Coordination between bZIP28 and HSFA2 in the regulation of heat response signals in Arabidopsis

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ABSTRACT

Heat stress can have detrimental effects on crop yield, causing devastating economic and societal impacts. In the last decade, many researches have uncovered multiple pathways involved in the heat stress response in plants.1-4 The function of heat shock transcription factors (HSFs) that control the heat stress response by binding to the heat shock response element (HSE) in the promoter of stress response genes has been established in multiple biological systems.5 Multiprotein bridging factor 1c (MBF1c) has been identified as another key regulator of heat stress response in plants.9,10 MBF1c might function as a transcriptional regulator that regulates expression of important heat response transcripts such as Dreh2A and Myb7. In addition, regulatory systems of reactive oxygen species (ROS) also play pivotal role in heat stress responses in plants. A cytosolic ascorbate peroxidase 1 (APX1) has been considered as one of the most essential regulators of cellular ROS level.8,9 Arabidopsis plants deficient in cytosolic APX1 were characterized by alteration in heat tolerance as well as growth suppression and higher sensitivity to oxidative stress.8,10 A second cytosolic APX isozyme, APX2 might be a key regulator of heat tolerance in plants.11-14 Expression of APX2 was shown to be induced by heat stress in an HSF3-dependent manner corresponding with the appearance of thermo-stable isofrom of APX that is required for heat tolerance.12 Recent studies proposed that unfolded protein response pathways in the endoplasmic reticulum (ER UPR) and in the cytosol (CPR) might be involved in heat sensing in plants.2,15,16 The ER UPR signaling pathways are known to be activated in cells in response to environmental stimuli that cause the impairment of protein stability in the ER; chemicals that induce the unfolding of proteins, osmotic, salinity and heat stress.17,18 Two transcription factors, basic leucine zipper 17 and 28 (bZIP17 and bZIP28) might play a key role in UPR-dependent heat sensing in plants.7 Activation of ER UPR involves translocation of bZIP17 and bZIP28 into the Golgi, where they are proteolytically cleaved. Cleaved bZIPs are then released into the nuclei and activate the ER chaperone genes as well as brassinosteroid signaling, which might be required for acclimation of plants to heat stress. In contrast to the ER UPR, CPR that is triggered by the presence of unfolded proteins in the cytosol, is primarily regulated by HSFA2.15 CPR was shown to induce novel alternative splicing of HSFA2 RNA that might modulate HSFA2 activity under abiostic stresses. In addition, the association of CPR with heat stress responses in plants could be also supported by the over representation of heat shock element (HSE) in the promoter of genes that are responsive to both heat stress and CPR induced by chemical inducer.

Both ER UPR and CPR might be involved in modulation of ROS signaling in plants. ER stress, induced by tunicamycin was able to enhance the expression of transcripts encoding ROS scavenging and producing enzymes, as well as ER-stress-related transcripts.19 In addition, exogenous H2O2 and ROS-producing reagents that are specific to different organelles, such as chloroplasts, mitochondria and peroxisomes enhanced expression of ER-stress-related transcripts including bZIP17 and bZIP28.20 Certain HSFs might function as molecular sensors that directly sense ROS and control the expression of oxidative stress response genes.21 The links between functions of HSFs and ROS scavenging systems could be also supported by the existence of HSE in the promoter of genes encoding APXs.

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Each of these putative sensors associated with UPRs is thought to activate a similar set of heat stress response genes leading to enhanced heat tolerance, but the relationship between these UPR pathways in the regulation of heat response pathways including ROS regulatory mechanisms and MBF1c-dependent pathways is still not understood. Here, we attempted to reveal the coordination between bZIP28 and HSFA2 by analyzing Arabidopsis plants lacking each of these UPRs regulators.

This study obtained knockout plants deficient in bZIP28 (bzip28-1; SALK_132285 and bzip28-2; SALK_114900) through the SIGnAL project. bzip28-1 or bzip28-2 contained a T-DNA insertion in the 1st or 2nd exon, respectively (Supplemental Fig. 1A). Although deficiency in expression of the full-length bZip28 transcript was confirmed both in bzip28-1 and bzip28-2 plants, expression of the transcript that includes the region encoding bZIP DNA binding domain was deficient only in bzip28-1 plants (Supplemental Fig. 1B).

To study the effects of the deficiency in bZIP28, a key regulator of ER UPR, on the responses of plants to heat stress, we measured chlorophyll concentration, fresh weight and electrolyte leakage of bzip28-1 and bzip28-2 plants exposed to 40°C heat stress for 4 days. No significant differences were observed in these growth and physiological parameters between the knockout and WT plants (Fig. 1). These results suggest that the deficiency in bZIP28 did not dramatically affect these growth and physiological responses of plants to heat stress, and that other heat response pathways might be activated to compensate for the deficiency in bZIP28.

Analyses of the heat response proteins (Fig. 2A) demonstrated that bzip28-1 plants accumulated higher level of cytosolic APX1, stromal/mitochondrial APX (s/mAPX) and thylakoid APX (TylAPX) proteins compared to WT plants. bzip28-2 plants also accumulated higher level of APX1 and s/mAPX proteins compared to WT plants, but, to a lesser extent compared to bzip28-1 plants. Accumulation of TylAPX protein was higher in bzip28-2 plants compared to WT plants under controlled conditions, but, this difference in the accumulation of TylAPX protein was not observed under heat stress. In contrast to APX proteins, higher accumulation of MBF1c protein, a transcriptional regulator required for basal thermotolerance, was detected in bzip28-2 plants compared to bzip28-1 and WT plants at 15 min following the application of heat stress. In addition, bzip28-2 plants also showed enhanced accumulation of HSP70 compared to WT and bzip28-1 plants under controlled condition and at 15 min following the application of heat stress. Furthermore, higher level of HSP101 protein was detected both in bzip28-1 and bzip28-2 plants compared to WT under heat stress. bzip28-1 plants showed higher level of HSP101 protein when compared to bzip28-2 plants.

We further analyzed expression of heat response transcripts encoding Apx2 and Cat2, ROS scavenging enzymes in the cytosol and the peroxisome, and Dreb2A and Myb7, transcription factors that might be regulated by MBF1c during heat stress (Fig. 2B). In contrast to the accumulation of APX proteins, transcript level of both Apx2 and Cat2 both in bzip28-1 and bzip28-2 plants was almost comparable with that in WT plants under controlled conditions and heat stress. Interestingly, bzip28-2 plants that accumulated higher level of MBF1c protein (Fig. 2A) showed enhanced expression of Dreb2A and Myb7 transcripts compared to WT plants at 15 min following the application of heat stress. Whereas significant differences in the expression of Dreb2A and Myb7 transcripts were not detected between WT and bzip28-1 plants. These results suggest that APXs-, MBF1c- and HSPs-dependent pathways might compensate for the deficiency in bZIP28 during heat stress.

Higher level of APX proteins in bzip28-1 plants prompted us to analyze H2O2 accumulation. bzip28-1 plants accumulated significantly higher level of H2O2 compared to WT at 10 min following the application of heat stress (Fig. 2C), suggesting that H2O2 might function as a signaling molecule to activate APXs-dependent heat response signals in bzip28-1 plants. On the other hand, H2O2 accumulation in bzip28-2 plants was almost comparable with that in WT plants during heat stress.

Although HSFA2 has been shown to function as a key regulator of unfolded protein responses in the cytosol (CPR) as well as oxidative stress responses in plants, coordination between bZIP28-dependent ER UPR and HSFA2-dependent CPR is still not clearly understood. We therefore investigated the expression of HsfA2 transcript and its alternative form that was shown to be induced by chemical CPR inducer (HsfA2 II) in bzip28-1 and bzip28-2 plants. Interestingly, expression of both HsfA2 and
HsfA2 II transcripts was significantly higher in bzip28-1 plants that showed clearly higher accumulation of APX proteins and \( \text{H}_2\text{O}_2 \) (Fig. 2A & C) compared to WT plants during heat stress (Fig. 2D). Whereas, level of HsfA2 and HsfA2 II transcripts in bzip28-2 was almost comparable with that in WT plants. HSFA2 might also contribute to activation of APXs-dependent heat response signaling in bzip28-1 plants.

To study the significance of HSFA2 in the regulation of APXs-dependent pathway, we analyzed the molecular and physiological responses of plants deficient in HSFA2 (hsfA2) to heat stress. Accumulation of all three types of APX proteins and expression of Apx2 and Cat2 transcripts were inhibited in hsfA2 plants compared to WT plants during heat stress (Fig. 3A, B), suggesting that HSFA2 might be required for the activation of ROS regulatory systems including APXs-dependent pathway. To further investigate the integration of HSFA2 with ROS signaling and bZIP28, we analyzed \( \text{H}_2\text{O}_2 \) accumulation and expression of bZIP28 transcript in hsfA2 plants. hsfA2 plants showed lower level of \( \text{H}_2\text{O}_2 \) compared with WT plants at 10 min following the application of heat stress (Fig. 3C). In addition, transcript level of bZIP28 was inhibited in hsfA2 plants compared to WT plants during heat stress (Fig. 3D). These results indicate that HSFA2 might be also required for modulation of \( \text{H}_2\text{O}_2 \) accumulation and expression of bZIP28 transcript during heat stress. Furthermore, hsfA2 plants showed significantly lower expression of Dreb2A and Myb7 transcripts compared to WT plants during heat stress (Supplemental Fig. 2A), despite the similar level of MBF1c protein accumulation between WT and hsfA2 plants (Fig. 3A), indicating the involvement of HSFA2 in the regulation of DREB2A and MYB7 during heat stress. Although the inhibition of several heat response pathways were observed in hsfA2 plants, deficiency in HSFA2 did not negatively affect heat tolerance in plants (Supplemental Fig. 2B-D). Chlorophyll concentration of hsfA2 plants was almost comparable with that of WT.
plants (Supplemental Fig. 2B). In addition, hsfA2 plants showed significantly higher fresh weight and lower electrolyte leakage compared to WT plants during heat stress (Supplemental Fig. 2C and D). Previous studies revealed the redundancy in the function of multiple HSFs during heat stress. Thus, other pathways might compensate for the deficiency in HsfA2. Existence of such other pathways could be at least partially supported by higher accumulation of HSP70 under the controlled condition and HSP101 at 15 min following the application of heat stress in hsfA2 plants compared to WT plants (Fig. 3A).

Despite the deficiency in a key regulator of heat response, heat tolerance in bzip28-1 and bzip28-2 was almost comparable with WT plants (Fig. 1), suggesting that other pathways might compensate for the deficiency in bZIP28 during heat stress. Indeed, we demonstrated that APXs-, MBF1c- and HSPs-dependent pathways were enhanced in bzip28-1 and bzip28-2 plants (Fig. 2). Accumulation of APX proteins was higher especially in bzip28-1 plants compared to WT plants during heat stress (Fig. 2A). Interestingly, bzip28-1 plants also demonstrated enhanced expression of HsfA2 and HsfA2 II transcripts accompanied by higher accumulation of H2O2 compared to WT and bzip28-2 plants (Fig. 2C, D). Although excess amount of ROS production can cause detrimental effects on plant cells, ROS also function as important signaling molecules that regulate acclimatory responses of plants to abiotic stresses including heat stress. Certain HSFs were proposed to function as molecular sensors that directly sense ROS and regulate the expression of oxidative stress response genes.

In addition, expression of HsfA2 transcript was shown to be highly responsive to H2O2. Thus, these findings together with the results of this study suggest that HsfA2 triggered by H2O2 signaling might activate APXs-dependent heat response pathways in bzip28-1 plants. Significance of HsfA2 in activation of the APXs-dependent heat responses could also be supported by lower accumulation of APX proteins in hsfA2 plants during heat stress (Fig. 3A). However, H2O2 accumulation was also lower in hsfA2 plants compared to WT plants during early response to heat stress (Fig. 3C). In addition, in contrast to the results of this study, protoplasts isolated from plants deficient in HsfA2 demonstrated higher accumulation of H2O2 during heat stress. These findings therefore indicate that feedback mechanisms in which HsfA2 modulate H2O2 production could exist, and that HsfA2-dependent pathways might flexibly control cellular H2O2 level depending on intensity of stresses, and types of cells or tissues. Furthermore, the level of bZip28 transcript was inhibited in hsfA2 plants compared to WT plants during heat stress (Fig. 3D). This finding together with the results obtained from the analyses of bzip28-1 plants demonstrated the possibility that HsfA2 might function upstream to two pathways; bZIP28- and APXs-dependent pathways, and disruption in bZIP28 could be complemented by the activation of HsfA2 and its downstream pathway involving APXs.

In contrast to APX proteins, higher accumulation of MBF1c protein was detected in bzip28-2 plants compared to WT and bzip28-1 plants under heat stress (Fig. 2A). Enhanced activity of MBF1c-dependent heat response in bzip28-2 could be also supported by higher expression of Dreb2A and Myb7 transcripts that might be regulated by MBF1c during heat stress (Fig. 2B). The differences in the enhanced heat response signals between bzip28-1 and bzip28-2 plants could be due to the difference in the positions of T-DNA insertion (Supplemental Fig. 1A). bzip28-1 plants contain T-DNA insertion upstream to the region corresponding to the bZIP DNA binding domain, whereas, bzip28-2 plants lack the region that does not include this domain. bZIP DNA binding domain in bZIP28 could, therefore, link to the regulation of ROS-dependent heat response signals. In addition, hsfA2 plants also showed lower level of Dreb2A and Myb7 transcripts compared to WT plants during heat stress, despite the similar level of MBF1c protein accumulation between WT and hsfA2 plants (Fig. 3D). These
results suggest that DREB2A and MYB7 might be also regulated by HSFA2 that functions independently of MBF1c during heat stress.

In this study, we demonstrated the possible integration between bZIP28 and HSFA2 in the regulation of ROS- and MBF1c-dependent heat stress response in Arabidopsis. To further test the hypothesis suggested in this study, double mutant deficient both in bZIP28 and HSFA2 or plants overexpressing these transcription factors should be analyzed. In addition, it should be interesting to investigate the integration of bZIP28- and HSFA2-dependent pathways with other putative heat sensors identified in recent studies.

In this study, A. thaliana ecotype Col-0 were grown on peat pellet (jiffy-7, Shippagan) under controlled conditions: 21°C, 16/8h light/dark cycle, 50 µmol m⁻² s⁻¹ in a growth chamber (LPH-241S, NK System, Tokyo, Japan). Knockout plants deficient in bZIP28 (At3g10800; SALK_132285 and SALK_114900) or HSFA2 (At2g26150; SALK_008978) were obtained through the SIGnAL project, and screened in homozygous form as previously described.9,26

Eighteen to twenty-one-day-old plants grown as described above were used in this study. For measurements of chlorophyll, fresh weight and electrolyte leakage, plants were subjected to heat stress for 4 days in a growth chamber (LH-80LED-DT, NK System, Tokyo, Japan) with the following temperature cycle: 6:00AM–10:00PM, 40 °C; 10:00PM–6:00AM, 21 °C. The 16-hour light period was imposed from 6:00AM to 10:00PM. For semi quantitative RT-PCR, qRT-PCR and protein gel blot analyses, plants were exposed to 40°C heat stress for 0, 15 and 45 min. For H₂O₂ measurement, plants were exposed to 40°C heat stress for 0, 5, 10 min.

Chlorophyll measurement was performed as previously described.28 Measurement of electrolyte leakage was performed as previously described29 using a conductivity meter (DS-71, Horiba, Tokyo, Japan). The accumulation of H₂O₂ was measured using Amplex Red (Molecular Probes, Invitrogen, http://www.invitrogen.com/) as previously described.29

RNA extraction was performed as previously described.24 First strand complementary DNAs (cDNAs) were produced after DNaseI treatment from 1 µg of total RNA with M-MuLV reverse transcriptase (New England Biolabs). Semi quantitative RT-PCR analysis was performed as previously described.6 qRT-PCR was performed in an optical 96-well plate with the ABI Prism 7000 system and the Thunderbird qPCR Master Mix (Toyobo, Tokyo, Japan). Threshold cycle values for target transcripts were calculated with the CT of primer used in this study are listed in Table S1. Protein extraction and protein gel blot analysis were performed as previously described.6 Antibodies that react with three different APX proteins (thylakoid APX [TylAPX], stromal/mitochondrial APX [s/mAPX] and cytosolic APX1) or MBF1c were used for protein gel blot analysis. Antibodies to detect HSP70 and HSP101 were purchased from Funakoshi Inc. (Tokyo, Japan).

**Acknowledgement**

This paper was supported by funding from Sophia University in Japan.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| APX          | ascorbate peroxidase |
| bZIP         | basic leucine zipper |
| CAT          | catalase        |
| CPR          | cytosolic unfolded protein response |
| DREB2A       | dehydration responsive element-binding protein 2A |
| ER           | endoplasmic reticulum |
| HSE          | heat shock element |
| HSF          | heat shock transcription factor |
| HSP          | heat shock protein |
| MBF1c        | multiprotein bridging factor 1c |
| MYB7         | myeloblastosis 7 |
| ROS          | reactive oxygen species |
| UPR          | unfolded protein response |

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