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LARGE-SCALE BIOLOGY ARTICLE

Tissue-Specific Transcriptomics Reveals an Important Role of the Unfolded Protein Response in Maintaining Fertility upon Heat Stress in Arabidopsis

Shuang-Shuang Zhang,[a,1] Hongxing Yang,[b,1] Lan Ding,[a] Ze-Ting Song,[a] Hong Ma,[a] Fang Chang,[a,2] and Jian-Xiang Liu[a,c,2]

[a] State Key Laboratory of Genetic Engineering, Collaborative Innovation Center of Genetics and Development, Institute of Plant Biology, School of Life Sciences, Fudan University, Shanghai 200433, China
[b] Shanghai Key Laboratory of Plant Functional Genomics and Resources, Shanghai Chenshan Plant Science Research Center, Chinese Academy of Sciences, Shanghai Chenshan Botanical Garden, Shanghai 201602, China
[c] State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou 310027, China

ORCID ID: 0000-0003-0791-1301 (J.-X.L.)

High temperatures have a great impact on plant reproductive development and subsequent fruit and seed set, but the underlying molecular mechanisms are not well understood. We used transcriptome profiling to investigate the effect of heat stress on reproductive development of *Arabidopsis thaliana* plants and observed distinct response patterns in vegetative versus reproductive tissues. Exposure to heat stress affected reproductive developmental programs, including early phases of anther/ovule development and meiosis. Also, genes participating in the unfolded protein response (UPR) were enriched in the reproductive tissue-specific genes that were upregulated by heat. Moreover, we found that the UPR-deficient *bzip28 bzip60* double mutant was sensitive to heat stresses and had reduced silique length and fertility. Comparison of heat-responsive wild type versus *bzip28 bzip60* plants identified 521 genes that were regulated by *bZIP28* and *bZIP60* upon heat stress during reproductive stages, most of which were noncanonical UPR genes. Chromatin immunoprecipitation coupled with high-throughput sequencing analyses revealed 133 likely direct targets of bZIP28 in Arabidopsis seedlings subjected to heat stress, including 27 genes that were also upregulated by heat during reproductive development. Our results provide important insights into heat responsiveness in Arabidopsis reproductive tissues and demonstrate the protective roles of the UPR for maintaining fertility upon heat stress.

INTRODUCTION

One of the major challenges to humanity is global climate change, which has great impact on plant growth and global food production (Lobell et al., 2011; Lesk et al., 2016). The global average temperature has risen by roughly 0.13°C per decade since 1950 according to the International Panel on Climate Change (2007), and the extreme annual daily maximum temperature is predicted to increase by ~1 to 3°C by the mid 21st century (International Panel on Climate Change, 2012). Based on mathematical modeling, cereal production in many regions of the world will most likely be affected, with an estimated loss of 6 to 7% yield per 1°C increase in seasonal mean weather and associated extreme heat disasters (Lesk et al., 2016). Unlike most animals, plants are sessile and must respond quickly to fluctuating environmental conditions at the chemical, molecular, cellular, and physiological levels (Bita and Gerats, 2013). To mitigate the negative effects of global warming on plant growth and development, a thorough understanding of the molecular mechanisms underlying plant thermotolerance is needed for sustainable yield production.

The responses of plants to elevated temperature and heat shock have distinct mechanisms. The bHLH transcription factor PIF4 plays a central positive role in the acclimation of *Arabidopsis thaliana* plants to elevated ambient temperatures below 29°C (Proveniers and van Zanten, 2013). In contrast, previous efforts have focused on understanding the heat shock response (HSR) in plants in the vegetative stage. At ~5°C above optimal growth conditions, changes in membrane fluidity and protein stability are observed (Bita and Gerats, 2013). Under these conditions, the classical HSR activates a cascade of heat shock factors (HSFs) and results in upregulation of several heat shock proteins (HSPs) (Bokszczanin and Fragkostefanakis, 2013). One of the most detrimental effects of heat shock is the accumulation of misfolded or unfolded proteins in both the cytosol and endoplasmic reticulum (ER), which in turn attenuates the interaction between molecular chaperones (i.e., HSPs) and transcription factors (e.g., HSFs). This allows the nuclear relocation of the transcription factors for the activation of downstream gene expression (Bokszczanin and Fragkostefanakis, 2013).

[1] These authors contributed equally to this work.
[2] Address correspondence to fangchang@fudan.edu.cn or jianxiangliu@fudan.edu.cn.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Jian-Xiang Liu (jianxiangliu@fudan.edu.cn).
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Accumulation of misfolded proteins in the ER leads to ER stresses and elicits the unfolded protein response (UPR), which is mediated by several membrane-associated transcription factors (Liu and Howell, 2010a). These membrane-associated transcription factors belong to either the basic leucine zipper (bZIP) or NAM, ATAF, and CUC (NAC) families and are found as quiescent forms attached to the ER or plasma membranes under normal growth conditions (Iwata and Koizumi, 2005; Liu et al., 2007b; Yang et al., 2014a, 2014b; Liu and Howell, 2016). Upon the accumulation of misfolded proteins in the ER, the Arabidopsis membrane-associated transcription factors bZIP17 and bZIP28 are activated via intramembrane proteolysis by the Golgi-resident protease S2P (Liu et al., 2007a, 2007b; Gao et al., 2008; Tajima et al., 2008; Che et al., 2010; Sun et al., 2015; Zhou et al., 2015). However, activation of the Arabidopsis bZIP60 protein and its orthologs in other plant species depends on unconventional mRNA splicing that is regulated by the ER-localized Inositol-requiring enzyme 1 (IRE1) proteins (Deng et al., 2011; Nagashima et al., 2011; Lu et al., 2012; Moreno et al., 2012). In the nucleus, the activated bZIP28 and bZIP60 proteins regulate downstream genes by recognizing and binding to specific *cis*-elements (Martínez and Chrispeels, 2003; Iwata and Koizumi, 2005; Kamauchi et al., 2005; Tajima et al., 2008; Iwata et al., 2009; Liu and Howell, 2010b; Sun et al., 2013b). Also, they direct deposition of H3K4 trimethylated histone marks to potentiate gene expression via interactions with COMPASS-like components in Arabidopsis (Song et al., 2015).

Although plant vegetative growth is sensitive to environmental stresses such as heat and drought, reproductive development and fertilization have been long considered as relatively more sensitive to these factors, often resulting in reduced crop yields (Peng et al., 2004; Liu et al., 2006; Barnabás et al., 2008; Liu and Bennett, 2011; Su et al., 2013). In general, male reproductive tissues are much more sensitive to high-temperature stress than female reproductive tissues at all stages of development (Hedhly, 2011). Male reproductive development in flowering plants begins with the initiation of stamen tissue from the floral meristem and the generation of meiotic and somatic cell layers followed by meiosis and tapetum development, which supports pollen development (Chang et al., 2011). Male meiosis produces microspores, which further develop into pollen grains following two mitotic divisions. Following anther dehiscence, the mature pollen grains are released and germinate on the stigma, allowing double fertilization to occur (Ma, 2005). However, the mechanism by which high temperature affects plant male reproductive development and fertility is poorly understood.

In addition, the role of the UPR in thermotolerance is not well studied in plants. During vegetative growth of Arabidopsis, heat shock induces the activation of both bZIP28 (Gao et al., 2008) and bZIP60 (Deng et al., 2011). However, the genes acting downstream of bZIP28 and bZIP60 during the HSR are largely not yet known. Among the genes induced by mild heat stress in wild-type Arabidopsis leaves, only a few genes were induced by the classical UPR inducer tunicamycin in a transcriptomic study (Sugio et al., 2009), indicating that bZIP28 and bZIP60 regulate different sets of genes during the HSR. Several UPR downstream genes are expressed at different stages of pollen development and during pollen tube growth in tomato (*Solanum lycopersicum*) and Arabidopsis (Fragkostefanakis et al., 2016), suggesting that the UPR is also important for reproductive development. It has been shown that the *bZIP60* promoter is highly active in Arabidopsis flowers, especially in microspores and tapetum cells, and that the *bZIP60* mRNA is constitutively spliced in anthers under normal growth conditions (Iwata et al., 2008; Deng et al., 2016). These results suggest that the UPR is important for thermotolerance both at the vegetative and reproductive stages; however, the underlying molecular mechanisms are largely unknown.

In this study, we compared the transcriptome profiles of rosette leaves, early and late stages of flowers of wild-type Arabidopsis plants that were subjected to heat stress during reproductive development. We discovered distinct heat stress response patterns between vegetative and reproductive tissues. We also found that *bzip28 bzip60* double mutant plants, which lack the two key UPR regulatory genes *bZIP28* and *bZIP60*, were sensitive to heat stress, having short siliques and reduced fertility. Comparison of gene expression profiles under heat stress condition between the wild-type and *bzip28 bzip60* mutant plants revealed the important roles of these two transcription factors in regulating genes associated with the HSR in reproductive tissues. Chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) revealed a number of direct targets of bZIP28 during the HSR at vegetative and reproductive stages in Arabidopsis.

RESULTS

Vegetative and Reproductive Tissues Have Distinct Expression Profiles in Response to Heat Stress

Recently, silique length was shown to be proportional to the number of seeds per silique and is a reliable parameter for the effect of heat stress on plant reproductive development (Bac-Molenaar et al., 2015). Wild-type Arabidopsis plants grown at normal temperature (22°C) were exposed to heat stress (37°C) for a short period (3 h) at the stage of late bolting (Figure 1A). Unopened floral buds at stage 10-12 (Smyth et al., 1990; Bac-Molenaar et al., 2015) and younger buds at stage 1-9 were tagged for further study. Immediately after heat stress, there was no obvious difference between the unstressed and stressed plants (Figures 1A and 1B). However, at maturity after recovery (10 d later) at normal growth temperature, the silique length corresponding to the first eight flowers (approximately stage 12 to stage 9 at the onset of stress) was reduced in plants that experienced heat stress (Figures 1C to 1E). We divided these siliques into three major categories: fully fertile (Type I), partially sterile (Type II), and completely sterile (Type III) (Figure 1F). The percentages of Type II and Type III siliques were much increased in plants exposed to heat stress (Figure 1F).

We collected rosette leaves (RLs), early flowers (EFs; stage 1-9), and late flowers (LFs; stage 10-12) (Figure 2A) immediately after heat stress at 37°C (3 h) from wild-type plants at the reproductive stage (Figure 1A) and isolated RNAs for RNA-seq analysis. The early flowers also included the inflorescence meristem and the top portion of the inflorescence stem. A total of 3435 heat upregulated genes and 3568 heat downregulated genes were detected in the three examined tissues (Supplemental Data Set 1). Among them,

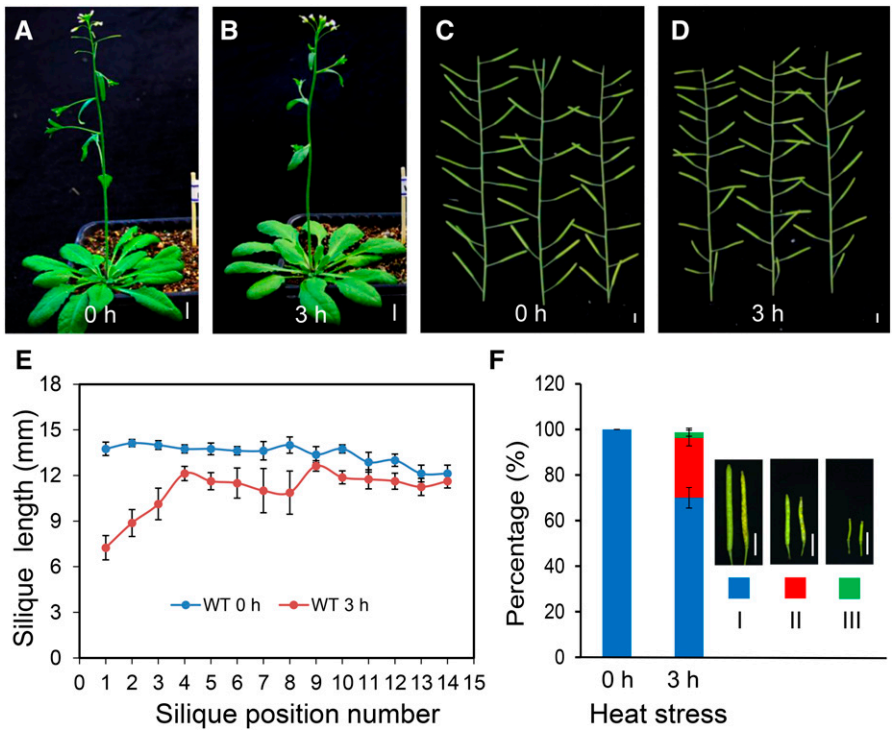

Figure 1. Impairment of Fertility by Heat Stress in Arabidopsis at the Reproductive Stage.

(A) and **(B)** Arabidopsis wild-type plants before and after exposure to heat stress (37°C, 3 h) at the stage of late bolting.
(C) to **(F)** Effect of heat exposure on silique development. Photos were taken at maturity 10 d after heat stress and silique lengths were measured. Silique position 1 (at the bottom) denotes the flower that was furthest along in development at the time of heat stress. In each plant, siliques were divided into three categories: fully fertile (Type I), partially sterile (Type II), and completely sterile (Type III), and the percentage was calculated. Bars in **(E)** and **(F)** indicate SE (*n* = 8 plants).
Bars = 1 cm in **(A)** and **(B)** and 5 mm in **(C)**, **(D)**, and **(F)**.

16.8% of the genes (577 out of 3435) were upregulated in common and 6.4% (229 out of 3568) were downregulated in common (Figures 2B and 2C). Gene Ontology (GO) enrichment analysis revealed that the group of commonly upregulated genes was enriched with genes involved in response to heat and heat acclimation, response to hydrogen peroxide, and protein folding/refolding (Figure 2D). Among commonly downregulated genes, genes involved in double-strand break repair via homologous recombination, defense responses, signaling pathways, and basic metabolic/catabolic processes were significantly enriched (Figure 2E).

The relatively modest heat shock conditions (35–38°C) that we used in our experiments were used in other recent studies, although relatively extreme temperature conditions (42–45°C) have also been widely used previously (Saidi et al., 2011; Liu et al., 2015). Thus, it is interesting to compare our results with previous results obtained from soil-grown rosette leaves under extreme heat stress conditions (44°C) (Suzuki et al., 2016). We found that ~40% of the genes upregulated in rosette leaves in our study were also upregulated in the study conducted by Suzuki and colleagues (Supplemental Figure 1A). A similar scenario was observed for downregulated genes (Supplemental Figure 1B). Hence, heat shock responses to different temperature regimes may have both common and distinct mechanisms. Further GO enrichment analysis showed that rRNA processing and modification genes were overrepresented in those genes specifically upregulated in our study (Supplemental Figures 1C to 1H).

To identify tissue-specific effects of heat treatment, we compared heat-responsive genes in different tissues. We found 27.5 and 23.2% of the upregulated genes were heat-responsive in rosette leaves and early flowers, respectively, whereas 40.1 and 25.2% downregulated genes were specifically inhibited by the heat stress in rosette leaves and early flowers, respectively. To define the tissue-specific heat-responsive gene expression programs, we adopted a more stringent criterion to filter out genes that showed statistically significant heat-responsive gene regulation in one tissue but had marginally significant changes in other tissues ($0.01 < q\text{-value} < 0.05$). In this way, we identified 475, 202, and 31 genes that were specifically upregulated in rosette leaves, early flowers, and late flowers, respectively (Figure 2B). In comparison, we obtained significantly larger number of tissue-specific downregulated genes in these tissues: 835 for rosette leaves, 314 for early flowers, and 55 for late flowers (Figure 2C). GO enrichment analysis revealed that among the specifically upregulated in rosette leaves, a number of RNA processing and modification related processes were found to be enriched,

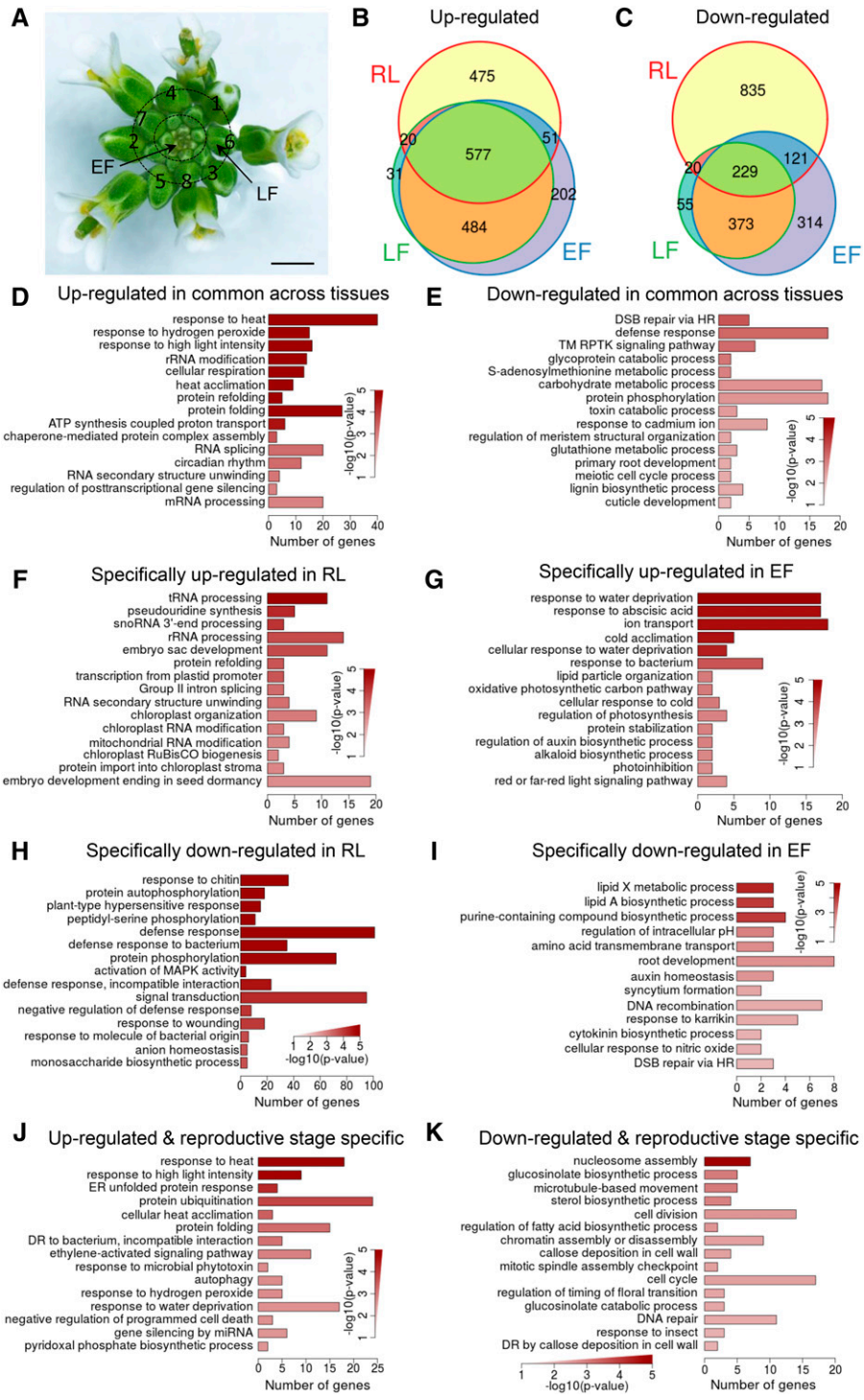

Figure 2. Heat Responsiveness in Arabidopsis Vegetative and Reproductive Tissues at the Reproductive Stage Determined by RNA-Seq Analysis.

(A) The Arabidopsis main inflorescence used for RNA-seq analysis. Flowers 1 to 8 at stage 10 to stage 12 were considered to be LFs (outer ring) and younger flowers at stage 1 to stage 9 were considered to be EFs (inner ring). RLs were also sampled at the same time. Bar = 2 mm.
(B) and **(C)** Venn diagrams showing the common and specific heat responsiveness of different Arabidopsis tissues.
(D) to **(I)** GO analysis of biological processes affected by heat. Shown are genes upregulated or downregulated by heat across all three tissues, specifically upregulated by heat in RL or EF, and specifically downregulated by heat in RL or EF in Arabidopsis.
(J) and **(K)** GO analysis of reproductive stage-specific genes upregulated and downregulated by heat in Arabidopsis.
DSB, double-strand break; HR, homologous recombination; TM, transmembrane; RPTK, receptor protein tyrosine kinase; RuBisCO, ribulose bisphosphate carboxylase complex; DR, defense response; floral transition, transition from the vegetative to the reproductive phase.

including tRNA processing, pseudouridine synthesis, snoRNA 3′-end processing, rRNA processing, Group II intron splicing, RNA secondary structure unwinding, chloroplast RNA modification, and mitochondria RNA modification (Figure 2F), suggesting the importance of posttranscriptional processing and modification of RNA molecules in the heat stressed vegetative tissues. In comparison, processes including response to water deprivation, response to abscisic acid, ion transport, cold acclimation, and cellular response to water deprivation were most significantly enriched among EF-specific upregulated genes (Figure 2G). GO enrichments were also quite different between RL-specific downregulated and EF-specific downregulated genes (Figures 2H and 2I).

To better understand different heat responses in between vegetative tissues (rosette leaves) and reproductive tissues (early and late flowers), we looked for genes that were simultaneously regulated in early and late flowers but not in rosette leaves. We obtained 484 and 373 genes that were significantly upregulated and downregulated in reproductive tissues, respectively, but not in vegetative tissues (Figures 2B and 2C). We considered these 857 genes to be reproductive tissue-specific genes and we considered the 1310 genes that were significantly upregulated or downregulated in vegetative tissues, but not in reproductive tissues, to be vegetative tissue specific genes. GO analysis revealed that heat upregulated reproductive tissue-specific genes were enriched in response to heat, endoplasmic reticulum unfolded protein response, protein ubiquitination, cellular heat acclimation, and protein folding (Figure 2J), whereas heat downregulated reproductive stage specific genes were enriched in nucleosome assembly (Figure 2K). Taken together, these transcriptome results demonstrate that heat-responsive programs in reproductive tissues have many active processes involved in water deprivation, abscisic acid response, protein misfolding, and degradation.

Transcriptional Cascades Are Prevalent in Reproductive Tissues in Response to Heat

The core regulators of the HSR are a group of transcription factors and their direct targets (HSFBs, DREB2A, and MBF1c; Ohama et al., 2016). To better understand the transcriptional regulatory networks in reproductive tissues as well as in vegetative tissues, we analyzed the expression patterns of genes encoding transcription factors (TFs) upon heat treatment. Interestingly, we identified 455 TF genes that were significantly regulated by heat in at least one tissue of Arabidopsis wild-type plants (Supplemental Data Set 2), accounting for 26.5% of the 1717 known TF-encoding genes in Arabidopsis. These heat-responsive TF genes belonged to 54 different TF families, representing diverse regulatory pathways. Among them, only a small number of heat-responsive TF genes were commonly regulated across all the three tissues examined (21 out of 217 TF genes upregulated; 15 out of 243 TF genes downregulated).

In comparison, significantly more heat responsive TF genes were specifically up- or downregulated in only one of the three tissues (129 upregulated genes, exact binomial test P < 2e-16; 188 downregulated genes, exact binomial test P < 2e-16; Figures 3A and 3B). Meanwhile, except for the genes commonly regulated across the three examined tissues, the two flower tissues shared a substantial number of heat responsive genes, but rosette leaves shared only a few heat-responsive genes with the two reproductive tissues (Figures 3A and 3B). These results suggested that different tissues recruit different transcriptional regulatory modules in responses to heat stress. Furthermore, TF genes were more likely upregulated by heat in early flowers than in rosette leaves [Figures 3A and 3B; Pearson's χ^2 test with Yate's continuity correction, χ^2 (1, n = 472) = 18.3, P = 2e-05]. Surprisingly, more HSF family transcription factors were upregulated by heat in reproductive tissues than that in vegetative tissues (Figure 3C; exact binomial test P = 0.002), and this was consistent with the heat responsive expressions of HSPs (Figure 3D; exact binomial test P = 0.004). Further analysis revealed that TF families other than HSF potentially participated in heat responses (Supplemental Figures 2A to 2D). For example, bZIP, NAC, and ERF family members were significantly upregulated by heat in flowers (Supplemental Figures 2 and 3).

Reproductive Developmental Programs Are Disturbed by Heat Stress

Plant reproductive development involves several key steps, including floral organ initiation and development, meiosis, and male and female reproductive development. The master regulators of floral organ development in Arabidopsis are mainly encoded by members of the MADS box gene family, especially those of the MIKC type, which have additional I, K, and C domains. We examined the expression of MADS box genes during the HSR. There was no significant expression alteration in flowers for the key floral organ identity genes, i.e., factors in the ABCE model, except for *SEPALATA4* (*SEP4*), which was significantly upregulated in early flowers by heat stress (Supplemental Data Set 1). Consistent with this, we observed that heat treatment significantly downregulated the floral repressor *AGL15* in early flowers (Supplemental Data Set 1).

To further examine the effects of high temperature stress on floral development, we examined the heat-responsive genes that have been demonstrated to be directly regulated by each of the three major floral organ identity genes, i.e., the A-function gene *AP1*, the B-function gene *AP3*, and the C-function gene *AGAMOUS* (*AG*) (Kaufmann et al., 2010; Wuest et al., 2012; Ó'Maoiléidigh et al., 2013). Among heat-responsive genes, we found that 4 out of the 11 AP1-activated targets and 8 out of 11 AP3-activated targets were heat-repressed in early flowers (Figure 4A). In comparison, a significantly higher proportion (24 out of the 29) of AG-activated and heat-responsive genes in early flowers were heat-repressed [Figure 4A; Pearson's χ^2 test with Yate's continuity correction, χ^2 (1, n = 63) = 12.5, P = 4e-04]. Hence, the floral developmental programs regulated by the C-function gene *AG* were more severely impaired by heat treatment. For heat-responsive genes that were directly repressed by AP1 or AP3 in early flowers, a small proportion was activated upon heat treatment (9 out of 34 for AP1; 4 out of 19 for AP3). Conversely, we observed significantly higher proportion of the heat-responsive genes that were directly repressed by AG were upregulated by heat stress in early flowers [9 out of 14; Pearson's χ^2 test with Yate's continuity correction, χ^2 (1, n = 67) = 6.2, P = 0.01] (Figure 4A). These results show that heat conditions have

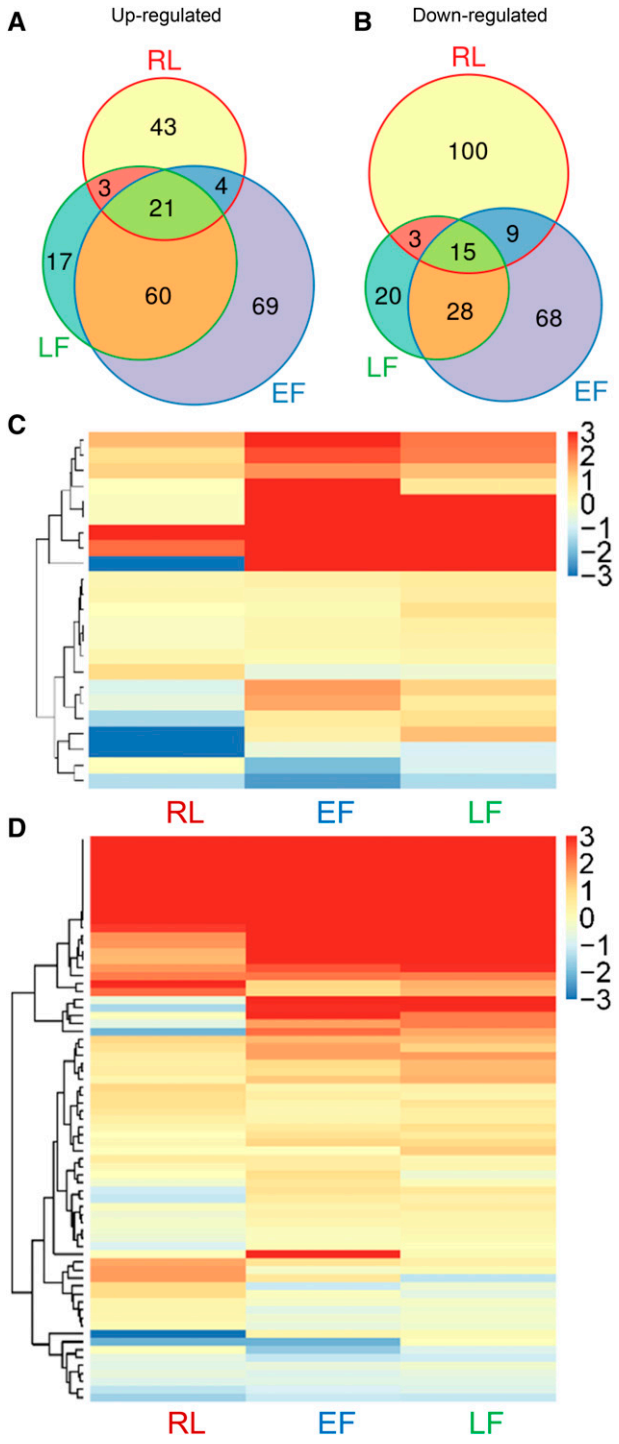

Figure 3. Heat Regulation of Transcription Factors in Vegetative and Reproductive Tissues of Arabidopsis at the Reproductive Stage.

(A) to **(B)** Heat responsiveness of putative transcription factors. Venn diagrams show numbers of genes upregulated **(A)** or downregulated **(B)** in RLs, EFs, and LFs of Arabidopsis wild-type plants at the late bolting stage. **(C)** and **(D)** Heat responsiveness of HSFs and HSPs. Heat maps show upregulated (red) and downregulated (blue) HSFs **(C)** and HSPs **(D)** in RLs, EFs, and LFs of Arabidopsis wild-type plants at late bolting stage.

a greater effect on the expression of floral C identity genes than do floral A or B. Among the ABC genes, the reproductive developmental programs downstream of AG seemed to be the most severely affected by the heat stress treatment.

In wild-type Arabidopsis plants, anther primordia are formed at flower stage 5, while that of the ovule starts at flower stage 8, and both tissues do not mature until late stages of floral development (after stage 12) (Smyth et al., 1990). Previously, we identified genes preferentially expressed in five representative reproductive tissues or cell types, i.e., anther, ovule, male meiocytes, female meiocytes, and pollen using previous data sets (Pina et al., 2005; Yang et al., 2011; Feng et al., 2012; Sánchez-León et al., 2012; Zhao et al., 2014; Zhang et al., 2015). To better understand the impacts of heat stress on reproductive development, we examined the heat responsiveness of these genes. Our results showed that 8 to 9% of genes preferentially expressed in anthers or ovules were repressed by heat in early flowers, and a significantly lower proportion (4 to 5%) of anther/ovule-specific genes were repressed by heat in late flowers (exact binomial test: P = 0.01 for anther data, P = 7e-07 for ovule data; Figure 4B). Similarly, more anther/ovule-preferential genes were activated by heat in early flowers than in late flowers (exact binomial test: P = 0.009 for anther data, P = 6e-04 for ovule data; Figure 4B).

Pollen grains begin to form at flower stage 10 and become almost completely mature at stage 12. The late flowers used in this study thus represent the duration of pollen development. We observed that over 3% of pollen-preferential genes were repressed by heat treatment in late flowers (Figure 4B). Examination of the pollen-preferential genes repressed by heat in late flowers showed that they included genes encoding invertases, pollen cell wall-related, and coat-related proteins (Supplemental Data Set 4). Thus, high temperature stress affected the maturation of pollen grains.

Meiosis is required for sexual reproduction. In Arabidopsis, meiosis in male meiocytes takes place during flower stage 9, while meiosis in female meiocytes occurs during flower stage 10, corresponding to early and late flowers used in this study, respectively. Over 7% of genes preferentially expressed in male meiocytes were repressed by heat in early flowering, and ~5% of female meiocyte-preferential genes were repressed by heat in late flowers (Figure 4B). GO enrichment analysis showed that among the genes specifically downregulated by heat in early flowers are the processes of DNA recombination and double-strand break repair via homologous recombination (Figure 2I; Supplemental Data Set 5). Among the heat-repressed reproduction-specific genes, we observed the enrichment of genes involved in microtubule-based movement, cell division, cell cycle, DNA repair, as well as in the mitotic spindle assembly checkpoint (Figure 2K; Supplemental Data Set 5). Because male and female meiocytes account for only a small proportion of the cells in each flower tissue, the influences of high temperature stress on meiosis might be relatively more dramatic. We checked the heat responsiveness of all genes with known functions in male meiosis. One-quarter of the known meiotic genes (24 out of 96) were downregulated by heat stress in early flowers, and eight of these were also downregulated in late flowers (Figure 4C; Supplemental Data Set 6).

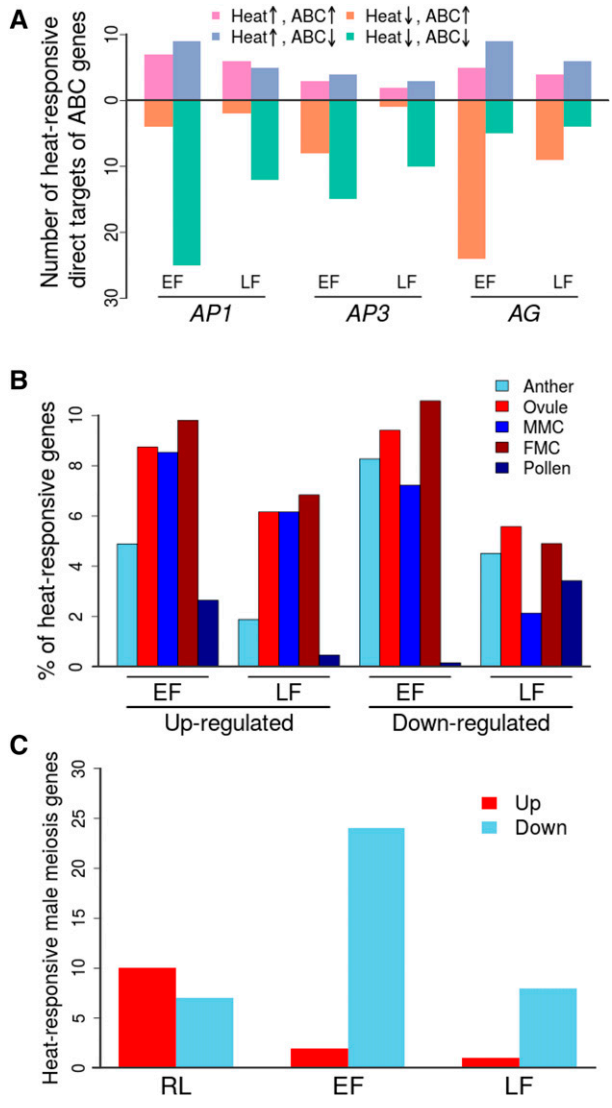

Figure 4. Effect of Heat Exposure on the Expression of Genes Associated with Plant Reproductive Development.

(A) Number of heat-responsive genes that are direct targets of ABC genes. Upward or downward arrows indicate the number of respective genes that were upregulated or downregulated either by heat or by one of the ABC genes.

(B) Percentage of heat-responsive genes preferentially expressed in reproductive tissues/cell types. MMC, male meiocytes; FMC, female meiocytes.

(C) Number of heat-responsive genes associated with male meiosis.

The UPR Is Important for Reproductive Development in Response to Heat Stress

Enrichment of genes participating in endoplasmic reticulum UPR among the heat upregulated reproductive stage specific genes (Figure 2J) prompted us to investigate the role of the UPR in the HSR. In Arabidopsis, two membrane-associated transcription factors, bZIP28 and bZIP60, are the major transcriptional regulators of the canonical UPR induced by the *N*-glycosylation inhibitor tunicamycin (Song et al., 2015), and both factors are important for the heat shock response (42°C) during the seedling stage (Gao et al., 2008; Deng et al., 2011). To examine the roles of *bZIP28* and *bZIP60* in maintaining fertility upon heat stress (37°C), we compared the heat sensitivity of wild-type, *bzip28 bzip60* double mutant, and *bzip28 bzip60* double mutant plants complemented with either *bZIP28* or *bZIP60* driven by the respective native promoter (Sun et al., 2013a) at the reproductive stage. Following exposure to heat stress at the reproductive stage, silique lengths in *bzip28 bzip60* double mutant plants were largely reduced compared with the wild-type plants (Figures 5A to 5C) but were not much reduced in the complemented transgenic plants (Figure 5D). These results demonstrated that *bZIP28* and *bZIP60* are important for thermotolerance at the reproductive stage. To investigate the downstream genes of *bZIP28* and *bZIP60* during the HSR, we examined the transcriptomes of *bzip28 bzip60* double mutant plants along with the wild-type plants. For comparison, we retrieved a list of canonical UPR genes induced in Arabidopsis seedlings (Song et al., 2015). We found that 10, 31, and 22 canonical UPR genes were upregulated by heat stress in rosette leaves, early flowers, and late flowers, respectively in the wild-type plants (Figure 6A). However, there were 5, 17, and 13 canonical UPR genes upregulated in rosette leaves, early flowers, and late flowers, respectively, in the *bzip28 bzip60* double mutant plants (Figure 6A), suggesting either that other transcription factors might also be involved in the regulation of some of these canonical UPR genes in the double mutant plants or that the promoters of these genes might contain other heat shock responsive *cis*-elements. More UPR genes were upregulated in flower tissues than in rosette leaves, but fewer UPR genes were downregulated in flowers than in leaves (Figure 6A; Supplemental Data Set 7). These results suggested that the UPR pathway mediated by bZIP28 and bZIP60 is important for heat responses in flower tissues as well as in vegetative tissues.

We then extended the analysis to all heat responsive genes and found that 587, 424, and 382 heat-responsive genes were upregulated in rosette leaves, early flowers, and late flowers, respectively, only in the wild-type plants, whereas 424, 322, and 206 heat-responsive genes were upregulated in rosette leaves, early flowers, and late flowers, respectively, only in the *bzip28 bzip60* double mutant plants (Figure 6B). We considered these specifically heat upregulated genes in the wild type in either of three examined tissues to be bZIP28/bZIP60-dependent heat-responsive genes ($\log_2$FC > 1 and q < 0.01 in wild-type plants, q > 0.01 in *bzip28 bzip60* plants; Supplemental Data Set 8). Among those commonly heat upregulated genes, fold changes of 269 genes in the *bzip28 bzip60* double mutant plants were dramatically decreased compared to the wild type (Figure 6C). We considered these genes together with the 252 bZIP28/bZIP60-dependent heat-responsive genes ($\log_2$FC > 1 and q < 0.01 in wild-type plants, q > 0.05 in *bzip28 bzip60* plants) to be the bZIP28/bZIP60-regulated heat-responsive genes (Supplemental Data Set 9). GO analysis of these genes revealed that response to heat, response to hydrogen peroxide, response to high light, and endoplasmic reticulum unfolded protein response were enriched (Figure 6D). Taken together, these results also suggest not only that bZIP28 and bZIP60 are important for fertility maintenance during the HSR but also that these two transcription factors

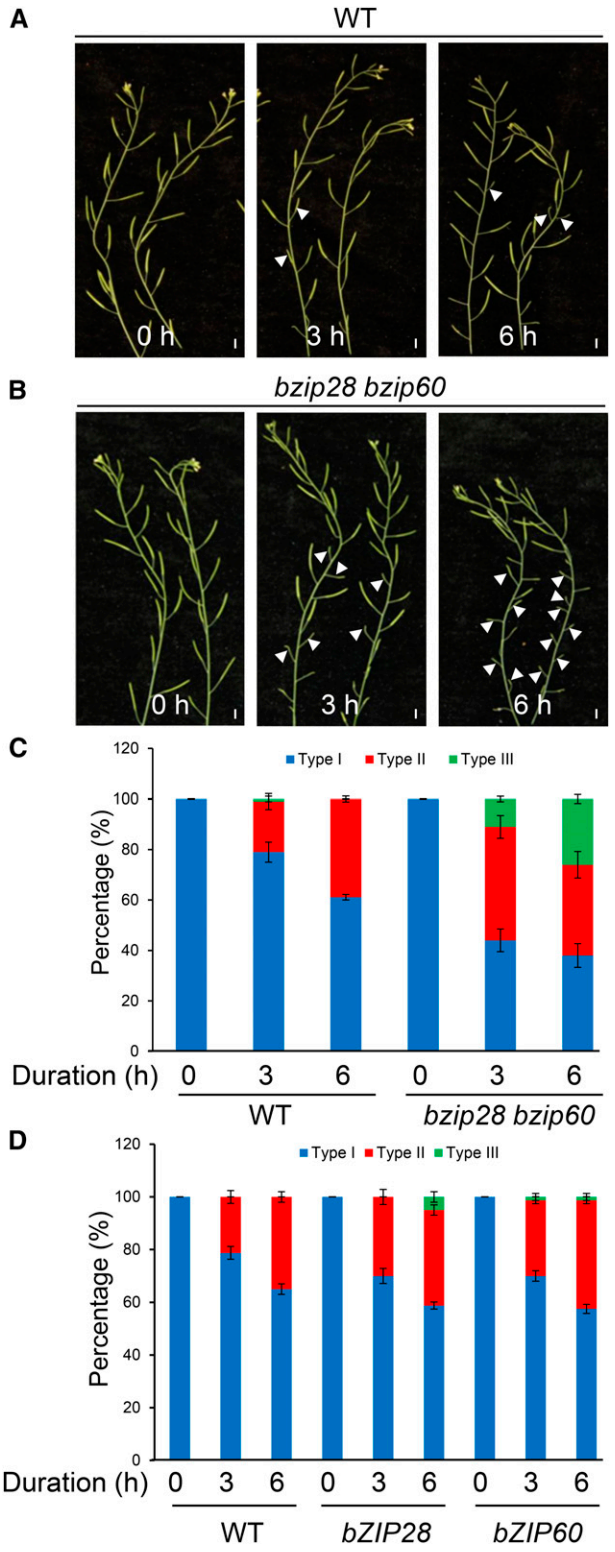

Figure 5. Role of UPR Regulators in Fertility Maintenance under Heat Stress at the Reproductive Stage.

(A) and **(B)** Effect of heat stress on inflorescence appearance. Arabidopsis wild-type (WT) plants **(A)** and *bzip28 bzip60* double mutant plants **(B)** following heat stress treatment for different times at the stage of late bolting. Bars = 5 mm.

(C) and **(D)** Effect of heat stress on fertility maintenance. Silique lengths were measured after recovery at each position of main inflorescence, and the percentage of each type of silique was calculated as in Figure 1F. The *bzip28 bzip60* double mutant plants were complemented with either *bZIP28* or *bZIP60* driven by the respective native promoter. Arrowheads point to some of the Type III complete sterile siliques. Bars depict SE (n = 10 plants in **[C]** and n = 8 plants in **[D]**).

regulate canonical UPR genes and other heat-responsive genes during the HSR in Arabidopsis.

bZIP28 Binds Directly to the Promoters of Heat-Responsive Genes

In a previous microarray experiment, 276 genes were upregulated by canonical UPR inducer tunicamycin in Arabidopsis seedlings, which was largely dependent on bZIP28 and bZIP60. Hereafter, we refer to these genes as bZIP28/bZIP60-dependent UPR genes (Song et al., 2015). However, the genome-wide direct target genes of bZIP28 or bZIP60 are unknown. To identify candidate bZIP28 direct targets, we performed ChIP-seq experiments with *MYC-bZIP28*-expressing plants (Liu et al., 2007b; Song et al., 2015) following previous methods for heat stress treatment (Gao et al., 2008). MYC-bZIP28 was enriched in the 2-kb upstream regions (relative to the transcription initiation sites) of a total of 133 genes in three replicates in heat stressed Arabidopsis seedlings comparing to the nonstressed plants (Supplemental Data Set 10). We considered these genes to be bZIP28 direct targets in Arabidopsis seedlings. Most of the proteins encoded by these bZIP28 direct target genes are located in the ER, Golgi apparatus, or in the secretory pathway (Supplemental Figure 4A). GO analysis showed that these proteins are involved in protein folding and response to endoplasmic reticulum stress (Supplemental Figure 4B). We compared these bZIP28 direct targets with those bZIP28/bZIP60-dependent canonical UPR genes. It was found that ~44% (58 out of 133) of the bZIP28 potential direct targets were also upregulated by the ER stress inducer tunicamycin (Supplemental Figure 4C), and among these, the promoters were enriched with the previously identified bZIP28 binding *cis*-element ERSE-II (Supplemental Figure 4D) (Liu and Howell, 2010a). We also compared these bZIP28 direct targets in seedlings with those HSR genes in rosette leaves, early flowers, and late flowers, respectively. The results showed that totally 27 target genes were upregulated by heat at reproductive stage, with 4, 25, and 14 genes in rosette leaves, early flowers, and late flowers, respectively (Figure 7A; Supplemental Data Set 10). These results suggested that bZIP28 regulates distinct HSR genes in different tissues in Arabidopsis. We also examined the heat responsiveness of these bZIP28 direct targets between the wild type and *bzip28 bzip60* double mutant. The results indicated that the upregulation of eight genes (*HSP70*, *CRT1A*, *ECA4*, *P24DELTA5*, *PDI9*, *SDF2*, *UTR3*, and a gene encoding a protein with unknown function) by heat were dependent on bZIP28/bZIP60 (Figure 7A). MYC-bZIP28 preferentially bound to the promoter regions of these eight genes (Figure 7B). We concluded that these eight

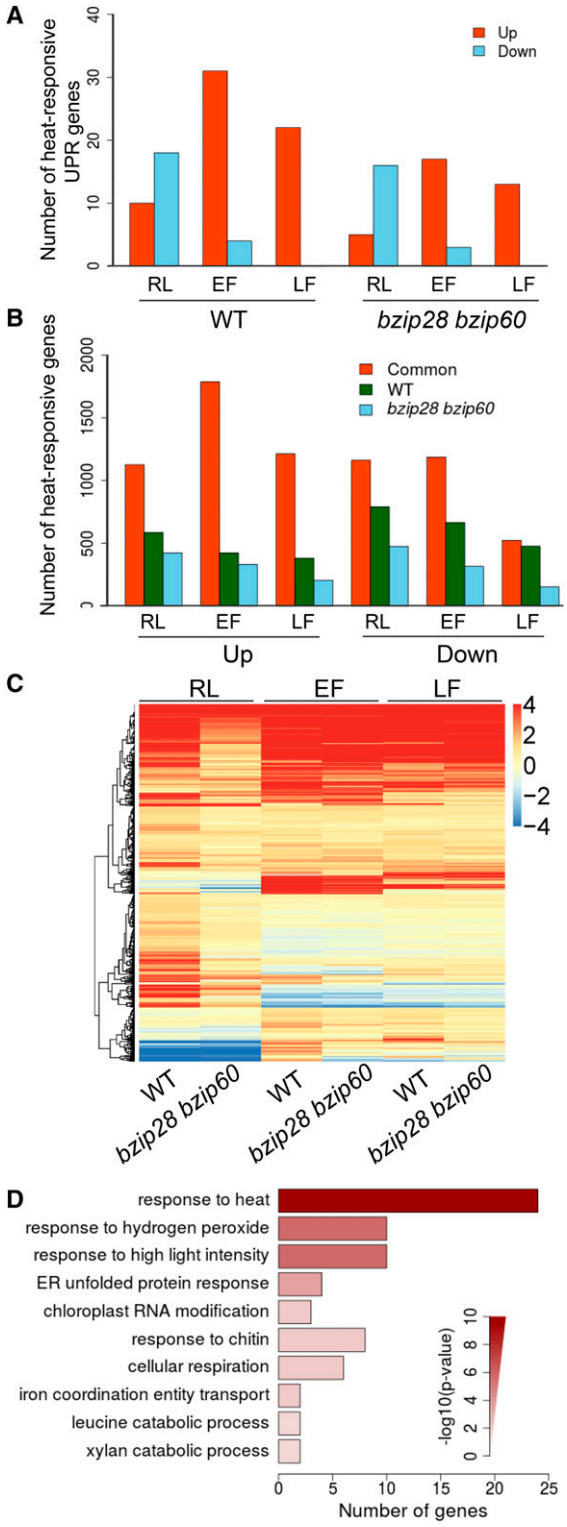

Figure 6. Heat-Responsive Genes Regulated by bZIP28 and bZIP60 at the Reproductive Stage.

(A) Responses of classical UPR genes to heat in vegetative and reproductive tissues in wild-type (WT) and *bzip28 bzip60* double mutant plants.

(B) Heat-responsive genes whose expression is dependent on bZIP28/bZIP60.

(C) Heat map showing heat responsiveness of genes partially dependent on bZIP28/bZIP60.

(D) GO analysis of heat-responsive genes regulated by bZIP28/bZIP60.

genes are direct targets of bZIP28 during the HSR at reproductive stage.

To learn whether the bZIP28 targets at 42°C are the same targets at 37°C, we also stressed *MYC-bZIP28*-expressing plant seedlings at 37°C (1 h) and conducted ChIP-qPCR for the aforementioned eight genes. Our results showed that bZIP28 bound to the promoter regions of all of these aforementioned eight genes (Supplemental Figure 5). We did not exclude those genes that were bound by MYC-bZIP28 but were upregulated by heat in both the wild type and *bzip28 bzip60* double mutant as the bZIP28 direct targets during the HSR at reproductive stage because other transcription factors may also regulate these genes during the HSR. Indeed, ERSE-II was enriched in the promoters of bZIP28-targeting heat-responsive genes (Figures 7C and 7D). We also did not exclude the possibility that bZIP28 has another subset of specific targets during the HSR at reproductive stage because attempts to perform ChIP-seq in *MYC-bZIP28*-expressing flowers were not successful. Nonetheless, several bZIP28-targeting heat-responsive genes in vegetative and reproductive tissues were revealed in this study.

To demonstrate the functional role of bZIP28 target genes in the HSR at the reproductive stage, we chose the UPR marker gene *BIP3* (Liu and Howell, 2016). RNA-seq data showed that *BIP3* was upregulated by heat stress in reproductive tissues in the wild-type plants, while such upregulation was abolished in the *bZIP28* and *bZIP60* double mutant plants (Figure 8A). We performed a phenotypic analysis of the HSR with a T-DNA knockout mutant of *BIP3* (Supplemental Figure 6). Compared with the wild-type plants, silique lengths were reduced in the *bip3* mutant plants when plants were heat stressed (37°C for 6 or 8 h) at the reproductive stage (Figure 8B). Although *HOT1* was not the direct target of bZIP28 (Figure 7B), it was highly upregulated in flower tissues both in the wild-type and *bzip28 bzip60* double mutant plants (Figure 8A). Knocking out *HOT1* in the *hot1* mutant plants (Supplemental Figure 6) also conferred heat sensitivity at reproductive stage (Figure 8C).

DISCUSSION

Many major crop plants, including cereals, are very sensitive to high temperatures at the reproductive stage (Barnabás et al., 2008). Over the past several decades, extensive studies have elucidated many of the molecular mechanisms of plant responses to high temperature during the vegetative stage (Liu et al., 2015). However, how reproductive tissues of plants respond to high temperature remains elusive. When plants are exposed to high temperatures during reproductive development, there are two particularly sensitive developmental stages: during meiosis and near the time of fertilization. In addition, several quantitative trait loci associated with thermotolerance at 35°C at the reproductive stage were identified based on the natural variations among different Arabidopsis ecotypes (Bac-Molenaar et al., 2015). However, the genetic basis of heat tolerance underlying these

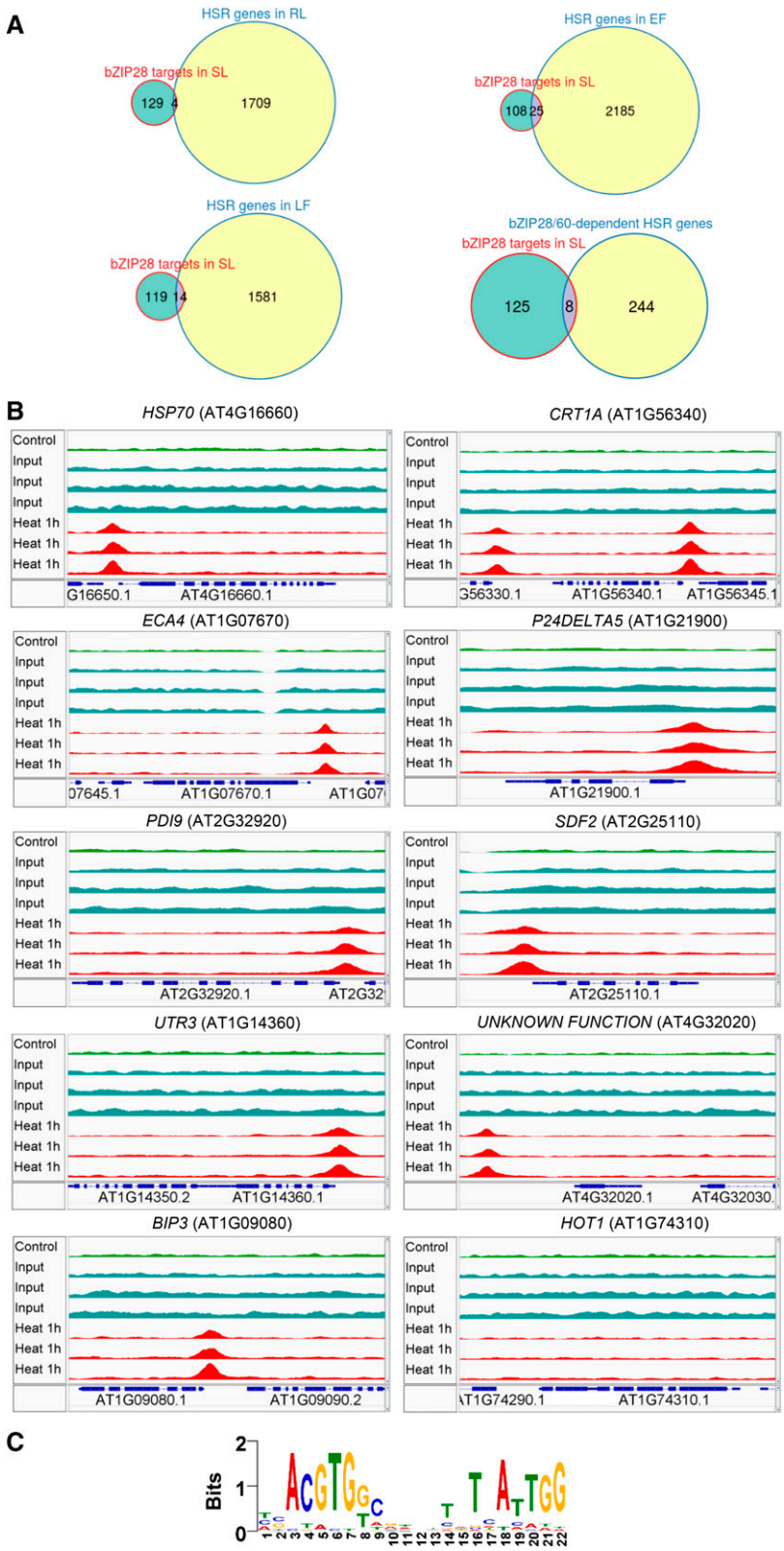

Figure 7. Direct Targets of bZIP28 under Heat Stress Conditions.

(A) Venn diagram showing the direct targets of bZIP28 in RLs, EFs, and LFs in response to heat stress at the reproductive stage. ChIP-seq data from *myc-bZIP28* expressing Arabidopsis plants stressed with heat at seedlings (SL) stage was compared with the RNA-seq data from the Arabidopsis wild-type plants stressed with heat at the late bolting stage and with the bZIP28/bZIP60-dependent heat-responsive genes.

quantitative trait loci is still largely unknown. Here, we have taken advantage of large-scale expression analyses and have examined the heat responsiveness of gene expression in both vegetative and reproductive tissues following exposure of Arabidopsis plants to heat stress at the reproductive stage. Hundreds of genes were specifically up- or downregulated in flower tissues by exposure to heat stress. Comparison of the wild-type plants and mutant plants defective in the ER stress response has revealed that the UPR mediated by the membrane-associated transcription factors bZIP28 and bZIP60 plays a vital role in maintaining fertility when plants experienced heat stress at the reproductive stage. During the heat stress response in different tissues, we found several direct targets of bZIP28, including canonical and noncanonical UPR targets. Our work provides a collection of candidate genes for providing thermotolerance at the reproductive stage. For example, knockouts of heat-responsive molecular chaperone genes *HOT1* and *BIP3* have conferred reduced fertility in Arabidopsis plants subjected to heat stress at reproductive stage (Figure 8).

Multiple Transcription Factor Families Regulate Transcription at High Temperature

The core regulators of the HSR in plants are members of the HSF transcription factor family, which regulate the expression of many genes encoding HSPs and other factors by recognizing palindromic binding motifs (5′-AGAAnnTTCT-3′) 5′ to the TATA box (Ohama et al., 2016). The Arabidopsis genome encodes at least 21 HSFs and 3 HSF-related proteins (Nover et al., 2001; Scharf et al., 2012). Fewer than half of these HSF transcription factors were upregulated by heat in at least one type of tissues in the wild-type plants (Figure 3C). However, under such heat stress conditions, a total of 217 transcription factor genes in 54 different families were significantly upregulated by heat in at least one type of tissue in the wild-type plants (Figure 3A). Besides HSFs, transcription factors in the ERF, NAC, MYB-related, bZIP, and bHLH families were also upregulated in early flowers during the HSR (Supplemental Figure 2). Although constitutively expressed transcription factors may be also important for gene expression during the HSR, these heat stress-induced transcription factors in reproductive tissues are likely important for dynamic transcriptional regulation of downstream genes during the HSR. Therefore, understanding the functions of these newly identified transcription factors in improving thermotolerance at the reproductive stage in plants is critical in future studies.

Heat Disturbs Developmental Programs in Reproductive Tissues and Affects Fertility Maintenance during the HSR

After the transition from the vegetative stage to reproductive growth, the shoot apical meristem becomes the inflorescence meristem and then floral meristem. Floral organs are then produced according to a canonical pattern of sepals, petals, stamens, and pistils, which are controlled by the complex gene networks (Pajoro et al., 2014). In our study, the floral promoting *SEP4* in the ABCE model (Ditta et al., 2004) was significantly upregulated by heat stress, while the floral repressor *AGL15* (Adamczyk et al., 2007) was significantly downregulated in early flowers by heat stress (Supplemental Data Set 1). By contrast, numerous genes downstream of *AP1*, *AP3*, and *AG* that were activated in normal growth temperatures were repressed in early flowers in response to heat stress (Figure 4A). These results suggest that the tightly controlled developmental program in reproductive tissues is disturbed by heat stress. Indeed, the expression of many genes related to early phases of anther and ovule development, pollen development, and meiosis was significantly delayed by heat stress in flower tissues (Supplemental Data Set 4).

Phytohormones such as auxin are important for plant reproductive development, and it has been reported that endogenous auxin levels and the expression of auxin biosynthetic genes are reduced in the anthers of both barley and Arabidopsis under heat stress conditions, causing male sterility (Sakata et al., 2010). By contrast, activation of auxin biosynthesis and accumulation of auxin indole-3-acetic acid have been observed in vegetative plant tissues upon exposure to high temperature (Gray et al., 1998). In our study, GO enrichment of regulation of auxin biosynthetic process genes was observed in upregulated genes in early flower tissues (Figure 2G), suggesting that the auxin biosynthetic pathway is important for maintaining fertility under heat stress conditions in Arabidopsis flowers. Drought, salt, and low temperature stresses increase the cellular level of the phytohormone abscisic acid (ABA; Xiong et al., 2002). However, ABA concentrations in heat-stressed plant leaves are very low (Zeevaart and Creelman, 1988). Using a transgene (*RD29A-LUC*), it was reported that low temperature and ABA treatments are synergistic in inducing the transgene expression. Although high temperature alone did not activate the transgene, it significantly increased the effects of ABA (Xiong et al., 1999). In this study, genes involved in ABA responses were enriched in upregulated genes only in early flower tissues (Figure 2G). These results indicate that ABA response is probably important for early flower development during heat stress. It is possible that high temperature stress has increased the sensitivity of flower tissues to endogenous ABA.

Key UPR Components Have Important Roles in Upregulating Heat-Responsive Genes in Arabidopsis

Environmental stresses may increase protein folding demand and denature correctly folded proteins in the ER, which triggers the UPR to mitigate the stress by upregulating downstream genes

Figure 7. (continued).

(B) Distribution of bZIP28 binding peaks on eight bZIP28/bZIP60-dependent heat responsive genes in the Integrated Genome Browser. Data for *BIP3* and *HOT1* were also included. For each gene model, blue boxes represent exons and blue lines represent introns. Aligned reads are indicated in red (heat-treated, three replicates), green (not heat-treated), and cyan (input, three replicates).

(C) Sequence logo for bZIP28 binding motif in the promoters of bZIP28-targeting heat-responsive genes (E-value = 4.9e-41). The height of each letter represents the frequency of the base at that position.

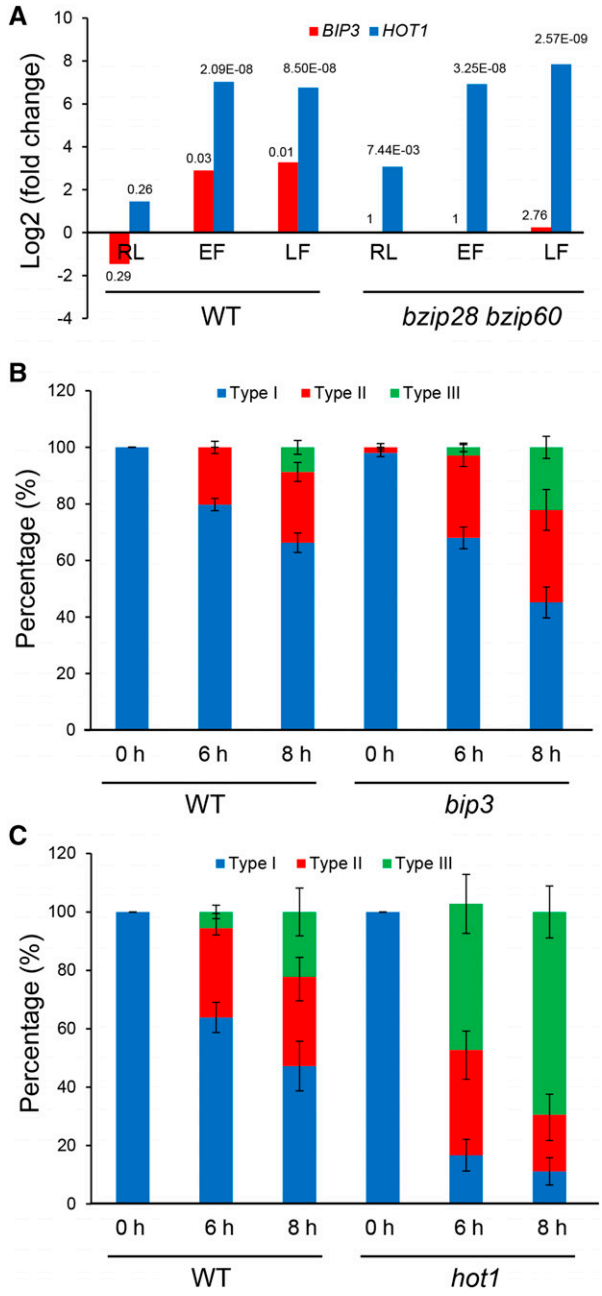

Figure 8. Reduced Fertility of Heat-Stressed Arabidopsis Mutant Plants.

(A) Responses of *BIP3* and *HOT1* to heat treatment in vegetative (RL) and reproductive tissues (EF and LF) at the late bolting stage in wild-type and *bZIP28/bZIP60* double mutant (*bzip28 bzip60*) seedlings. Q-values are indicated above or below the bars.

(B) and **(C)** Silique lengths of *bip3* and *hot1* mutant plants after heat stress treatment at the reproductive stage. Silique lengths were measured after recovery at each position of main inflorescence, and the percentage/ratio of each type of silique was calculated as in Figure 1F. Bars depict SE ($n = 8$ in **[B]** and $n = 9$ in **[C]**).

encoding factors involved in protein folding and ER-associated protein degradation (Liu and Howell, 2010a, 2016). Membrane-associated transcription factors bZIP28 and bZIP60 are the master regulators of the canonical UPR induced by tunicamycin in Arabidopsis (Song et al., 2015). Both bZIP28 and bZIP60 are activated by a 42°C heat shock in seedlings (Gao et al., 2008; Deng et al., 2011). However, their direct targets under heat stress condition are largely unknown. In this study, at least 133 genes are considered to be direct targets of bZIP28 during the HSR (42°C) in Arabidopsis seedlings (Supplemental Data Set 10). Among them, 44% of them (Supplemental Figure 4C) are canonical UPR targets of bZIP28 or bZIP60 (Song et al., 2015), suggesting that bZIP28 has both canonical and novel targets during the HSR in Arabidopsis seedlings. These results indicated that additional activators other than bZIP28 might be required for the expression of these novel target genes under heat stress conditions or that additional repression factors might be recruited on the promoters of these novel targets under canonical UPR conditions.

Because bZIP28 is relocated from the ER to the nucleus in response to heat stress (Gao et al., 2008), it is reasonable to consider these novel bZIP28 targets as noncanonical UPR genes during the HSR, although it is difficult to directly detect the accumulation of misfolded proteins in the ER during heat stress. Indeed, upregulation of many canonical UPR genes by heat stress is dependent on *bZIP28* and/or *bZIP60* (Figures 6A and 6D), and bZIP28 binds to the promoters of several canonical UPR genes, both of these findings suggest that the UPR is elicited during the HSR in which bZIP28 is involved. It is also possible that certain bZIP28 direct targets under heat stress conditions are not the ones under canonical UPR conditions induced by tunicamycin. Understanding the biological functions of these canonical and noncanonical targets of bZIP28 during the HSR is needed in the future. We found here that bZIP28 and bZIP60 also play important roles in thermotolerance at the reproductive stage, as mutation of both genes conferred heat sensitivity of silique length and fertility (Figures 5A to 5D). One of the bZIP60-regulated canonical UPR genes, *BIP3*, was previously reported to be involved in the HSR in Arabidopsis seedlings (Deng et al., 2011). In this study, we found that *BIP3* was also a direct target of bZIP28 (Figure 7B; Supplemental Data Set 10), and our genetic analysis demonstrated that knockout of *BIP3* conferred heat sensitivity in terms of silique length and fertility at reproductive stage (Figure 8B).

Furthermore, RNA-seq analysis revealed that at least 521 genes were differentially regulated between wild-type and *bzip28 bzip60* double mutant plants at the reproductive stage (Supplemental Data Set 9). Combined analysis of RNA-seq and ChIP-seq data sets suggested that other transcription factors shared direct targets with bZIP28/bZIP60 during the HSR at the reproductive stage. In previous reports, the membrane-associated transcription factor bZIP17, a paralog of bZIP28, was activated in a similar manner to bZIP28 in response to ABA and salt stress in Arabidopsis seedlings (Liu et al., 2007a; Zhou et al., 2015). In the canonical UPR, *bZIP17* and *bZIP28* were barely affected while *bZIP60* was rapidly upregulated by tunicamycin treatment in Arabidopsis seedlings (Tajima et al., 2008). However, all the three bZIP transcription factor genes were dramatically upregulated by

heat in Arabidopsis flower tissues (Supplemental Data Set 1), suggesting that bZIP17 may be also involved in the HSR in reproductive tissues. We and other colleagues have demonstrated that bZIP17 has a negligible role in regulating canonical UPR downstream genes in Arabidopsis seedlings (Tajima et al., 2008; Song et al., 2015). However, bZIP17 is activated in response to heat shock (42°C) in seedlings (Che et al., 2010). Thus, bZIP17 possibly shares direct targets with bZIP28/bZIP60 in Arabidopsis during the HSR at the reproductive stage. Due to technical difficulties, we could not carry out ChIP-seq analysis of bZIP60 targets. Nonetheless, our work has revealed a collection of HSR genes regulated by bZIP28/bZIP60 and has found several canonical and noncanonical direct targets of bZIP28 during heat stress responsiveness in Arabidopsis. These findings are important for understanding the role of the UPR components in the heat stress response in plants.

The Responsiveness of Arabidopsis to High Temperature at the Reproductive Stage Is Tissue Specific

Although all plant tissues are sensitive to heat stress, reproductive tissues are especially susceptible to high temperatures, and this often results in yield reduction (Hatfield et al., 2011). Previously, several experiments investigated the genome-wide responsiveness of Arabidopsis seedlings to heat stress (Rizhsky et al., 2004; Swindell et al., 2007; Barah et al., 2013). However, few studies have examined the molecular mechanisms of the response to heat at the reproductive stages. In this study, vegetative and reproductive tissues were exposed to high temperature at the same time, and tissue-specific responsiveness to heat stress was observed in terms of both the number of heat-regulated genes and the GO enrichments in the RNA-seq analysis (Figure 2). Analysis of transcription factor families revealed that the expression of different sets of transcription factors was affected by heat stress in different tissues (Figure 3). Further ChIP-seq analysis demonstrated that the direct targets of bZIP28 were differentially expressed in different tissues after exposure to heat stress (Figure 7). One of the Arabidopsis HSP100/CLPB cytosolic chaperone genes, *HOT1*, was previously found to be important in acquired thermotolerance in vegetative tissues (Lee et al., 2005). This gene was more upregulated in reproductive tissues than that in vegetative tissues (Figure 8A). *BIP3*, encoding an ER-resident chaperone, was upregulated by heat stress only in reproductive tissues (Figure 8A). Knockout of either *HOT1* or *BIP3* (Supplemental Figure 6) dramatically reduced silique length and fertility of mutant plants after exposure to heat stress at reproductive stage (Figures 8B and 8C). Our work has emphasized the importance of investigating the functional roles of heat stress-responsive genes that are specifically or more affected in flower tissues in plants.

METHODS

Plant Material and Growth Conditions

All *Arabidopsis thaliana* materials used in this study used the Col-0 ecotype. The *bzip28 bzip60* double mutant was obtained by crossing the *bzip28* single mutant to the *bzip60* single mutant (Sun et al., 2013a). The genetic complementation materials were prepared as described previously. About 1 kb of promoter sequences of *bZIP60* or *bZIP28* was first cloned into the pCAMBIA1300 vector, and then the coding sequences of *bZIP60* or bZIP28 were inserted to generate the complementary constructs pC60 or pC28, which were introduced into the *bzip28 bzip60* mutant plants by floral dip methods (Sun et al., 2013a). Mutants of *bip3* and *hot1* were obtained from the Arabidopsis Biological Resource Center. For phenotype analysis and RNA-seq experiments, seeds were sown into commercial soilless mixes which include peat, vermiculite, and perlite after being stratified at 4°C for 2 d, and cultivated under standard growth conditions (22°C, 16/8-h day/night photoperiod, 60% humidity). For ChIP-seq experiments, seeds were sown directly on half-strength Murashige and Skoog medium (with 1.2% sucrose and 0.7% agar, pH 5.7) and stratified at 4°C for 2 d, then transferred to an illuminated (100 μmol m^{-2} s^{-1}) incubator at 22°C with a 16/8-h day/night photoperiod.

Analysis of the Heat Shock Phenotype

Heat shock phenotype analyses were conducted at the reproductive stage. After the appearance of the first flowers, plants were subjected to heat treatment in a growth room at 37°C with comparable light intensity conditions for different times as indicated in the text, while the other control plants were grown at 22°C. Before heat treatment, the flowers selected were furthest along in development and were marked with colored threads/tags. After several hours of heat shock, plants were transferred to 22°C and continued to grow for 10 d. Then, the main inflorescence starting from the tag was removed for photography and later analysis. The silique next to the tagged thread was labeled as position number 1, and 10 to 14 siliques in sequence were used to determine the length. Meanwhile, the condition of seeds was observed under a microscope. Siliques were further divided into three major categories: fully fertile (Type I, >10 mm in length), partially sterile (Type II, 5–10 mm in length), and completely sterile (Type III, <5 mm in length). A Student's *t* test or two-way ANOVA was used to evaluate the significance of phenotypic difference.

RNA-Seq Analysis

For RNA-seq analysis, flowering plants were treated at 37°C for 3 h, and the control plants were kept at 22°C. After heat shock, EFs (stage 1-9), LFs (stage 10-12) (Smyth et al., 1990), and RLs were collected and immediately frozen in liquid nitrogen. Flowers from 20 plants or leaves from 8 plants were pooled for each sample, and three biological replicates were used in the study. Plant materials were ground in liquid nitrogen, and total RNA was extracted with Trizol (Invitrogen). The cDNA libraries were constructed following Illumina standard protocols and sequenced with Illumina HiSeq 3000 by Genergy Bio. RNA-seq reads were aligned to the Arabidopsis reference genome (version TAIR10) using TopHat (version 2.0.13) (Kim et al., 2013) after filtering out low-quality (lowest base score < 30) reads using SolexaQA (version 3.1.3). Uniquely aligned reads were counted for each annotated gene using the program htseq-count from the python package HTSeq (Anders et al., 2015). Differential gene expression was evaluated using the R package edgeR (Chen et al., 2014).

In brief, the read count data for all genes and samples were imported to edgeR once to perform global normalization, calculation of the common dispersion factor, and then estimation of gene-specific dispersion parameters (i.e., tag-wise dispersion) (Chen et al., 2014). Differential gene expression between each pair of heat-stressed plants and controls were evaluated by calling exact test (Chen et al., 2014). Raw P values were adjusted for multiple comparison effects using the q-value (false discovery rate) method (Storey and Tibshirani, 2003). The cutoff for significant differential expression was set as >2 absolute fold change (FC) and q-value < 0.01. We used the software package topology-based GO scoring

(topGO, version 2.26.0) of the R package to conduct gene ontology enrichment analysis, with the gene-to-GO association data obtained from the GO database (submitted by TAIR at 4/1/2016). Genes preferentially expressed in anther, ovule, male meiocytes, and female meiocytes were retrieved from the respective publications (Yang et al., 2011; Ma et al., 2012; Zhao et al., 2014). RNA-seq data for pollen were retrieved (Loraine et al., 2013), with pollen-preferential genes defined as those with averaged FPKM (fragments per kilobase of transcript per million of mapped reads) > 1 in pollen and 0.1 in seedlings, rosette leaf, and early flower.

ChIP-Seq Assays

For ChIP-seq, 13-d-old seedlings of *Pro35S:MYC-bZIP28* plants were subjected to heat stress with 42°C for 1 h. The stressed materials and nonstressed controls were fixed with 1% formaldehyde for 2 × 10 min under vacuum, and then glycine was added to a final concentration of 0.125 M to stop the reaction. The fixed materials were frozen in liquid nitrogen for ChIP experiments. ChIP assays were performed using procedures described previously (Song et al., 2015). Protein A-agarose beads (Millipore) and an anti-MYC antibody (Sigma-Aldrich) were used to precipitate the DNA. ChIP-DNA and input-DNA libraries were constructed by Genergy Bio. Briefly, DNA samples were end-repaired, followed by A-base addition and ligation with adapters. After PCR enrichment, the DNA library was quantified with Qubit (Thermo Fisher), followed by cBot cluster generation (Illumina) and library sequencing with an Illumina HiSeq 3000. Raw reads were preprocessed to remove adaptors using an in-house developed Perl script, to trim low-quality bases (score <30) and to filter out short reads (<50 bp) using SolexaQA (version 3.1.3). Preprocessed reads were aligned against the Arabidopsis reference genome using Bowtie (version 2.2.3) (Langmead and Salzberg, 2012). Sequence alignments were then filtered to retain only uniquely aligned reads. This was followed by duplicate redundancy removal using the rmdup command of SAMtools (version 1.2.1). Peak-calling was performed using MACS (version 1.4.2) (Zhang et al., 2008), and between-sample differential bindings were evaluated using MAnorm (Shao et al., 2012). For each biological replicate, peak regions showing >2-fold increase in binding strength (as measured by normalized M values) in the heat-stressed samples compared with controls, and with P values <0.01 were defined as potential binding regions of bZIP28. For each peak region, the nearest transcriptional start site was identified and the corresponding gene was considered as the potential target. The final set of bZIP28 binding regions constitutes the significant peaks associated with the same target genes across all the three biological replicates. Motifs significantly overrepresented in the identified binding region of the potential direct targets of bZIP28 were discovered and displayed using MEME (Bailey and Elkan, 1994), with the parameters set as: -dna–revcomp-nmotifs '20' -evt '0.05' -minsites '5′ -maxw '25' -maxsize '200000'. For ChIP-qPCR, 13-d-old seedlings of *Pro35S:MYC-bZIP28* plants were subjected to heat stress with 37°C for 1 h. ChIP was performed using the same protocol as for ChIP-Seq, and DNA enrichment was examined with qPCR. Briefly, the DNA level of each gene in three biological replicates was normalized to that of *ACTIN* measured in the sample with SYBR Green PCR master mix (Bio-Rad) in a multiplex quantitative PCR system (CFX 96 Touch; Bio-Rad). DNA levels were calculated with a ΔCt (threshold cycle) method. Primers for ChIP-qPCR were designed and listed in Supplemental Table 1.

Accession Numbers

RNA-seq and ChIP-seq data from this article can be found in Gene Expression Omnibus under the accession numbers GSE94015 and GSE94146, respectively. Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: *AG* (AT4G18960), *AGL15* (AT5G13790), *AP1* (AT1G69120), *AP3* (AT3G54340), *BIP3* (AT1G09080), *bZIP28* (At3g10800), *bZIP60* (AT1G42990), *CRT1A* (AT1G56340), *ECA4* (AT1G07670), *HOT1* (AT1G74310), *HSP70* (AT4G16660), *P24DELTA5* (AT1G21900), *PDI9* (AT2G32920), *SDF2* (AT2G25110), *SEP4* (AT2G03710), *UNKNOWN FUNCTION* (AT1G14360), and *UTR3* (AT4G32020). Germplasm used included *bzip28* (SALK_132285), *bzip60* (SALK_050203), *bip3* (SALK_024133), and *hot1* (CS16284).

Supplemental Data

Supplemental Figure 1. Comparison of Heat-Regulated Genes in Soil-Grown Arabidopsis Leaves Stressed at 37°C versus 44°C.

Supplemental Figure 2. Heat-Responsiveness of Different Transcription Factors in Arabidopsis Vegetative and Reproductive Tissues at the Reproductive Stage.

Supplemental Figure 3. Heat Responsiveness of the ERF Transcription Factors.

Supplemental Figure 4. Direct Targets of bZIP28 in Response to Classical ER Stress in Arabidopsis Seedlings.

Supplemental Figure 5. Enrichment of bZIP28 at Promoters of Target Genes under Heat Stress.

Supplemental Figure 6. Characterization of *bip3* and *hot1* Mutant Plants.

Supplemental Table 1. Primers Used for ChIP-qPCR.

Supplemental File 1. ANOVA Tables.

Supplemental Data Set 1. Heat Stress-Regulated Genes in Vegetative and Reproductive Tissues at the Reproductive Stage in Arabidopsis.

Supplemental Data Set 2. Heat Stress-Regulated Transcription Factors in Vegetative and Reproductive Tissues at the Reproductive Stage in Arabidopsis.

Supplemental Data Set 3. Heat Stress-Regulated Downstream Targets of ABC Genes in Arabidopsis.

Supplemental Data Set 4. Heat Stress-Regulated Genes Associated with Anther/Ovule Development in Arabidopsis.

Supplemental Data Set 5. Heat Stress-Regulated Genes Associated with Meiosis in Arabidopsis Reproductive Tissues.

Supplemental Data Set 6. Heat Stress-Regulated Genes with Known Functions during Male Meiosis in Arabidopsis.

Supplemental Data Set 7. Heat Stress-Regulated UPR Genes at the Reproductive Stage in Arabidopsis.

Supplemental Data Set 8. bZIP28/bZIP60-Dependent Heat-Responsive Genes at the Reproductive Stage in Arabidopsis.

Supplemental Data Set 9. bZIP28/bZIP60-Regulated Heat-Responsive Genes at the Reproductive Stage in Arabidopsis.

Supplemental Data Set 10. bZIP28 Direct Targets during the HSR in Arabidopsis Seedlings.

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AUTHOR CONTRIBUTIONS

J.-X.L. and F.C. conceived the project. S.-S.Z., L.D., and Z.-T.S. performed the experiments. H.Y. carried out the bioinformatics analysis. J.-X.L., S.-S.Z., H.Y., and F.C. designed the experiments and analyzed the data. J.-X.L., H.Y., and S.-S.Z. wrote the manuscript. J.-X.L. and H.M. revised and finalized the article.

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