Acta Physiol Plant (2017) 39:67
DOI 10.1007/s11738-017-2351-7

ORIGINAL ARTICLE

Arabidopsis heat stress transcription factors A2 (*HSFA2*) and *A3* (*HSFA3*) function in the same heat regulation pathway

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Received: 4 December 2015 / Revised: 6 October 2016 / Accepted: 6 January 2017 / Published online: 4 February 2017
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Abstract Heat stress transcription factors (HSFs) play an essential role in the adjustment of plants to high temperatures. These molecules have evolved complicated mechanisms that rely on interactions between different HSFs and other heat stress-related genes [such as *bZIP28*, *multiprotein bridging factor 1c* (*MBF1c*), *calmodulin-binding protein kinase 3* (*CBK3*)] in response to different heat stresses (such as occasional or successive high temperatures). In the present study, phenotypic, gene expression and yeast two-hybrid assays revealed that *HSFA2* and *HSFA3* function in the same heat regulation pathway. The single mutants, *hsfa2* and *hsfa3* as well as double mutant *hsfa2* and *hsfa3,* exhibited heat-sensitive phenotypes in acquired thermotolerance after a long recovery time (ATLR) but not in basic thermotolerance and acquired thermotolerance after a short recovery time (ATSR). The expression of *HSP18.1-CI* and *HSP25.3-P* was down-regulated in single and double mutants of *hsfa2* and *hsfa3* under successive heat stress in ATLR assays. In addition, HSFA2 interacted with HSFA3 at the protein level in yeast two-hybrid assays. These results demonstrated dynamic alterations in the expression of *HSFA2*, *HSFA3* and other heat-related genes in ATLR assays, providing new insights into the relationship between *HSFA2* and *HSFA3*; this information will refine the *HSF* network in the regulation of heat stress response.

Keywords *HSFA2* · *HSFA3* · Acquired thermotolerance after long recovery time · Gene expression · Yeast two-hybrid assay

Introduction

As sessile autophytic organisms, plants have an advantage regarding food-seeking but struggle against adverse environment stresses, such as high temperature. High temperatures impair plant growth and development, and extreme thermal stress even promotes programmed cell death (Vacca et al. 2004). For survival, plants have developed delicate mechanisms to overcome this adversity. The heat stress transcript factor (HSF) family is one of the major regulation groups that help plants to adapt to elevated high temperatures. Compared with the small *HSF* family in Drosophila or vertebrates, plants possess large *HSF* families, and more than 21 *HSFs* have been identified in Arabidopsis, while 24 *HSFs* have been identified in tomato, and 25 *HSFs* have been identified in rice (Scharf et al. 2011). Based on the presence of the conserved DNA-binding domain (DBD) and oligomerization domain (OD), HSF family proteins can be divided into A, B and C groups. In tomato, three main *HSFs* (*HSFA1*, *HSFA2* and *HSFB1*) regulate the expression of heat responsive genes and plant thermotolerance, and these proteins are

Communicated by A Chandra.

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Electronic supplementary material The online version of this article (doi:10.1007/s11738-017-2351-7) contains supplementary material, which is available to authorized users.

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modulated through *HSP70* and *HSP90* (Hahn et al. 2011). In Arabidopsis, at least ten *HSFs* participate in the heat stress response, and most of these molecules have recently been summarized (see reviews Scharf et al. 2011; von Koskull-Doering et al. 2007). Previous studies have reported that *HSFA1s* acts as master regulator in the early heat response (Liu and Charng 2012), while *HSFA2* and *HSFA3* play important roles in prolonged heat stress in Arabidopsis (Schramm et al. 2008).

Potential heat shock elements (HSEs) have been identified in the promoter regions of all *HSFs*, indicating that *HSFs* may be auto-regulated or regulated through other *HSFs* (Nover et al. 2001). Indeed, HSFA1d and HSFA1e showed the transient transcriptional activation of the HSE1 motif localized at 188 bp upstream of the *HSFA2*. Interestingly, *HSFA2* was suppressed only in *hsfa1e* and *hsfa1d* mutants, not in *hsfa1a* or *hsfa1b* single mutants or the *hsfa1a hsfa1b* double mutant, indicating that *HSFA2* can only be regulated through *hsfa1e* and *hsfa1d* (Nishizawa-Yokoi et al. 2011). However, another study reported that HSFA1a and HSFA1b interact with HSFA2 via bimolecular fluorescence complementation (BiFC), indicating that these factors may assist HSFA2 in heat regulation at the protein level (Li et al. 2010). HsfA2 was not only mediated through *HSFA1s* but also other heat-related genes that participate in heat regulation. Mitogen-activated protein kinase 6 (MPK6) phosphorylate the 249 threonine of HSFA2, which is indispensable for the nuclear localization of HSFA2, and recent studies have revealed that *MPK6* was highly induced under heat stress (Evrard et al. 2013). A mutant of *fk506-binding protein 62* (*fkbp62*, also named as *rof1*) was sensitive to acquired thermotolerance after long recovery time (ATLR). Interestingly, the expression of small *HSPs*, located downstream of *HSFA2*, were dramatically suppressed in *fkbp62* in response to stress and during the recovery time in ATLR assays. The ROF1-HSP90.1 complex showed nuclear localization after heat stress, which depends on the interaction between HSP90.1 and HSFA2, indicates that *ROF1* participates in the *HSFA2-sHSP* pathway (Meiri and Breiman 2009). Unlike *rof1*, the *rof2* mutant enhances plant thermotolerance, which also required HSFA2 during the recovery time in the ATLR assay. However, ROF2 hetero-polymerizes with ROF1 to participate in the function of the ROF1-HSP90.1-HSFA2 complex. Moreover, the transient co-expression of ROF2, ROF1 and HSFA2 suppresses the expression of *sHSPs*, suggesting that ROF2 functions as a negative feedback regulator of HSFA2 in plant thermotolerance (Meiri et al. 2010).

In addition to *HSFA2*, the regulation network of *HSFA3* was also well characterized. The *dehydration responsive element binding protein 2* (*DREB2*) was highly induced under high temperatures and salt and drought stresses (Liu et al. 1998; Sakuma et al. 2006a). Transiently expressed DREB2A or DREB2B directly binds to the *DRE1* and *DRE2* motifs in the promoter region of *HSFA3*, and both factors can intensively activate the expression of *HSFA3*. Furthermore, *HSFA3* was remarkably promoted in *DREB2A CA* and *DREB2C* overexpression seedlings (Sakuma et al. 2006b; Chen et al. 2010), and the down-regulation of *HSP18.1-CI* and *HSP25.3-P* in *dreb2a* and *dreb2c* depends on *HSFA3* (Chen et al. 2010; Schramm et al. 2008). Both mutant strains and the *DREB2A*, *DREB2C* and *HSFA3* overexpression lines exhibited similar performances on basic heat resistance and seed germination after heat stress (Yoshida et al. 2008; Chen et al. 2010). These data revealed that *DREB2s* directly controls *HSFA3* in the heat regulation pathway.

Although intensive studies on the individual regulation networks of *HSFA2* and *HSFA3* have been reported, the interaction between these factors has only been hypothesized in a previous study (Schramm et al. 2008). In the present study, the direct interaction between *HSFA2* and *HSFA3* was confirmed through genetic analysis, gene expression analysis and yeast two-hybrid assays. The potential roles of these factors in heat regulation pathways were also discussed in the present study. These results provide valuable information for constructing a fine heat regulation network in Arabidopsis, which is useful and referable in crop research and breeding.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis thaliana* Col-0 ecotype was used in the present study, and *hsfa2* (SALK_008978), *hsfa3* (SALK_011107) and *hsp101* (CS16284) mutants were obtained from the Salk Institute. The double mutant *hsfa2 hsfa3* was constructed by crossing *hsfa2* with *hsfa3*. T-DNA insertions were examined as previously described (http://signal.salk.edu/tdnaprimers.2.html), and the primers are listed in Table S1. The plant growth conditions were maintained according to Li et al. (2012). Briefly, these mutants and wild-type seedlings were grown on nutrient composites (Pei Lei, China) or plates containing half-strength Murashige and Skoog medium (Sigma-Aldrich, St. Louis, MO, USA) with 1% (w/v) sucrose and 0.8% (w/v) agar. All materials were cultivated in a greenhouse at 22 °C with a 16-h light/8-h dark photoperiod and a relative humidity of 70% under an illumination density of 230–300 $\mu Em^{-2}\ s^{-1}$.

Thermotolerance assays

Thermotolerance assays were conducted as previously described (Larkindale et al. 2005). The newly harvested dry seeds of each line were surface sterilized with ethanol

for 1 min, followed by commercial bleach for 3 min, and rinsed 3–4 times with sterile H_2O. The sterilized seeds were subsequently planted on plates and stratified for 3 days. For the basal thermotolerance (BT) assay, the seeds (plates) were heated at 45 °C in a water bath for the indicated time. For the acquired thermotolerance (AT) assays, 3- or 7-day-old seedlings were first acclimated from 38 °C for 90 min, recovered at 22 °C for 2 h, and subsequently treated at 45 °C for 150 min (ATSR). Alternatively, the plants were recovered at 22 °C for 2 days and subsequently treated at 45 °C for 1 h (ATLR). Five days after treatment, the expansion of the hypocotyl out of the seed coat was considered germination, and plants that remained green and producing new leaves were scored as survived (Larkindale et al. 2005). The data were expressed as the mean ± standard error (SE) (n = three biological replicates, 30 seeds per plant were analyzed for each replicate).

RNA preparation and RT-PCR examination

For RNA extraction, fresh seedlings (root included) were sampled from 3-day-old plants grown on plates after heat stress for the indicated times. Total RNA was prepared using TRIZOL reagent (Invitrogen, USA). For each sample, 5 μg of RNA was digested with 1 μl of DNase (Thermo, USA) to exclude residual DNA and subsequently used for reverse transcription using the TransScript First-Strand cDNA Synthesis Super Mix (TransGen, China). Reverse transcription PCR (RT-PCR) was performed using 2 × Taq PCR MasterMix (RTC3104-03, Tiangen, Beijing). The following thermal cycle conditions were used: 94 °C for 4 min, followed by 26–40 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min and a gradual decrease in the temperature to 25 °C. *A. thaliana Actin 7* (*Act7*) was used as the internal control. The gene-specific primer sequences are listed in Table S1.

Vector construction and yeast two-hybrid assay

The Matchmaker Two-Hybrid System (Clontech, USA) was used for the yeast two-hybrid assay. Fragments of *HSFA2*, *HSFA3*, *HSFA2 OD*, *HSFA3 OD*, *HSFA1a OD* and *HSFA1b OD* were amplified from an Arabidopsis cDNA library using the corresponding primers as listed in Table S1, and these fragments were subsequently inserted into the pGBKT7 and pGADT7 (Clontech, USA) vectors using the restriction sites indicated in Table S1. The yeast two-hybrid assay was performed with co-transformation following the manufacturer's instructions. Briefly, the yeast cells of strain AH109 were co-transformed with the bait and prey vectors using a lithium acetate-based protocol and grown on synthetic dextrose media (SD). The transformants were first spread onto SD media lacking leucine and tryptophan (SD/-Leu/-Trp), and subsequently the co-transformed positive colonies were grown on SD media lacking leucine, tryptophan, adenine and histidine (SD/-Leu/-Trp/-Ade/-His) to detect the activation of the *HIS3* and *ADE2* reporter genes. A prey vector containing the SV-40 large T-antigen (pGAD-T) and a bait vector containing murine p53 (pGBKT7-53) were used as a positive control system (Clontech, USA), and a bait vector containing human lamin C (pGBKT7-Lam) was used as a negative control (Clontech, USA).

The β-galactosidase assay was performed according to the colony-lift filter assay (Laporte et al. 1999). Yeast cells grown on SD/-Leu/-Trp/-Ade/-His plates within 4 days were imprinted onto a filter (Whatman No. 5). After two cycles of freeze/thawing in liquid nitrogen, the filter was incubated on another filter presoaked with Z buffer/X-gal solution (45 mM $Na_2HPO_4·7H_2O$, 40 mM $NaH_2PO_4·H_2O$, 10 mM KCl, 1 mM $MgSO_4·7H_2O$, 0.8 mM X-gal and 0.04 mM β-mercaptoethanol) at 30 °C. The photographs were captured 2 h after incubation.

Statistical analysis

Significant differences in the germination and survival rates between the mutant and wild-type seedlings were determined using independent samples t tests. Differences were considered statistically significant at the $P < 0.05$ and $P < 0.01$ levels.

Results

HSFA3 and *HSFA2* regulate plant ATLR in the same pathway

HSFA2 is acquired for the extension of acquired thermotolerance (Charng et al. 2007), and the potential regulation between *HSFA2* and *HSFA3* has been hypothesized in a previous study (Schramm et al. 2008). However, little evidence of how these two genes interact and the pathway involving these genes has been reported. To this end, we constructed an *hsfa2 hsfa3* double mutant by crossing *hsfa2* with *hsfa3*, and all mutants were confirmed as homozygous through the detection of T-DNA insertion fragments (Fig. 1). The newly produced double mutant, the *hsfa2* and *hsfa3* single mutants, wild-type plants, and the well-studied heat-sensitive mutant *hsp101*, which was served as a positive control for heat treatment, were submitted to a series different heat stress strategies, including seed germination against BT, seedling survival against ATLR and ATSR. The seeds from these plants were sown on the same plates as indicated (Fig. 2a,

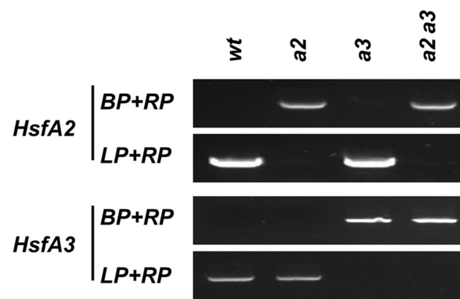

Fig. 1 Genotyping of *hsfa2*, *hsfa3* and the *hsfa2 hsfa3* double mutant. *BP* represents the T-DNA border primer, *LP* represents the left T-DNA border primer and *RP* represents the right genomic primer. *BP* + *RP* were used to identify homozygous mutants, while *BP* + *LP* were used to identify wild-type seedlings; when both results were positive, the mutant was heterozygous

including BT and AT assays). For the BT assay, all plants exhibited similar seed germination rates under normal conditions (Fig. 2b, c). Compared with the control, germination was significantly delayed in all plants following heat stress; however, most of these plants can survive with similar germination rates, except *hsp101*, which was decreased to 10% of the wild-type (Fig. 2d, e).

Similar to the germination rate, no significant difference was detected in the survival rate among the mutant and wild-type seedlings under normal conditions (Fig. 3a, b). The mutants and wild-type seedlings were harmed at varying degrees in ATSR and ATLR treatments. However, *hsfa2*, *hsfa3* and *hsfa2 hsfa3* were indistinguishable from wild-type plants in ATSR, while the survival rate of the positive control, *hsp101*, decreased to 17.8% of wild-type seedlings (Fig. 3c, d). An obvious difference between the *hsf* mutants and wild-type seedlings was observed in the ATLR assays. Compared with the wild-type, the *hsfa2*, *hsfa3*, *hsp101* and *hsfa2 hsfa3* double mutants showed impaired heat resistance, with more yellow leaves and reduced survival rates (Fig. 3e, f). The survival rates of *hsfa2*, *hsfa2 hsfa3* and *hsp101* decreased to almost half that of wild-type; however, *hsfa3* was not significantly different from the wild-type seedlings (Fig. 3e, f). To confirm the impaired performance of the mutants that underwent ATLR, plants at different ages were densely planted, and the results could be reproduced in a planting density-independent manner when tested with 3 day- and 7 day-old seedlings (Fig. S1a–f). Taken together, the phenotype of the *hsfa2 hsfa3* double mutant was similar to that of the

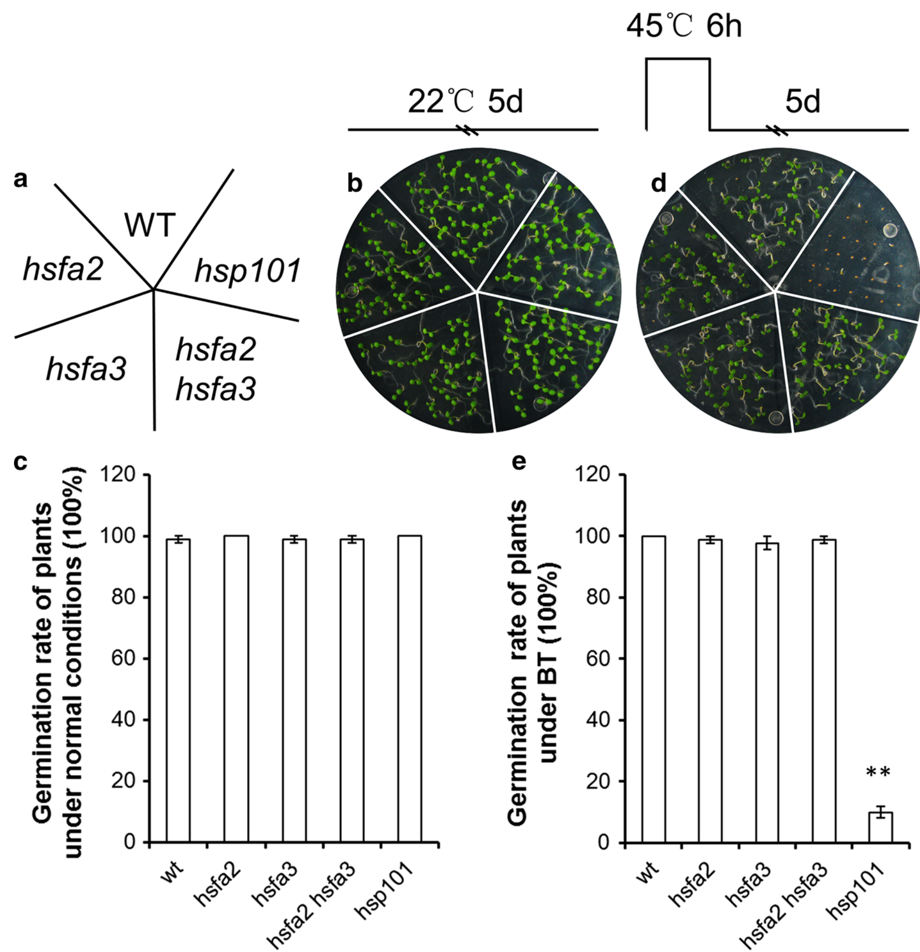

Fig. 2 Phenotypes of *hsfa2*, *hsfa3* and *hsfa2 hsfa3* after the BT test. **a** A diagrammatic drawing showing the positions of all plant seedlings (Fig. 3, Fig. S1). **b**, **c** Seed germination and statistical analysis of all mutants under normal conditions. **d**, **e** Seed germination and statistical analysis of all mutants after heating at 45 °C for 5 h. Photographic and statistical analyses were performed at 5 days after treatment. The data were collected from three independent biological repeats and expressed as the mean ± SE (standard error). Significant differences between wild-type and mutant plants at $^{**}P < 0.01$ levels, based on a *t* test

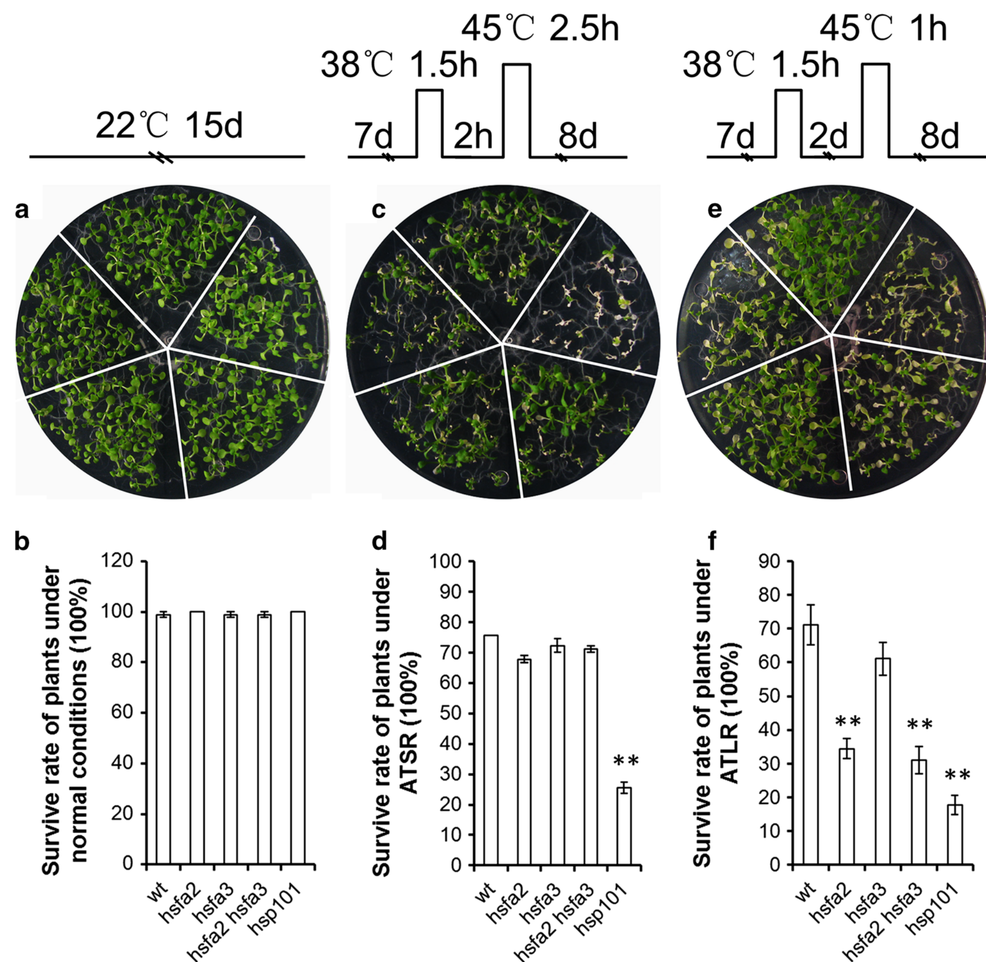

Fig. 3 Phenotypes of *hsfa2*, *hsfa3* and *hsfa2 hsfa3* after ATSR and ATLR assays. **a** Seedling survival rate and statistical analysis **b** of all the seedlings under normal conditions. **c** Seedling survival rate and statistical analysis **d** of all the seedlings after ATSR. **e** Seedling survival rate and statistical analysis **f** of all seedlings after ATLR. Photographic and statistical analyses were performed 15 days after sowing in control and ATSR and 17 days after sowing in ATLR. The data were collected from three independent biological repeats and expressed as the mean ± SE. Significant differences between wild-type and mutant plants at $^{**}P < 0.01$ levels, based on a *t* test

hsfa2 and *hsfa3* single mutants after heat stress, similar to *hsfa2*.

Expression analysis of heat stress responsible genes in *hsfa2* and *hsfa3* mutants

HSFA2 and *HSFA3* co-regulated a subset of genes in transcriptional profiling studies (Table S2) (Nishizawa et al. 2006; Yoshida et al. 2008). Among these genes, *HSP18.1-CI* and *HSP25.3-P* are regulated by *HSFA2* during the recovery time or prolonged heat stress in ATLR (Charng et al. 2007). We subsequently monitored the expression of these two genes with *HSP101* in the 3-day-old seedlings of *hsfa2*, *hsfa3*, *hsfa2 hsfa3* and *hsp101* at different time points using an ATLR assay (Fig. 4a). RT-PCR revealed that the transcript levels of *HSP18.1-CI* and *HSP25.3-P* were dramatically increased within 30 min of the first heat stress treatment, and subsequently maintained for 2 h in all mutants (Fig. 4b A–C); the levels of both genes returned to normal after 48 h of recovery, and no significant difference was detected in all mutants (Fig. 4b D). In response to a second heat stress, both *HSP18.1-CI* and *HSP25.3-P* were induced to a slightly weaker degree in wild-type than in the first heat stress. However, during the long recovery time and the second heat stress, the transcript levels of *HSP18.1-CI* and *HSP25.3-P* were hardly induced in *hsfa2* and *hsfa2 hsfa3* and was relatively lower in *hsfa3* than in the wild-type; this effect lasted to the end of the heat treatment (Fig. 4b E, F). The results indicated that the decreased expression of *HSP18.1-CI* and *HSP25.3-P* may directly correlate with the thermotolerance deficiency in *hsfa2*, *hsfa3*, *hsfa2 hsfa3* mutants during the long recovery time.

However, the expression patterns of *HSP18.1-CI* and *HSP25.3-P* in *hsp101* were similar to that of wild-type (Fig. 4b A–F), suggesting that the changes in the expression of these genes did not induce the heat-resistance deficiency in *hsp101*. Moreover, the *HSP101* transcript levels were not changed in *hsfa2* and *hsfa3*, and the levels of *HSFA2* and *HSFA3* were not altered in *hsp101*, indicating that *HSP101* may not be required for heat sensitivity in *hsfa2* and *hsfa3* mutants (Fig. 4b).

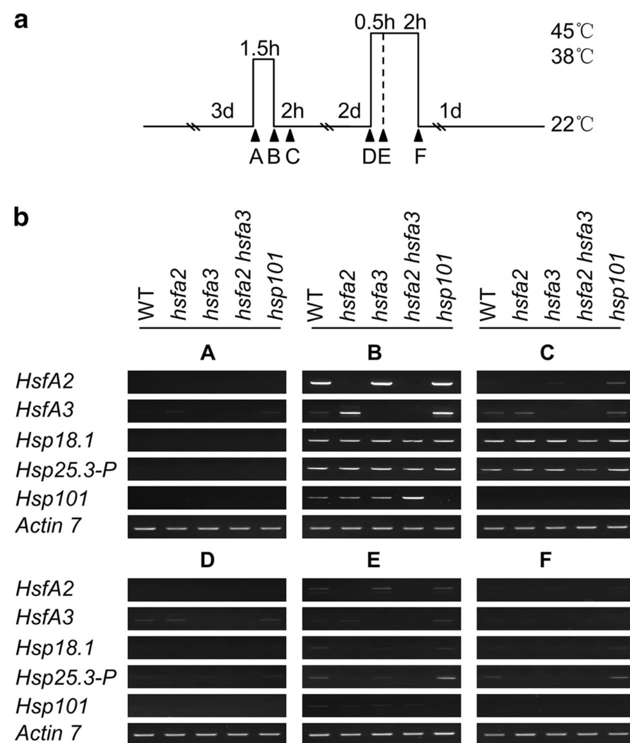

Fig. 4 Changes in the expression of heat-related genes in *hsf* mutants. **a** Schematic diagram showing the heat stress regimen. The *arrows* indicate the time points when the samples were harvested (*A–F*). **b** RT-PCR analysis of *HSPs* in the seedlings of WT and *hsfa2*, *hsfa3*, *hsfa2 hsfa3* and *hsp101* mutants. *A–F* indicate the time points described in **a**. *Actin 7* was used as the internal control

HSFA2 interacts with HSFA3, HSFA1a and HSFA1b in vitro

In most of the sampling points of the heat stress treatment (Fig. 4a), *HSFA3* expression was higher in the *hsfa2* mutant than in wild-type, regardless of heat treatment (Fig. 4b A–F), suggesting a potential direct interaction between *HSFA2* and *HSFA3*. To provide more confident evidence, a yeast two-hybrid assay was performed. The full-length and truncated coding sequences of the oligomerization domains (OD) of *HSFA2*, *HSFA3*, *HSFA1a* and *HSFA1b* were cloned into *pGBKT7* and *pGADT7*, respectively. The interactions were examined through co-transformation and β-galactosidase staining. Both nutritional deficient screening and β-galactosidase staining revealed a strong interaction between HSFA2 and HSFA3 (Fig. 5), and the OD fragments of HSFA2 and HSFA3 were sufficient for the interaction of these two proteins (Fig. 5). The yeast two-hybrid results provided solid proof of the interaction between HSFA2 and HSFA3. Moreover, in the *HSF* family, the *HSFA1* subfamily plays a dominant role in heat regulation; thus, to investigate whether HSFA2 directly interacts with the central factor HSFA1, yeast two-hybrid analysis was conducted with the combination of BD-HSFA2 with AD-HSFA1a OD and AD-HSFA1b OD, respectively. HSFA2 also exhibited a strong interaction with HSFA1a and HSFA1b in nutrition deficiency screening and β-galactosidase staining assays (Fig. 5).

Discussion

Genetic analysis with mutants is a useful method to study the relationship of two or more different genes. When the genes function in the same regulatory pathway, their double mutant should exhibit a similar phenotype and gene expression profile as the single mutants; in contrast, the phenotype of the double mutant may be a combination of the two single mutants (Luo et al. 2005). In the present study, the single and double mutants of *HSFA2* and *HSFA3* showed similar phenotypes in response to heat stress (Fig. 3, S1), indicating that the two genes function in the same heat regulation pathway. Genetic and expression analyses further suggested that *HSFA3* may be downstream of *HSFA2*, reflecting its weaker heat-sensitive phenotype and co-regulation of the same heat-related genes in acquired thermotolerance (Fig. 4). Moreover, the direct interaction between HSFA2 and HSFA3 at the protein level is consistent with the results of the phenotypic and expression analyses (Fig. 5).

However, when a gene family has multiple members in the whole genome and these factors have similar gene functions, studying double or multiple mutants is an effective strategy to evaluate genetic redundancy. Success stories have previously been reported in the analysis of *HSFA1* genes. The *hsfa1a hsfa1b* double mutants were insensitive in basic thermotolerance and slightly weaker in acquired thermotolerance assays (Lohmann et al. 2004); the survival rate of the *hsfa1d hsfA1e* double mutants decreased after an additional acquired thermotolerance treatment (Nishizawa-Yokoi et al. 2011). In addition, the quadruple mutant, *hsfa1a hsfa1b hsfa1d hsfa1e* completely lost the capacity for heat resistance (Liu et al. 2011). This information suggests that the four members of *HsfA1* genes function redundantly. In the present study, the double mutant *hsfa2 hsfa3* did not show more heat sensitivity than the *hsfa2* or *hsfa3* single mutant (Fig. 3, S1), suggesting that the functions of these genes are not completely redundant. The transcription profiles showed a tight relationship with the phenotype, and only 4% of heat responsive genes were affected in *hsfa1a hsfa1b* double mutants (Lohmann et al. 2004), while more than half of these genes were affected in the quadruple mutants (Liu et al. 2011). This effect was not observed for the *hsfa2 hsfa3* double mutant, as the induction of *HSP18.1-CI* and *HSP25.3-P* in the double mutant was similar to that in the *hsfa2* and *hsfa3*

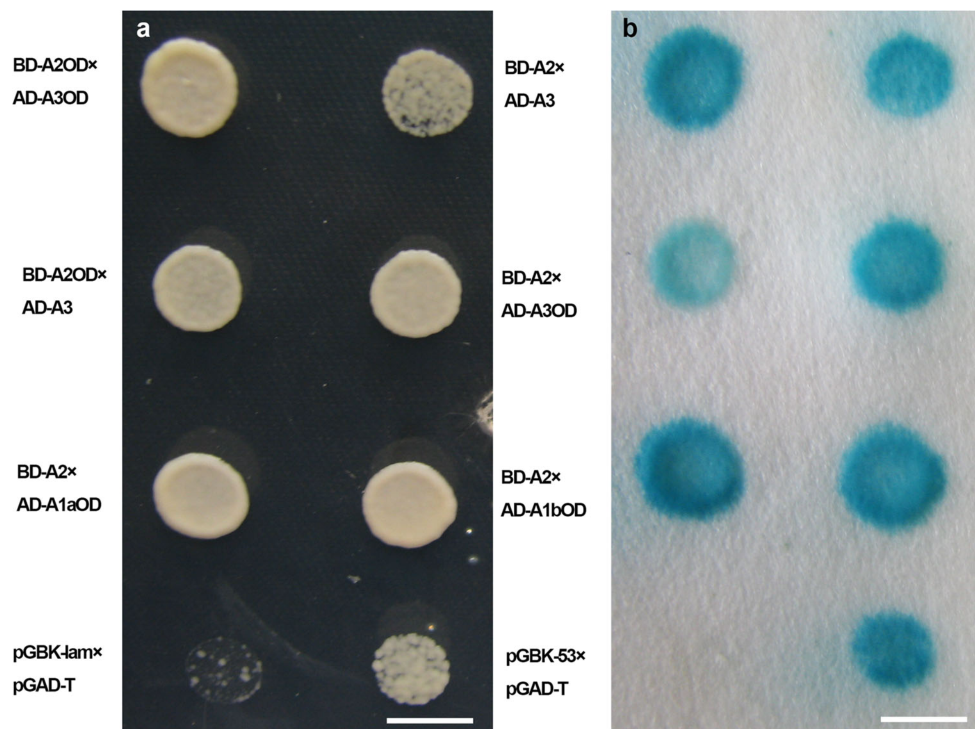

Fig. 5 Yeast two-hybrid assays. Nutrition deficiency screening (**a**) and β-galactosidase staining assays (**b**). Each combination is indicated beside the clones in (**a**), and the staining (**b**) was imprinted from (**a**). BD-A2OD, AD-A3OD, BD-A2, AD-A3, AD-A1aOD and AD-A1bOD represent pGBKT7-HSFA2 Oligomerization Domain, pGADT7-HSFA3 Oligomerization Domain, pGBKT7-HSFA2, pGADT7-HSFA3, pGADT7-HSFA1a Oligomerization Domain and pGADT7-HSFA1b Oligomerization Domain, respectively. The images of the nutrition deficiency screening were obtained at 3 days after inoculating on SD-Trp/-Leu/-Ade/-His media, and β-galactosidase staining was imaged at 2 h after incubation at 30 °C

single mutants after heat stress (Fig. 4b D–F). These results are consistent with the results showing that *HSFA2* and *HSFA3* function in the same pathway, and *HSFA2* plays a dominant role over *HSFA3*.

HSFA1 is considered the master regulator in response to heat stress in *Arabidopsis* (Liu et al. 2011); another important *HSF* gene, *HSFA2*, regulates plant ATLR (Charng et al. 2007). The results of a genetic complementary analysis revealed that *HSFA2* functions downstream of *HSFA1* (Liu and Charng 2013). However, whether other *HSF* genes are associated with the HSFA1a-HSFA2 regulatory pathway remains unknown. In the present study, the results of the yeast two-hybrid assay revealed intense interactions between HSA1s-HSFA2 and HSFA2-HSFA3 (Fig. 5), confirming the results of a previous study showing that *HSFA2* may work in the same pathway as *HSFA1s* (Liu and Charng 2013). However, the OD region is sufficient for the interaction between *HSFA2* and *HSFA3*, *HSFA1a*, *HSFA1b*, respectively; they may be the key domain for the oligomerization between different *HSFs*, as similar results have also been reported in studies of *HSF4* and *HSF5* (Baniwal et al. 2007).

However, according to previous studies, none of the single or double mutants of *HSFA2* and *HSFA3* exhibited a more sensitive phenotype than the *hsfa1s* quadruple mutants, which also confirmed the leading role of *HSFA1* in plant heat regulation. While the expression analysis revealed the independence between *HSP101* and *HSFA2* or *HSFA3* compared with wild-type, neither *HSFA2* nor *HSFA3* transcripts were affected in *hsp101*, and the *HSP101* transcript in *hsfa2* and *hsfa3* remained unaffected (Fig. 4). Thus, *HSFA2* and *HSFA3* may function in parallel pathways with *HSP101* in regulating heat stress.

The expression of *HSFAs* was not only suppressed by *HSFBs* (Ikeda et al. 2011) but also by *HSFAs*, e.g., the expression of *HSFA4a* and *HSFA4c* can be specifically suppressed by *HSFA5* (Baniwal et al. 2007). In the present study, the expression of *HSFA3* was enhanced in the *hsfa2* mutant, particularly after heat treatment at 37 °C for 1 h (Fig. 4b B). Interestingly, this effect seemed independent of heat stress treatment because at most sample points (including the normal condition, Fig. 4b A) of the heat stress treatment, *HSFA3* was up-regulated at varying degrees in the *hsfa2* mutant, suggesting that *HSFA2* may suppress the expression of *HSFA3*. Alternatively, *HSFA3* may have a complimentary effect to the *hsfa2* mutant. However, the results of the functional analysis of the *hsfa3* single mutant in the present study conflicted with those of

previous studies (Schramm et al. 2008; Chen et al. 2010). A transcriptional profiling study reported that *HSFA3* was down-regulated 9.4-fold in a 4-week-old *hsfa2* mutant line after successive heat stress treatments (42 °C for 3 h, 20 °C for 21 h, 42 °C for 1 h) (Schramm et al. 2006), while in the present study, *HSFA3* was enhanced in *hsfa2* at different sample points of ATLR assays. These contradictory results may reflect the different development stages and different heat stress methods, as heat resistance differed with plant development stages and heat stress regimen, even with the same material (Yu et al. 2014). However, at the end of the second heat stress treatment, both *HSFA2* and *HSFA3* transcripts were too negligible for detection using reverse transcript PCR analysis. Moreover, *HSFA3* was reported to regulate seed germination under high temperatures and plant ATSR (Schramm et al. 2008). However, no significant differences were observed in basic thermotolerance (Fig. 2) and ATSR assays (Fig. 3c, d) in the present study. In contrast, we observed a reduction of *HSFA3* in plant ATLR (Fig. 3d, e). Interestingly, the expression of *HSP18.1-CI* and *HSP25.3-P* in *hsfa2*, *hsfa3* and the *hsfa2 hsfa3* double mutant was indistinguishable from that in wild-type seedlings in the first heat stress of ATLR but was significantly suppressed in the second heat stress (Fig. 4b B–F), suggesting that *HSFA3* may primarily function in ATLR regulation. Thus, these results showed dynamic alterations in the expression of *HSFA2* and *HSFA3* and related *HSP* genes in ATLR assays, making the heat regulation network of *HSFs* more explicit.

Author contribution statement X. L. and E. Y. designed the experiment, X. L., W. W. J. W. and Y. C. performed the experiments and analyzed the data, and X. L., E. Y., W. L. and B. M. drafted the manuscript. All authors participated in the discussion.

Acknowledgements This work was financially supported through a grant from the Special Foundation from Guizhou Academy of Agriculture Science ([2013] 003), the Provincial Natural Science Foundation of Guizhou Province (Qian J [2015]2080), and the Provincial Natural Science Foundation of Guizhou Province (Qian J LKN [2013] 04).

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