The ability of an organism to acclimate to its environment is a key determinant in its global distribution and capacity to compete with other organisms. The heat stress response, a highly conserved environmental and developmental program in eukaryotic and prokaryotic organisms, is an important component of the acclimation response of plants. Previous studies have shown that heat shock transcription factors play an important role in thermotolerance in plants and other organisms, controlling the expression of different heat shock proteins and detoxifying enzymes. In contrast, although several other pathways, involving ethylene, salicylic acid (SA), and trehalose, were recently shown to play a central role in thermotolerance in plants, a key regulator of these responses was not identified. Here we report that the highly conserved transcriptional co-activator, MBF1c (multiprotein bridging factor 1c), is a key regulator of thermotolerance in Arabidopsis thaliana. MBF1c protein accumulates rapidly and is localized to nuclei during heat stress. MBF1c is required for thermotolerance and functions upstream to SA, trehalose, ethylene, and pathogenesis-related protein 1 during heat stress. In contrast, MBF1c is not required for the expression of transcripts encoding HSFA2 and different heat shock proteins. Interestingly, MBF1c interacts with TPS5 (trehalose phosphate synthase 5), which is also heat-inducible, and mutants deficient in TPS5 are thermosensitive. Our results provide evidence for the existence of a tightly coordinated heat stress-response network, involving trehalose-, SA-, and ethylene-signaling pathways, that is under the control of MBF1c.

Thermotolerance is an essential component of the acclimation response of different organisms (1–6). It is generally divided into acquired thermotolerance (i.e. the ability to acquire tolerance to otherwise lethal heat stress) and basal thermotolerance (i.e. the inherent ability to survive temperature above the optimal growth) (5). Previous studies have shown that heat shock transcription factors (HSFs) play an important role in thermotolerance in plants and other organisms, regulating heat shock proteins (HSPs) as well as different acclimation and detoxification proteins (7–10). In contrast, although several other pathways, involving ethylene, salicylic acid (SA), and trehalose, have recently been proposed to play an important role in thermotolerance in plants (5, 11–15), a key regulator of these responses was not identified.

MBF1 (multiprotein bridging factor 1) is a highly conserved transcriptional co-activator involved in the regulation of diverse processes, such as endothelial cell differentiation, hormone-regulated lipid metabolism, central nervous system development, and histidine metabolism (16–19). MBF1 proteins from different organisms interact with transcription factors, such as c-Jun, GCN4, and ATF1, or with different hormone receptors and link them with the TATA-binding protein (16–20). The flowering plant Arabidopsis thaliana contains three different genes encoding MBF1, all of which can complement MBF1 deficiency in yeast (21). MBF1a (At2g42680) and MBF1b (At3g58680) are developmentally regulated (22). In contrast, the expression of MBF1c (At3g24500) is elevated in Arabidopsis in response to pathogen infection, salinity, drought, heat, hydrogen peroxide, and application of the plant hormones abscisic acid or SA (21–23).

Here we report that MBF1c is a key regulator of thermotolerance in Arabidopsis. MBF1c is required for basal thermotolerance and functions upstream to SA, trehalose, and ethylene during heat stress. In contrast, MBF1c is not required for acquired thermotolerance or the expression of HSFA2 and different HSPs. Interestingly, MBF1c associates with a heat-induced protein thought to be involved in trehalose biosynthesis (TPS5 (trehalose phosphate synthase 5); At4g17770) and is localized to the nuclei of cells during heat stress.

**EXPERIMENTAL PROCEDURES**

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^9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3 and Table 1.

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under controlled growth conditions, mbf1c, tps5-1, and tps5-2 did not display any visible phenotype (not shown). Plant transformation, null mutant rescue, and selection were performed as described in Refs. 24–27. GFP or GFP fused in-frame to the C-terminal of MBF1c was expressed in plants under the control of the 35 S CaMV promoter, using a pGreen-based vector, and visualized using a Nikon Eclipse E400 epifluorescence microscope (Nikon Corp., Tokyo, Japan) or an Olympus, IX 81 FV 1000 confocal microscope (Olympus, Tokyo, Japan), with 4',6-diamidino-2-phenylindole as control for nuclei (28). Leptomycin B (2 μM) was used to inhibit nuclear export (29). T3 homozygous lines obtained from three individual transformation events, a homozygous null mutant, and two T3 complemented homozygous null mutants were used for all heat stress studies of MBF1c, and two independent homozygous null mutants were used for all heat stress studies of TPS5 (5, 23, 26). Basal and acquired thermotolerance were measured as described in Refs. 35. The effects of trehalose (1 mM), 1-aminocyclopropane-1-carboxylic acid (ACC) (10 μM), or SA (0.1 mM) on mutant survival were studied as described in Refs. 11 and 30–32, using a 2-h treatment at 45 °C.

For the analysis of transcript, protein, and metabolite accumulation, 2-week-old plants were subjected to 38 °C, 100 μmol m⁻² s⁻¹, and sampled at 0, 10, 20, 30, and 60 min. Control plants kept at 21 °C were sampled in parallel. All experiments were performed in triplicates and repeated at least three times. Statistical analysis was performed as described in Ref. 23.

Molecular and Biochemical Analysis—RNA and protein were isolated and analyzed according to Refs. 24–26. cDNA probes corresponding to the following genes were used for RNA gel blots: PR1, At2g14610; TPS5, At4g17770; TPS11, At2g18700; Zat12, At5g59820; Zat7, At3g46080; APX1, At1g07890; HSFA2, At2g26150; HSP70, At3g12580; HSP90, At5g52640; HSP101, At1g74310. RT-PCR was performed according to Ref. 33, using the following primers for TPS5: 5'-TGTCAGCCAAAAGAGCTTATGGAAC, 3'-ATCATC-ACGACGCAGAGATGAAA. Metabolic analysis was performed as described in Refs. 23, 32, and 34 (supplemental materials). Yeast two-hybrid analysis was performed at the University of Wisconsin Molecular Interaction Facility, as described in Ref. 35. Testing for protein-protein interaction by growth on selection media and β-galactosidase activity was performed as described in Ref. 35. Analysis of microarray data, available on the World Wide Web, was performed as previously described (36). The following annotation for TPS was used: TPS1, At1g78580; TPS2, At1g16980; TPS3, At1g17000; TPS4, At4g27550; TPS5, At4g17770; TPS6, At1g60620; TPS7, At1g06410; TPS8, At1g07290; TPS9, At1g23870; TPS10, At1g60140; TPS11, At2g18700.

Protein Purification and Antibody Production—The coding region of MBF1c was expressed in Escherichia coli BL21 Gold cells using the pCAL-n vector (Stratagene, La Jolla, CA). MBF1c expression was induced by 1 mM isopropyl 1-thio-D-galactopyranoside in 1 liter of liquid LB medium for 3 h at 37 °C. Cells were harvested, and MBF1c was purified from inclusion bodies (37), resuspended in 1 M Tris-HCl (pH 8.0), and separated by 12% SDS-PAGE. MBF1c protein was cut from gels and used for the production of rabbit polyclonal antibodies (AnaSpec Inc., San Jose, CA).

Results

Characterization of MBF1c Protein in Arabidopsis—MBF1c protein rapidly accumulated in Arabidopsis leaves during heat stress (Fig. 1A). In contrast, MBF1c protein was not detected in wild type plants grown under controlled conditions or in a null
mutant of MBF1c (mbf1c; SALK_083813). MBF1c protein was detected in transgenic plants constitutively expressing MBF1c (35S::MBF1c), grown under controlled conditions, or subjected to heat stress (Fig. 1A). The size of MBF1c protein detected by protein blots in Arabidopsis plants precisely corresponded to the predicted molecular weight of the MBF1c open reading frame (16.4 kDa; not shown).

To examine subcellular localization of MBF1c, MBF1c-GFP fusion protein (C-terminal fusion) and control GFP protein were expressed in transgenic plants under the control of the 35S CaMV promoter (35S::MBF1c-GFP and 35S::GFP, respectively). When transgenic plants with constitutive expression of MBF1c-GFP were grown under controlled conditions, MBF1c-GFP fusion protein was mainly localized to the cytosol (Fig. 1, B–D). In contrast, when plants were subjected to heat stress (1 h, 38 °C), MBF1c-GFP protein was mainly detected in nuclei, whereas the localization of the GFP control protein was not altered (Fig. 1, B–D). This response, observed with three independent transgenic lines expressing MBF1c-GFP at different levels (not shown), was not effected by leptomycin B (2 μM), an inhibitor of nuclear export, suggesting that nuclear localization of MBF1c during heat stress is a result of enhanced nuclear import (not shown).

Thermotolerance of Knock-out Plants Deficient in MBF1c Expression—A null mutant of MBF1c (mbf1c; SALK_083813) was isolated and subjected to heat stress. mbf1c did not accumulate MBF1c protein in response to heat stress (Fig. 1A). Wild type, mbf1c, and 35S::MBF1c seedlings were directly subjected to heat stress (41, 43, or 45 °C) for 2 h to measure basal thermotolerance or treated at 38 °C for 1.5 h, allowed to recover for 1 h at 21 °C, and subjected to 45 °C for 2 h (38 → 45 °C) to measure acquired thermotolerance. Seedlings were also kept at 45 °C until all wild type plants died (45 °C L). The basal thermotolerance phenotype of mbf1c plants at different times (min) following heat stress (38 °C) was measured to determine the rate of recovery (Fig. 2B). This response, observed with three independent transgenic lines expressing MBF1c-GFP at different levels (not shown), was not effected by leptomycin B (2 μM), an inhibitor of nuclear export, suggesting that nuclear localization of MBF1c during heat stress is a result of enhanced nuclear import (not shown).

**Transcript Expression in MBF1c Null Mutants during Heat Stress**—Transcript expression of MBF1c was enhanced as early as 10 min following heat stress application (Fig. 3). In contrast, MBF1c transcript was not detected in mbf1c plants. Compared with wild type, transcript expression of HSFA2, several different HSPs, and cytosolic APX1 (ascorbate peroxidase 1) was not...
altered in mbf1c plants during heat stress (Fig. 3). In contrast, PR-1 (pathogenesis-related protein 1) transcript expression was not detected in mbf1c plants during heat stress, and the expression of transcripts encoding the reactive oxygen species response zinc finger protein Zat12 (24–26) was enhanced in mbf1c plants compared with wild type plants during heat stress. In contrast to Zat12 and compared with wild type plants, the expression of Zat7, which functions as a repressor (35), decreased in mbf1c plants during heat stress (Fig. 3). The rapid expression of MBF1c during heat was not suppressed in mutants deficient in SA (npr1) or chloroplast-to-nuclei retrograde signaling (gun1, abf4, and tylapx; supplemental Fig. 1).

Interaction of MBF1c with TPS5—a yeast two-hybrid screen was performed for the full-length cDNA clone of MBF1c. As prey, we used a library constructed from Arabidopsis seedlings subjected to different abiotic stresses (35). Approximately 18 million clones were screened via mating. Of these, 52 tested positive for interaction. Isolates were then validated by retransformation and one-on-one mating, followed by selection and growth and β-galactosidase assays (35) (supplemental Table 1). At4g17770 (TPS5); also contains an intact C-terminal trehalose phosphatase domain) and two proteins (At3g12960 and At1g43170) that are most likely yeast two-hybrid artifacts (35) were identified.

MBF1c could interact with TPS5 in vivo in yeast (Fig. 4A) as well as in vitro (supplemental Fig. 2). Transcript expression of TPS5 was too low to be detected by RNA blots in wild type and mbf1c plants during heat stress (Fig. 3). RT-PCR analysis was therefore used and showed a transient increase in TPS5 expression during heat stress in wild type plants (Fig. 4B). In contrast, TPS5 expression was high in mbf1c plants in the absence of stress but declined during heat stress (Fig. 4B). Interestingly, the steady-state transcript level of TPS5 was elevated in 35S::MBF1c plants in the presence or absence of heat stress (Fig. 4C). In silico analysis of all TPS transcripts in Arabidopsis (36, 38) revealed that TPS5 is the only TPS transcript elevated during heat stress in Arabidopsis (Fig. 4D). Moreover, two independent TPS5 null mutants (SALK_144791, tps5-1; SALK_007952, tps5-2) were deficient in basal (45 °C) but not acquired (38 → 45 °C) thermotolerance (Fig. 4E).

Involvement of MBF1c in Trehalose Metabolism during Heat Stress—Trehalose accumulated in wild type plants during heat stress and in 35S::MBF1c plants grown under controlled conditions (Fig. 5, A and C). In contrast, trehalose did not accumulate...
during heat stress in mbf1c plants (Fig. 5A), suggesting that MBF1c functions upstream to trehalose accumulation during heat stress. We therefore examined whether trehalose will rescue mbf1c plants as well as tps5-1 and tps5-2 mutants deficient in ethylene (ein2-1) and SA (sid2) (39) signaling (Fig. 6A). mbf1c and ein2 plants were both deficient in basal thermotolerance, supporting our previous studies on MBF1c and ethylene signaling (23). In contrast to the results obtained with mbf1c or ein2 plants, trehalose did not alter the heat tolerance of transgenic plants that constitutively express MBF1c (35S::MBF1c; Fig. 6D), suggesting that the trehalose-dependent enhancement of thermotolerance observed in mbf1c, tps5-1, tps5-2, and ein2-1 and sid2 plants is already activated in 35S::MBF1c plants. This possibility is also supported by the findings that trehalose improved the basal thermotolerance of wild type plants during heat stress and brought it to the level found in 35S::MBF1c plants (Fig. 5C).

**MBF1c Functions Upstream to SA during Heat Stress**—PR-1 expression was similar in mbf1c and wild type plants 3 h following SA application (supplemental Fig. 3), suggesting that MBF1c is not directly required for PR-1 expressing. SA transiently accumulated in wild type plants during heat stress and
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accumulated in 35S::MBF1c plants grown under controlled conditions (Fig. 5, B and C). In contrast, SA did not accumulate in mbf1c plants during heat stress (Fig. 5B), suggesting that MBF1c is required for SA accumulation during heat stress in Arabidopsis. SA (0.1 mM) was able to rescue mbf1c plants as well as a mutant deficient in SA accumulation (sid2) and tps5-1 and tps5-2 (Fig. 6B). sid2 and mbf1c plants had a similar level of deficiency in basal thermotolerance, supporting our hypothesis that MBF1c functions upstream to SA during heat stress. In contrast to the effect of SA on the basal thermotolerance of sid2, SA failed to rescue the ein2 mutant from heat stress (Fig. 6B). This result suggested that ethylene functions downstream to or independent of SA in the basal thermotolerance pathway of Arabidopsis. SA did not enhance the tolerance of transgenic plants that constitutively express MBF1c (35S::MBF1c) to heat stress (Fig. 6D), and these plants were found to accumulate SA when grown under controlled conditions (Fig. 5C).

SA Functions Upstream to Ethylene during Heat Stress—ACC (10 μm), an ethylene precursor, rescued mbf1c, sid2, tps5-1, tps5-2, and a mutant deficient in ethylene accumulation (ain1-1) (40) from heat stress (Fig. 6C). In contrast, ACC had no effect on 35S::MBF1c plants (Fig. 6D). PR-1 expression could not be detected in sid2 plants during heat stress (Fig. 7A). In contrast, PR-1 expression was detected in ein2 plants during heat stress. Our results therefore support a model in which MBF1c functions upstream to SA accumulation that functions upstream to ethylene and PR-1 during heat stress. The expression of PR-1 during heat stress does not appear, however, to be dependent on ethylene. PR-1 expression is suppressed in mbf1c and sid2 plants during heat stress (Figs. 3 and 7A). In contrast, PR-1 expression was not suppressed in tps5 plants (Fig. 7B). This finding suggested that PR-1 expression is independent of TPS5 and that SA, ethylene, and PR-1 function separately from TPS5 during heat stress (Fig. 7C). In contrast to PR-1 expression, expression of HSFA2 was elevated in tps5 mutants during heat stress (Fig. 7B), suggesting that tps5 plants are attempting to compensate for their enhanced thermosensitivity (Fig. 4E) by overactivating the heat shock response pathway.

**DISCUSSION**

A simplified model depicting the proposed role of MBF1c in regulating thermotolerance in Arabidopsis is shown in Fig. 7C. Our data support a model in which MBF1c functions upstream to trehalose, SA, and ethylene. Although our results support a model in which SA and ethylene function independently of trehalose (Fig. 7), it is not entirely clear at this point whether this model is completely accurate or whether a linear model, such as MBF1c → SA → ethylene → trehalose, is more suitable.

Similar to HSFs, MBF1 proteins are highly conserved in different organisms, including human, fly, yeast, and plants (7, 16–19). Our findings, therefore, raise an interesting possibility that MBF1 proteins from other organisms are also involved in regulating responses to temperature stress. The intimate relationship identified in our study between MBF1c and trehalose metabolism during heat stress (Figs. 4–7) could also be studied in other organisms, because trehalose is thought to play a key signaling and defense role during different environmental stress conditions (12, 15, 41–44). At least in plants, it is generally believed that trehalose functions as a signaling molecule (12, 15, 42, 45, 46). Previous studies have focused on TPSs as key regulators of trehalose signaling (12, 15). Although TPS5 belongs to a large gene family, only transcripts encoding TPS5 were found to specifically accumulate during heat stress, and mutants of TPS5 were deficient in basal thermotolerance (Fig. 4). These findings point to a high degree of specificity in the function of different TPS genes in Arabidopsis and suggest that specific TPS genes could have specialized roles in trehalose signaling during different growth conditions or environmental stresses.

Several lines of evidence support the hypothesis that MBF1c and TPS5 interact. The expression of TPS5 and MBF1c is co- Elevated during heat stress (Fig. 4D). TPS5 and MBF1c were found to interact in yeast and in vitro (Fig. 4A and supplemental Fig. 2), null mutants for TPS5 and MBF1c are both sensitive to basal, but not acquired, thermotolerance (Figs. 2 and 4E). MBF1c null mutant has abnormal expression of TPS5 and does...
not accumulate trehalose during heat stress (Figs. 4B and 5A), 35S::MBF1c plants accumulate transcripts encoding TPS5 and trehalose (Figs. 4C and 5C), and trehalose can rescue mbf1c plants from heat stress (Fig. 6A). Nonetheless, additional studies are required to examine the possibility that MBF1c regulates trehalose metabolism via its interaction with TPS5.

MBF1c is thought to be a transcriptional co-activator. It was shown to complement MBF1 deficiency in yeast (21), suggesting that at least in yeast it could mediate transcription of GCN4-controlled genes. The localization of MBF1c protein to nuclei during stress (Fig. 1B) and the presence of a conserved helix-turn-helix DNA-binding domain in MBF1c (22), strengthen the possibility that MBF1c functions in plants as a transcriptional regulator. We previously performed transcriptome analysis of 35S::MBF1c plants, grown under controlled growth conditions, and found that a large proportion of the transcripts elevated in 35S::MBF1c plants were associated with ethylene, SA, and trehalose (23). These findings support our current analysis of mbf1c plants and suggest that MBF1c could function as a transcriptional regulator that enhances the expression of selected transcripts in plants in the absence of stress. It is therefore possible that even the low levels of MBF1c protein found in the nuclei of 35S::MBF1c-GFP plants, grown under controlled growth conditions (Figs. 1, B–D), could activate ethylene, SA, and trehalose responses.

Our analysis of gain- and loss-of-function MBF1c plants provides strong evidence for a link between MBF1c and trehalose metabolism, suggests a role for MBF1c as a central regulator of thermostolerance in Arabidopsis, and demonstrates that MBF1c functions upstream to SA, PR-1, ethylene, and trehalose during heat stress. Because the expression of MBF1c is enhanced in current analysis of gain- and loss-of-function MBF1c plants, grown under controlled growth conditions (Figs. 1, 25–231), could activate ethylene, SA, and trehalose responses.

In support of this possibility, 35S::MBF1c plants were found to be more tolerant to heat and/or osmotic stresses as well as to bacterial infection (23).

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