (Conviron) with the indicated photoperiod at ∼20 °C. The total leaf number was scored at the time of bolting. For hypocotyl measurement, seeds were stratified for 3 d and then grown on 1× MS (no sucrose) vertical plates under short days in a growth chamber (Percival) at ∼20 μE intensity at the indicated temperature. The seedlings were scanned on day 6, and the hypocotyls were measured using ImageJ software. For RNA isolation, seedlings were grown in growth chambers (Percival) with lights at 60–80 μE intensity. Flowering and growth experiments were performed two or three times, yielding similar results.

Molecular Cloning and Constructs. For the Y1H screen, the −673/−328-bp region of the *PRR7* promoter was subcloned in pENTR/TOPO-D (Gateway; Invitrogen) and then cloned in pGLacZi (63). Primers are listed in Table S2. The primers for site-directed mutagenesis of pENTR-proPRR7 were designed using QuikChange Primer Design (Agilent Technologies), and PCR was performed with PfuTurbo DNA polymerase (Agilent Technologies), followed by *Dpn*I digestion and cloning. The HSEmut constructs were verified by sequencing. The full-length promoter of *HsfB2b* (3,082 bp, including the 5′ UTR) and *PRR7* (1,096 bp, including the 5′ UTR), respectively, was amplified with ExTaq polymerase (TaKaRa) from gDNA, subcloned in pENTR/TOPO-D, and fused to *LUC* in the Gateway-compatible destination vector pFLASH (64). The *proUBQ10:Hsf-GFP* (*Hsf-ox*) constructs were generated by multisite cloning in R4pGWB504 (65), the *Hsf* ORFs subcloned in pENTR/TOPO-D and *proUBQ10* (646 bp upstream of ATG; At4g05320) in pDONRP4P1R (Invitrogen).

Luciferase Imaging. Details are provided in *SI Materials and Methods*.

Gene Expression Analysis. Details are provided in *SI Materials and Methods*.

Yeast One-Hybrid Screen. The pGLacZi-proPRR7 and HSEmut constructs were integrated into the genome of the yeast strain YM4271 using NcoI. The yeast one-hybrid screen and HSEmut assays were performed as described previously (23, 66). Ortho-nitrophenyl-β-galactoside (ONPG) served as the substrate for measurement of β-galactosidase activity.

***Arabidopsis* Gene Identifiers.** *Arabidopsis* gene identifiers were as follows: *BBX25*, At2g31380; *BBX29*, At5g54470; *CCA1*, At2g46830; *HsfB2b*, At4g11660; *PIF4*, At2g43010; *PRR7*, At5g02810; *RVE7*, At1g18330; *UBQ10*, At4g02310.

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