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阿拉伯芥轉錄因子 HSFA1a/b/d 及 HSFA2 對一

群熱逆境記憶效應基因調控之研究

Transcription Factors HSFA1a/b/d and HSFA2
Mediate the Memory Response of a Group of Heat
Stress Response Genes in Arabidopsis

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致謝

跟臺北的其他地方比，南港真的是陰雨不斷，但這就是我成長的地方。當我上小學時，我無法理解學校旁邊的神祕建築群是什麼，只知道周邊有許多的矮房子，和大片雜草叢生的土地，直到年紀漸長，我才知道建築群是中央研究院。而周邊圍繞著眷村，隨著眷村拆除並改建高樓，我也到了找尋實驗室的階段。這裡要先感謝謝明勳老師，沒有他，我不會進入中研院，加入常老師的實驗室，並理解到自己是巨大科研體系下的小小螺絲釘，不在行適應環境的我能存留下來。必然要先感謝愷倫，我幾乎所有實驗的基本功都是由她指導的，她驚人的耐心，熟練的實驗技巧，及崇高的人格，可說是實驗室的守護神，希望妳未來回加拿大後，能在製藥科學上有傑出的成就。接著要感謝學長暐迪，除了給我很多重大的幫助之外，你的工作能力和學術基礎都讓我敬佩，你的個性更是團隊的潤滑劑，這點是我永遠追不上的。此外，亭穎、郁婷、宗遠、偉民、祥欽，你們對我曾經做過的幫助，我都有記在心裡，這裡一併致謝。接著當然要感謝我的指導老師，老師對於學術研究的熱忱，及治學態度的嚴謹，充分詮釋了一位科學家應有的態度，老師對實驗室的經營也十分用心，實驗室的管理者並非簡單的工作，我可以感覺到老師把實驗室每一個人都當作未來的科學家在看待，而不是實驗室的匆匆過客。接下來，在我高中及大學階段，兩項無意中進入我人生的東西，如今都是我最珍惜的物件，它們對完美一詞下了註解，並賦予我面對困難的勇氣，在此向所有相關之人士致謝。最後要感謝我的父母，隨著年紀漸長，我才知道要養育下一代有多麼不容易，那是無怨無悔，願意犧牲一切的付出，謹在此表達我的感激之情。在碩士生涯中，我理解到，也許人類永遠無法真正了解宇宙，但是為了求知所跨出的每一小步，都提升了我們對自然萬物的敬畏之情，也許，這就是過去頂尖的科學和哲學家，想要傳達給我們的訊息吧。

Abbreviations

ChIP, Chromatin immunoprecipitation

DMSO, Dimethyl sulfoxide

GDM, Geldanamycin

HS, Heat stress

HSE, Heat shock element

HSFA1, Heat shock factor A1

HSFA2, Heat shock factor A2

HSMR, Heat stress memory response

HSP, Heat shock protein

HSR, Heat stress response

LAT, Long-term acquire thermotolerance

ROF1, Rotamase FK506-binding protein1

RT-PCR, Reverse transcription-polymerase chain reaction

SAT, Short-term acquire thermotolerance

SUMO, Small ubiquitin-like modifier
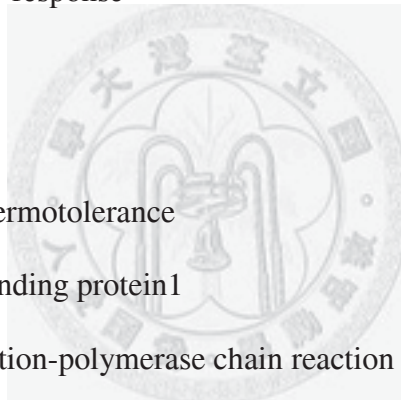
摘要

植物有許多應對環境逆境的策略，其中之一是對逆境的記憶能力，過去的研究發現，植物可以對不同的環境刺激進行記憶，然而，植物是否對熱逆境也有記憶則並不清楚。利用微陣列技術分析基因表現後發現，阿拉伯芥中有一小群基因受到反覆的熱處理後，其基因表達量會與只受到單一熱處理後有顯著差異，此現象進一步被及時定量聚合酶連鎖技術確認，我們把這個現象稱為熱逆境記憶反應，分析後，發現其中的維生素 C 過氧化酶(APX2)及肌醇磷酸合成酶(MIPS2)，其基因表達量在 HSFA2 過量表現株中上升，而在 HSFA1s 四重剔除株中下降，於是我們試圖去闡明 HSFA2 與 HSFA1s 跟熱逆境記憶的關係，比較野生株及 *HSFA2* 剔除株後，發現 *HSFA2* 剔除株中的熱逆境記憶效應完全消失，證實了 HSFA2 在記憶效應的重要性。然而，ROF1 及 HSP90 兩個已知會結合 HSFA2 的蛋白質，皆不影響熱逆境記憶效應。利用 HSFA1s 的三重及四重突變株實驗，證實 HSFA1a/d 也對熱逆境記憶效應十分重要。儘管過去研究指出 HSFs 會被 SUMO 化，但是比較 E3 SUMO ligase 突變株 *siz1* 後，並沒有發現其會影響熱逆境記憶效應。最後，存活率測試發現熱逆境記憶效應能極大的提升植物面對致死熱逆境之下的存活率，證實熱逆境記憶效應確實有其重要的生理功能。

Abstract

Plants have many strategies to cope with environmental stresses, and stress memory is one of them. Previous studies show that plants can respond to and remember various stimuli. However, it was not clear how plants remember the experience of heat stress. In this study, the results of microarray analysis showed that a small group of genes in Arabidopsis were induced by heat treatment to higher or lower expression level in seedlings that had a prior exposure to high temperature three days ago than those that had not. This phenomenon was called heat stress memory response (HSMR) herein. The HSMR of two of these genes, *ascorbate peroxidase 2* (*APX2*) and *myo-inositol phosphate synthase 2* (*MIPS2*) were further confirmed by quantitative RT-PCR. Since *APX2* and *MIPS2* were shown to be up-regulated by overexpression of heat shock transcription factor A2 (HSFA2) and down-regulated in heat shock factor A1 quadruple knockout (KO) mutant, the functions of HSFA2 and HSFA1 in HSMR were investigated. In the *HSFA2* KO mutant, HSMR was greatly diminished, suggesting that HSFA2 plays an important role in HSMR. However, rotamase FK506-binding proteins and heat shock protein 90 that bind to HSFA2 were dispensable for HSMR. Studies on the quadruple and triple KO mutants of the four HFSA1 genes suggest that HSFA1a and HSFA1d were the key players of HSMR. In eukaryotes, sumoylation regulates the activities of HSFs. However, HSMR of the T-DNA KO mutant of *SIZ1,* which encodes a small ubiquitin-like modifier E3 ligase, was not significantly altered. Finally, thermotolerance assay showed that HSMR was associated with significantly enhanced survival rates of seedlings under severe high temperature, which underscores the importance of this type of stress memory.

1. Introduction

1.1 Memory response in plants

Memory is an important biological function that organisms need for fitness in facing environmental changes. Unlike animals, plants do not have central nerve system to remember things. Nevertheless, researchers have shown that plants can memorize environmental stimuli by other means. The best known example of memory response in plants is the phenomenon of vernalization in *Hyoscyamus niger* (Schmitz and Amasino, 2007). The biennial plant requires both vernalization and long days to flower. The vernalized plants will grow vegetatively for several months under short days and flower immediately after shifting to long days (Lang and Melchers, 1947), which indicates that the plants "remember" a prior exposure to cold. It becomes clear that posttranslational modifications of histones in the chromatin of flowering repressor loci are part of the molecular mechanism for the memorize of winter (Sung and Amasino, 2005). Recent studies showed that Arabidopsis can sense and "remember" different light wavelengths which in terms affect the immune response (Szechynska-Hebda et al., 2010). Another research showed that plants can "remember" previous treatment of salicylic acid or its analog and influence systemic acquired resistance and related genes expression after pathogen induction (Jaskiewicz et al., 2011). Other examples of plant memories to specific environmental cues and stresses are given in recent review articles (Bruce et al., 2007; Trewavas, 2009). These examples demonstrate that the capacity of plants to remember perceived stimuli from the environment should be common in various physiological aspects.

So far, whether plants can remember the experience of heat stress (HS) is not clear.

Actually, acquired thermotolerance or heat acclimation can be viewed as a learning process in plants. They "learn" from a prior non-lethal heat treatment, which leads to survival of plants after exposure to otherwise lethal high temperature. In the past, research mainly focuses on the HS response of genes and their roles in thermotolerance (Kotak et al., 2007; Wahid et al., 2007). Since memory is an important component of learning behaviors, it is likely that plants can retain memories of prior exposures to heat. The memory to heat could be very important to plants as they are often subjected to several rounds of HS during their life span. However, this area has not been explored. The elucidation of such memory effect in plants can provide in depth understanding of how plants adapt to HS in nature.

1.2 Plant HS response

The response of organisms to adversely high temperatures is called HS response (HSR). In eukaryotic cells, many genes encode different types of molecular chaperones that function in the refolding of denatured proteins, and proteins that alter the metabolic processes in cells for protection from free radical damage (Wu, 1995). Many of these genes are heat responsive and controlled by heat shock transcription factor (HSF). In animals, only one HSF is found in invertebrates and four in vertebrates (Akerfelt et al., 2010). In plants, more HSFs are found in their genomes (Nover et al., 2001; Shiu et al., 2005).

In Arabidopsis genome, there are 21 HSF genes, which can be grouped into three major classes—A, B, and C (Nover et al., 2001). Most of the class A HSFs have transcription activator functions; class B HSFs play a role in the repression of

transcription; class C is unknown in function. The number of HSFs and members in each class are different among plant species. For example, there are four members in the *HSFA1* group in Arabidopsis—*HSFA1a* (At4g17750), *HSFA1b* (At5g16820), *HSFA1d* (At1g32330), *HSFA1e* (At3g02990)—but only one member, *HSFA1a*, in rice. By contrast, there is only one member in *HSFA2* group in Arabidopsis, but five members, *HSFA2A-E*, in rice (Kotak et al., 2004).

The existence of a temperature response receptor in plants is unknown. Upon exposure to elevated temperature, the concentration of Ca^{2+} in cytoplasm of Arabidopsis cells is increased within 15 min (Gong et al., 1998). The calcium-binding protein calmodulin then activates the calmodulin-binding protein kinase 3 (AtCBK3), which phosphorylates HSFA1a to increase its binding activity to the promoter heat shock element (HSE) *in vitro* (Liu et al., 2005; Liu et al., 2008). Various assays carried in our lab of *hsfA1* quadruple knockout (KO) mutant (A1QK) revealed that HSFA1s are important for seed maturation, seedling development, and tolerance to numerous abiotic stresses such as heat, salt and oxidative stress (Liu et al., 2011).

HSFA2 (At2g26150) is a heat-inducible HSF regulated by HSFA1a/B/D (Liu et al., 2011). HSFA2 affects many stress responses in Arabidopsis including heat, anoxia, oxidative, and high light stresses (Nishizawa et al., 2006; Charng et al., 2007; Banti et al., 2010; Nishizawa-Yokoi et al., 2010), which shows that HSFA2 is one of the key factors operating in stress response network in Arabidopsis. Recent studies carried out in our lab demonstrated that *hsfA2* T-DNA KO line compromised long-term acquired thermotolerance (LAT) but not short-term acquired thermotolerance (SAT) in plants, which suggests that HSFA2 has special role in the extension acquired thermotolerance

(Charng et al., 2007).

Previous reports show that the activity of HSFA2 is regulated by other factors, such as peptidyl prolyl cis/trans isomerase (ROF1) and HSP90, which are relocated from cytosol to nucleus with HSFA2 and are required for the extension of acquire thermotolerance (Meiri and Breiman, 2009).

1.3 Do plants remember the experience of heat? And how?

In this study, I attempted to address this question by first identifying genes with transcriptional memory response in the model plant, Arabidopsis. If the response of a gene to HS revealed by its transcript level is substantially altered by a prior exposure to the similar heat treatment, I call this type of genes as HS memory response genes. This approach is similar to a recent study on drought stress memory response (Ding, 2012). Then, I performed genetic analysis on possible regulatory components of this memory response. Results of my experiments uncovered a new phenomenon, suggesting a new mechanism existed for plants adapting to multiple HS. Our data also showed a specific group of genes regulated by HS memory response, and that HSFA1 and HSFA2 play a crucial role in this response.

2. Materials and Methods

2.1 Plant materials and growth condition

The Arabidopsis (*Arabidopsis thaliana*) Col-0, *HSFA2* (At2g26150) T-DNA insertion line SALK_008978, *SIZ1* (At5g60410) T-DNA insertion line SALK_058033, *HSFA1d* T-DNA insertion line SALK_022404 and *HSFA1e* T-DNA insertion line SALK_094943 (Alonso et al., 2003) were obtained from the Arabidopsis Biological Resource Center (ABRC; Ohio State University). *HSFA2* complementation line was produced previously (Charng et al., 2007). *HSFA1* triple KO lines and *HSFA1b/E* double KO lines were created previously by crossing the *HSFA1a/1b* and *HSFA1d/1e* double KO lines (Liu et al., 2011), *HSFA1a/1b* was provided Dr. Fritz Schöffl (University of Tübingen, Germany) (Lohmann et al., 2004; Busch et al., 2005). *HSFA1d/1e* double KO was created by crossing the *HSFA1d* and *HSFA1e* KO lines. The seeds were sterilized with 70% ethanol and 1% bleach, then sown on 1/2 Murashige and Skoog (MS) medium containing 1% sucrose and 0.8% agar. All seeds were placed in the dark, at 4 °C for 2-3 days to synchronize germination before growing in the culture room, in which temperature was maintained at 22 °C with 16h/8h light-dark cycle (100 μmol s^{-1} m^{-2}).

For HSP90 inhibition, geldanamycin (Sigma, G3381) dissolved in dimethly sulfoxide (DMSO) was added to the 1/2 MS medium to a final working concentration of 1.5μM.

2.2 Heat treatment

All heat treatments were conducted in the dark in water bath at indicated temperature and duration as previously described (Charng et al., 2006). The plates

containing Arabidopsis seedlings were sealed by water-proof tape to prevent water penetration. Samples for protein or RNA extraction were collected at indicated times and frozen by liquid nitrogen immediately. Survival rates were calculated after 8-d-recovery at normal growth condition following thermotolerance assay treatment.

2.3 Total RNA extraction, microarray analysis, and quantitative PCR

Total RNA was extracted according to the method described previously (Onate-Sanchez and Vicente-Carbajosa, 2008). One hundred mg of seedlings were homogenized and lysed by adding 300 μL of lysis solution (2% SDS, 68 mM sodium citrate, 132 mM citric acid, 1 mM EDTA). Protein and DNA were precipitated and removed by adding 100μL precipitation solution (4 M NaCl, 16 mM sodium citrate, 32 mM citric acid) and centrifuged at 16,000 x g at 4°C for 10 min. RNA was precipitated by adding 300 μL isopropanol and centrifuged at 16,000 x g at 4°C for 4 min, then treated with DNase1 for 30 min at 37°C to remove residual genomic DNA. RNA was quantified by using ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). One μg of total RNA was used as template to synthesize cDNA for PCR by M-MLV reverse transcriptase (Promega, Madison USA) according to manufacturer's protocol.

Microarray experiments were performed by the Affymetrix Gene Expression Service Lab of Academia Sinica. Data were normalize by algorithm robust multi-array average (RMA) (Irizarry et al., 2003) available from Remote Analysis Computation for Gene Expression Data (RACE) web site (http://race.unil.ch/).

For quantitative PCR, ABI 7300 Real-Time PCR System was used. For each

reaction, 20 μL of reaction mixtures contain 12.5 ng of sample cDNA, SYBR green

PCR master-mix, and 200 nM gene-specific primers (Table 1). The reaction was started

with an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for

15 s and 60°C for 60 s (Liu et al., 2011). Expression of *ACTIN2* (At3g17870) was used

to normalize the expression level of target genes by subtracting cycle threshold (CT)

value of *ACTIN2* from the CT value of target genes. Each transcription levels were

means of three repeats ± SE.

2.4 Protein extraction and immunoblotting

Total protein was extracted from Arabidopsis seedlings with buffer (60 mM Tris-HCl

pH8.5, 2% SDS, 2.5% glycerol, 0.13 mM EDTA, and 1% protease inhibitor cocktail).

Protein was quantified by DC Protein Assay kit (Bio-Rad Hercules, CA, USA) with

bovine serum albumin as standards. Proteins were separated on 4%-12% SDS-PAGE

(NuPAGE, invitrogen, Carlsbad CA, USA) and transferred to nitrocellulose membrane

for antibody detection.

Polyclonal antibodies against Arabidopsis HSFA2 were obtained from rabbit

immunized with synthetic peptide 'HLLKNIKRRRNMGLQNVN' which was done by

LTK Biotechnology (Taoyuan, Taiwan). Antibody against tubulin was purchased from

Sigma (T5168).

3. Results

3.1 Microarray analysis to identify HS memory response genes

Previously, few studies have focused on how plants respond to repeated mild heat treatments (Schramm et al., 2006). It was not clear how a prior HSR affects the second ones. Here, I employed Arabidopsis microarray to address this question using transcriptional changes as criteria. To this end, Arabidopsis seedlings grown on agar plates were subjected to one (H1) or repeated once and twice (H2 and H3) heat treatments at $37^{o}C$ for 1 h (Fig. 1). For H2 and H3 treatment, two and three heat treatments were separated by a 72-h-recovery time at $22^{o}C$, which, according to our experience, was long enough to avoid interference of the transcripts remaining from prior heat treatment. The ratio of gene expression in response to two HS treatment versus one HS treatment was determined using normalized microarray data. The threshold was set to 2-fold increase and decrease to determine whether the gene expressions were affected by prior heat treatment. Our data showed that 66 genes were up-regulated and 33 genes down-regulated in sample with H2 treatment as compared to H1 treatment (Fig. 2A). I focused on the up-regulated genes and verified the microarray results by quantitative PCR for two selected genes (Fig. 2B). I call these small subset of genes affected by a prior heat treatment as 'HS memory response' genes, as their response to heat apparently was affected by a prior HS experience remembered by the plant cells, and more times prior HS does not further boost HS memory response genes (Fig. 2B) . The identities of the 66 up-regulated HS memory response genes are listed in Table 2. Only 29 genes were up-regulated by single heat treatment.

3.2 HSFA2 is required for the HS memory response

In order to find which factors are responsible for such memory response, I searched for transcription factors that are known to regulate the aforementioned 66 genes. It was shown that ascorbate peroxidase 2 (*APX2*) and myo-inositol phosphate synthase 2 (*MIPS2*) are down-regulated in Arabidopsis *hsfA2* KO line and up-regulated in *HSFA2* overexpression (OE) line (Nishizawa et al., 2006; Schramm et al., 2006), suggesting that HsfA2 is responsible for the HS memory response of these genes. To test this hypothesis, I compared the responses of these genes to H1 and H2 treatments in WT, *hsfA2* KO, and *hsf*A2 complementation lines. I found that the memory responses of these two genes were dramatically reduced in the *hsfA2* KO mutant, but rescued in the *hsfA2* complementation (COM) line (Fig. 3). I further used microarray to determine whether HSFA2 was also required for the HS memory response of other genes. The result showed that the memory responses of most genes, 61 out of 66, were substantially lost in the *hsfA2* KO mutant (Fig. 4), suggesting that HSFA2 is a major component in the HS memory response.

3.3 HSFA1a/b/d regulate HS memory response

HSFA2 is transcriptionally regulated by HSFA1a/b/d, which are the master regulators of HSR in Arabidopsis (Liu et al., 2011). To understand the function of HSFA1s in the memory response, I measured the expression of *APX2* and *MIPS2* in *HSFA1* quadruple KO (*QK*) and four *HSFA1* triple KO (TK) lines with each containing intact copy of *HSFA1a*, *b*, *d*, or *e* only and named *aTK*, *bTK*, *dTK*, and *eTK*, respectively (Liu et al., 2011). The result showed that in *eTK* line, the expression of

HSFA2, *APX2* and *MIPS2* remained at low level even after repeated heat treatment (Fig. 5A). The results for *QK* were the same as that for *eTK* (data not shown). This is consistent with the previous findings that HSFA1e is not involved in HSR (Liu et al., 2011). In *aTK*, *bTK*, and *dTK*, the expression of *HSFA2* was substantially induced by H1 or H2 treatments (Fig. 5A). However, the heat inducibility of *APX2* and *MIPS2* was nearly lost in response to single heat treatment in these triple KO mutants. Interestingly, the HS memory response of these two genes could be attained with only one HSFA1 present, except that HSFA1b alone was not sufficient for the HS memory response of *MIPS2* (Fig. 5A).   HSFA1a and HSFA1d were the major players, and the HS memory response could be induced to a level comparable to that of the WT when both HsfA1a and HsfA1d are present (Fig. 5B).

3.4 A prior experience of HS enabled a faster and stronger HS response

To further characterize the HS memory response, time-course analysis was employed to study the transcript levels of *APX2* and *MIPS2* of 7-d-old seedlings incubated at $37^{\circ}C$ for various periods with or without a prior heat treatment applied 3 d earlier. The transcript levels of *APX2* and *MIPS2* in WT increased over time at $37^{\circ}C$, and the transcripts increased much faster in the seedlings that had experienced HS than in the ones that had not (Fig. 6A). Such difference was not prominent in the *hsfA2* KO mutant, which is consistent with the previous results (Fig. 3). To see whether the transcripts of *APX2* and *MIPS2* eventually reach the same levels with or without prior heat treatment by extending incubation time at $37^{\circ}C$, the heat treatment time was extended up to 120 min. The results showed that the transcript levels of *APX2* and

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MIPS2 no longer increased after 60 min of incubation at 37°C in WT without a prior heat treatment and were much lower than that of the samples with experience of HS (Fig. 6B). These results suggest that the prior experience of HS enables a faster and stronger response in the transcription of *APX2* and *MIPS2* to a subsequent HS.

3.5 HS memory response was not associated with a higher level of HSFA2 protein

Since HSFA2 is a pivotal factor in HS memory response, it was of interest to know whether the memory response is associated with the quantity of HSFA2. The microarray and quantitative RT-PCR data suggest that the transcription of *HSFA2* was not subjected to the regulation of HS memory response. To see whether it responds to repeated heat treatment faster, a time course study was performed to analyze the transcript level of *HSFA2*, and the answer was negative (Fig. 7A). Another possibility was that HSFA2 was stable up to 72 h after induced by heat. To examine this hypothesis, western blot analysis with HSFA2 specific antibody was performed. Results show the HSFA2 protein was induced by heat treatment at 37°C, but decreased over time during recovery at 22°C. After 72 h recovery following the heat treatment, HSFA2 was undetectable in western blot (Fig. 7B). I also analyzed HSFA2 protein level immediately after heat treatments. Data show the protein levels of HSFA2 were low after 1 h of heat treatment at 37°C, and further increased after 2 h of heat treatment. The protein levels were not substantially different between single or repeated heat treatments (Fig. 7C). Therefore, the HS memory response was not due to an obviously higher level of HSFA2 protein in the sample with experience of HS.

To precisely define the relation of HSFA2 level and memory response, I used

repeated heat treatments with different recovery times at room temperature. HS regime with 2 h of recovery time sharply increased the transcript level of *APX2* and *MIPS2*, and the relative transcript level rose to a peak in 24 h of recovery and slightly decreased over 3 d. In contrast, the transcript level did not boost up in a continuous 4-h heat treatment without recovery time. These results confirmed that the memory response is triggered by recovery at room temperature and independent of the quantity of HSFA2 (Fig. 8). In *hsfA2* KO line, transcription level dramatically decreased after 4-h HS, and cannot be rescued by the two hour recovery time (Fig. 8), suggesting that HSFA2 negatively regulate the attenuation of HS response.

3.6 SUMO E3 ligase SIZ1 was not critical for HS memory response

Previous studies showed that Arabidopsis HSFA2 is post-translationally modified by sumoylation (Cohen-Peer et al., 2010; Miller et al., 2010). In animals, sumoylation of HSFs regulates DNA binding and *HSP* expression (Goodson et al., 2001; Hong et al., 2001; Hietakangas et al., 2003; Hilgarth et al., 2003). In Arabidopsis, heat-shock induces conjugation of SUMO1/2 to proteins (Kurepa et al., 2003; Miura et al., 2005), and SIZ1, the SUMO E3 ligase facilitates basal thermotolerance (Yoo et al., 2006). To see whether protein sumoylation mediated by SIZ1 is involved in the HS memory response, I studied the *siz1-2*, a T-DNA KO mutant (Miura et al., 2005). The results show that the high expression of *APX2* and *MIPS2* induced by H2 treatment were slightly affected in *siz1-2* (Fig. 9), suggesting that SIZ1-mediated sumoylation does not play a critical role in the HS memory response.

3.7 Two regulators of HSFA2, ROF1 and HSP90, were not involved in memory response

HSP90 was shown to interact and regulate the activity of HSF in mammalian and plant cells (Ali et al., 1998; Zou et al., 1998; Yamada et al., 2007; Hahn et al., 2011; Yoshida et al., 2011). Recently, it was shown that ROF1 binds HSP90.1 and facilitates the activity of HSFA2 (Meiri and Breiman, 2009), suggesting that ROF1 and HSP90 might be required for HS memory response. To test this possibility, T-DNA KO mutant of *ROF1* and HSP90 specific inhibitor, geldanamycin (Whitesell et al., 1994), were used.　In the *ROF1* KO line, the high expression of *APX2* and *MIPS2* induced by H2 treatment were not affected (Fig. 10). Treatment of geldanamycin also did not abolish the HS memory response (Fig. 11). These results suggest that ROF1 and HSP90 are not involved in regulating the HS memory response.

3.8 HS memory response significantly enhances thermotolerance

Among the 66 HS memory response up-regulated genes, some may encode enzymes involved in protection against severe HS, such as APX2 that catalyzes the removal of hydrogen peroxide (Panchuk et al., 2002; Larkindale and Vierling, 2008). The HS memory response probably confer an enhanced performance in acquired thermotolerance. To test this hypothesis, two short-term acquired thermotolerance, SAT1 and SAT2, assays were performed. In SAT1 assay, only one heat acclimation treatment was performed 2 h before a severe HS challenge. In SAT2 assay, two acclimation treatments, separated by 72 h, were applied 2 h before the severe HS challenge, which evaluated the effect of HS memory response (Fig. 12).　Fig. 12 shows

that the short-term acquired thermotolerance level of WT was significantly higher in

SAT2 than in SAT1 assay, suggesting that HS memory response enhances the capacity

of acquired thermotolerance.   However, such effect was substantially lost in the *hsfA2*

KO mutant, which is consistent with the role of HSFA2 in HS memory response shown

in Section 3.2. A complementation line expressing a wild-type copy of *HSFA2* in the

KO line restore this effect. Despite I usually used APX2 and MIPS2 for marker to

observe HSMR, but the survival rate were not different from WT (Data not Shown).

4. Discussion

4.1 HS memory as one type of stress memories

The concept of stress memory of plants has been clearly defined in a recent review (Bruce et al., 2007). However, the term "stress imprint" was proposed to replace "stress memory" in order to distinguish it from the memory of animals. They define that 'a plant "stress imprint'' as a genetic or biochemical modification of a plant that occurs after stress exposure that causes future responses to future stresses to be different.' However, the definition can be applied essentially to all organisms. The stress memory of plants is not a distinctive phenomenon. Two major mechanisms were proposed to be involved in stress memories of plants. One is associated with accumulation of proteins or transcription factors; the other is associated with epigenetic change. In my study, evidences of HS memory and its physiological significance in Arabidopsis are provided. The results show that Arabidopsis seedlings could remember a prior exposure to high temperature and alter the transcriptional response of a small subset of genes to a later heat stress condition, leading to an enhanced thermotolerance. The HS memory response probably is formed by one of the two mechanisms mentioned above, which will be discussed in the following sections.

4.2 HS memory response genes

In this study, I identified a group of genes displayed memory response to repeated HS (Table 2). Not many studies have been conducted to elucidate their functions in heat tolerance. APX2 has been reported to scavenge hydrogen peroxide in plant cells with the housekeeping APX1 (Koussevitzky et al., 2008). It was shown to confer acquired

thermotolerance in Arabidopsis (Larkindale and Vierling, 2008). MIPS2 is involved in the phosphorylation of myo-inositol in phytate synthesis. It is known to have plant pathogen resistance function, which cannot be replaced by MIPS1 (Murphy et al., 2008). Interestingly, although HSFA1e does not act as transcription activator for HS response (Liu et al., 2011). It is a HS memory response gene regulated by HSFA2 (Table 2). 23 genes were not responsive to single heat treatment, but substantially up-regulated after repeated HS. 22 genes were up-regulated by single heat treatment and were further induced to higher level after repeated HS (Table 2). It is not clear why these genes are specifically employed in HS memory response in Arabidopsis. Interestingly, most of the canonical heat shock protein genes were not subjected to memory regulation.

4.3 The roles of HSFA1 and HSFA2 in HS memory response

HS memory response requires HSFA2, but the accumulation of HSFA2 protein was not responsible for the memory response. In addition to HSFA2, HSFA1a/b/d are also required because in *HSFA1 eTK* or *QK* mutant HSFA2 could not be induced by heat. However, induction of HSFA2 was required but not sufficient. In *hsfA1* triple KO mutants, *aTK*, *bTK*, and *dTK,* HSFA2 was induced by single heat treatment to similar levels, but HS memory response was attained in *aTK* and *dTK* lines, but weaker in *bTK* line (Fig 5), suggesting that HS memory response is not regulated by HSFA2 alone. In tomato, HSFA1 and HSFA2 can form hetero-oligomeric superactivator complex after HS (Chan-Schaminet et al., 2009). It is likely that a complex formed by HSFA1a/b/d and HSFA2 is involved in the memory response. Further studies and experimentations need to be performed to confirm this possibility.

Previous research show that ROF1 binds HSP90.1 and facilitates the activity of HSFA2 in LAT (Meiri and Breiman, 2009), Nevertheless, both HSP90 inhibitor, geldanamycin, and *ROF1* KO lines did not affect the HS memory response, suggesting that the mechanisms of LAT and HS memory response are different.

Although geldanamycin is known to inhibit HSP90 through binding its ATPase activity site, it is still not confirmed whether geldanamycin would cross inhibit other proteins that also contains ATPase domain, thus there lacks a direct method to assess HSP90's function. Prior work has reported that HSP90 can regulate plant growth, and the seedling growth becomes abnormal in HSP90-RNAi lines (Sangster et al., 2008). However, I did not observe any differences in the appearance of seedlings between DMSO control and geldanamycin treated seedlings (data not shown), so whether HSP90 affects HSR is still not totally clear.

Previous report shows that HSFA2 affects long-term but not short-term acquired thermotolerance (Charng et al., 2007), these data was in agreement to microarray data showing that *hsfA2* KO line does not influence most of heat response genes too much after a short term HS (Schramm et al., 2006). Nevertheless, when *HSFA2* KO line were recovering at room temperature or were treated with prolonged HS, the transcription level of heat-induced genes decreased faster compared with WT (Charng et al., 2007), suggesting that HSFA2 functions to maintain HSR. Here I showed a novel function of HSFA2 that assists plants in remembering prior heat acclimation, and leads to more efficient HSR when facing another heat challenge, even in short term HS (Fig 6A). The result also show HS memory response only needs very few amount of HSFA2 (Fig 7C). The survival rate of our thermotolerance assay confirmed that HS memory response can

further enhance thermotolrance (Fig 12). Interestingly, *HSFA2* KO line also show

increase survival rate when given repeated HS, despite the increased extent was lower

than that of WT (Fig 12), suggesting a HSFA2-independent HS memory response. It is

not clear which factors affect memory response in *HSFA2* KO line. Microarray data

show only a few genes preserve HS memory response in *HSFA2* KO line (AT1G52690;

AT3G02480), and the biological functions of these genes are unknown. I can not

exclude some of memory response at post-transcriptional level.

In thermotolerance assay, a change in transcription level is a common phenotype to

confirm specific gene responses toward certain stimuli, such as memory response

toward HS. Because many genes involved in HS memory response do not show change

in transcription level when only given a single HS (Table 2), they are often overlooked

in their function in thermotolerance. In this study, our data showed that HS response is

consisted of not only those genes induced upon a single HS, but also include those that

'remembers' prior sublethal HS and retain memories of it to prepare for the coming

challenge. These two groups of genes do not overlap entirely, and this discovery shows

that HSR is more complex than our former understanding.

4.4 Chromatin modifications and memory response

As mentioned above, one of the mechanism that form stress memory is epigenetic

change. Many genes are regulated by chromatin modifications, which can precisely

regulate specific genes without altering gene sequence (Schmitz and Ecker, 2012), and

as such, information is stored on genes to prepare for the next stimulation. Because

memory response initiated immediately after heat treatment (Fig 6A), it is sensible to

hypothesize that HS memory response is regulated by modifications of chromatin structure. The recent study show that specific genes can retain memory of drought stress with H3K4me3 modification on their promoters (Ding et al., 2012). It remains to be seen whether similar modifications are involved in HS memory response.

5. Perspectives

   In this study, a small group of genes were found to have memory response.   Next step is to figure out the common principle how they are regulated by HSMR. It is possible that these memory response genes contain consensus *cis*-element for HSMR in their promoters, which could be predicted by bioinformatic tools such as *Athena*. Promoter deletion assay could be performed to identify which region is important for memory response.   Chromatin immunoprecipitation (ChIP) assay is another strategy for discovering the important chromatin modifications on the promoters of HSMR genes. Because protein level of HSFA2 is not linearly correlated with the HS memory response, it is possible that memory response is also regulated by downstream genes of HSFA2, including transcription factors or enzymes. Comparing the proteomes between *HSFA2* KO and WT or screening for genetic mutants may be suitable approaches to further identify new components of HS memory response. The latter approach relies on developing a high throughput method in identifying mutants that are not able to remember the experience of HS.
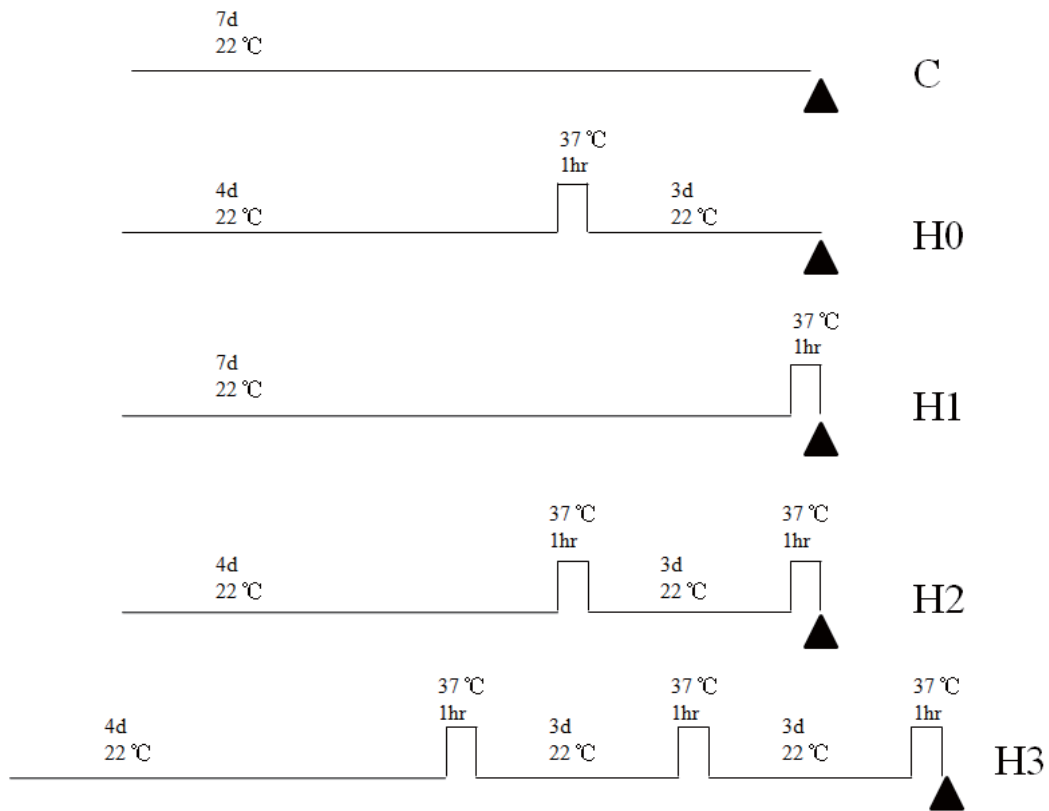
Tables and figures

Fig. 1 Schematic diagrams of HS regimes used in this work. C, a non-HS control.

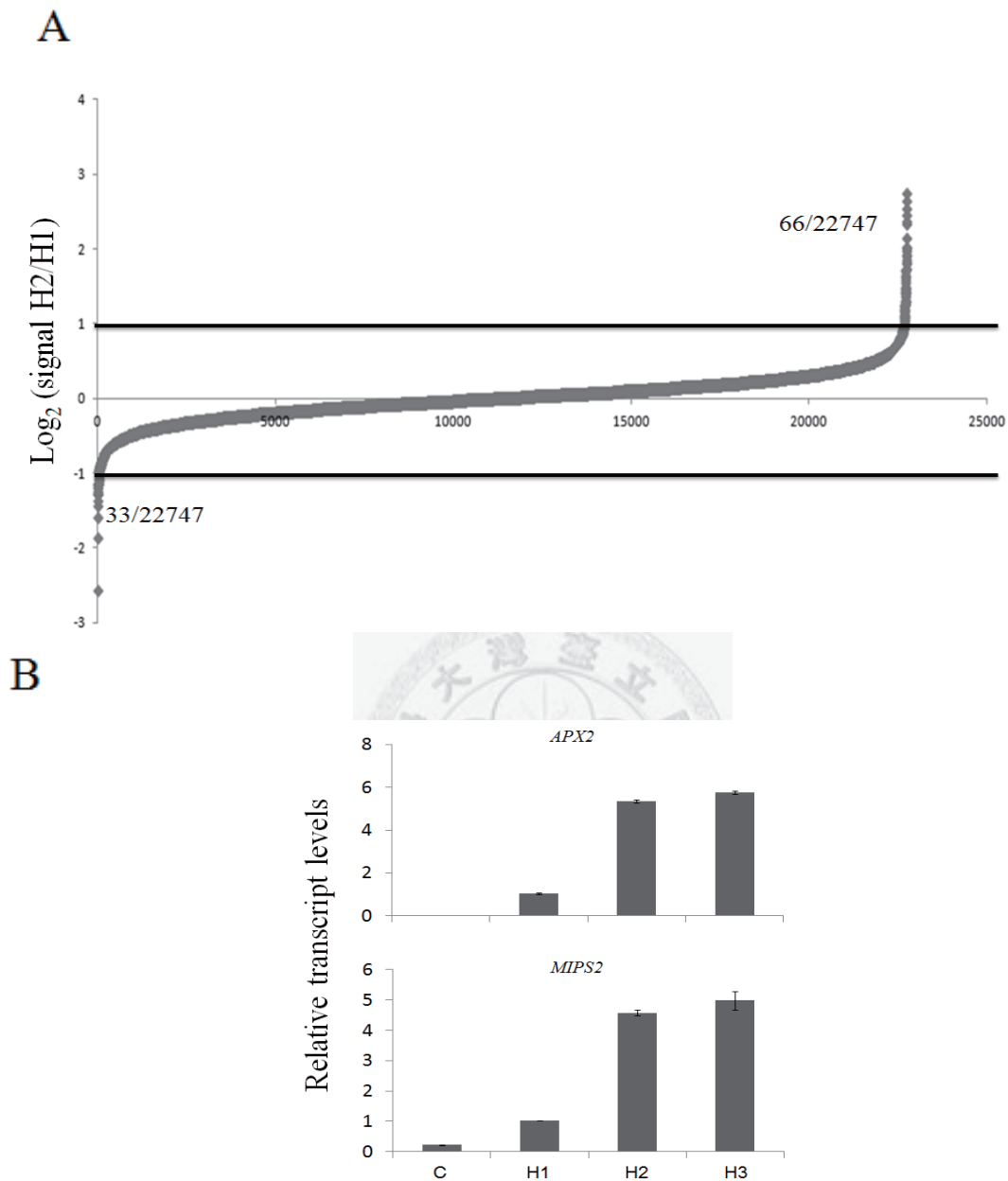The black triangles indicate the time of sample harvesting.

Fig. 2 Identification of HSMR genes by microarray analysis and confirmation by RT-PCR. (A) The seedlings were given H1 or H2 treatments as indicated in Fig. 1. The vertical axis represents the ratio of microarray signals of the two heat treatments. The ratio of each gene was arranged from the lowest to the highest along the horizontal axis for 22,747 gene probes on the ATH1 chip. (B) Quantitative RT-PCR analysis of *APX2* and *MIPS2* in seedlings after C, H1, H2 and H3 treatments.
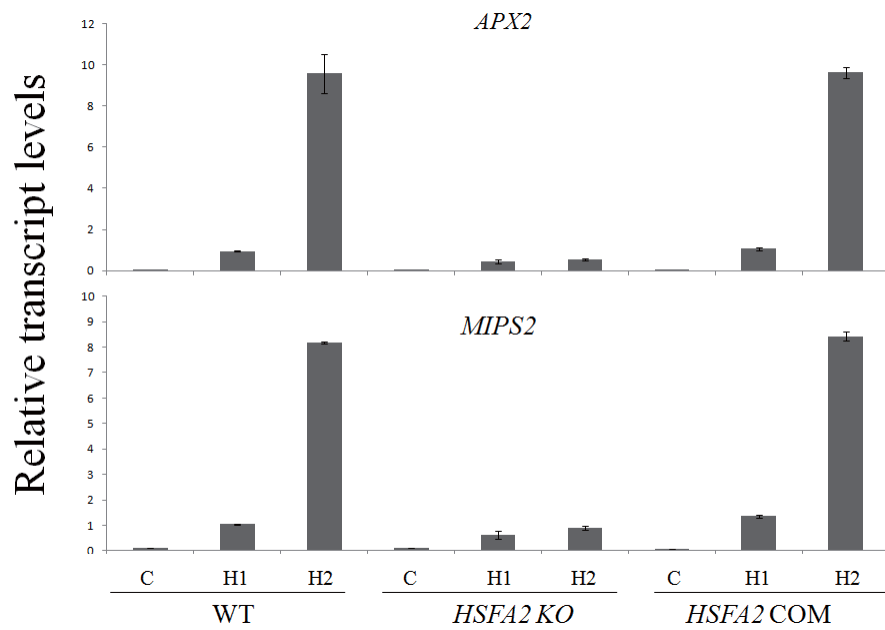
Fig. 3 Analysis the HSMR of *APX2* and *MIPS2* in *HSFA2* KO and complementation (COM) lines. 7-d-old seedlings were given C, H1, or H2 treatments, and the transcription level of *APX2* and *MIPS2* were analyzed by RT-PCR; *ACTIN2* was used for normalization.
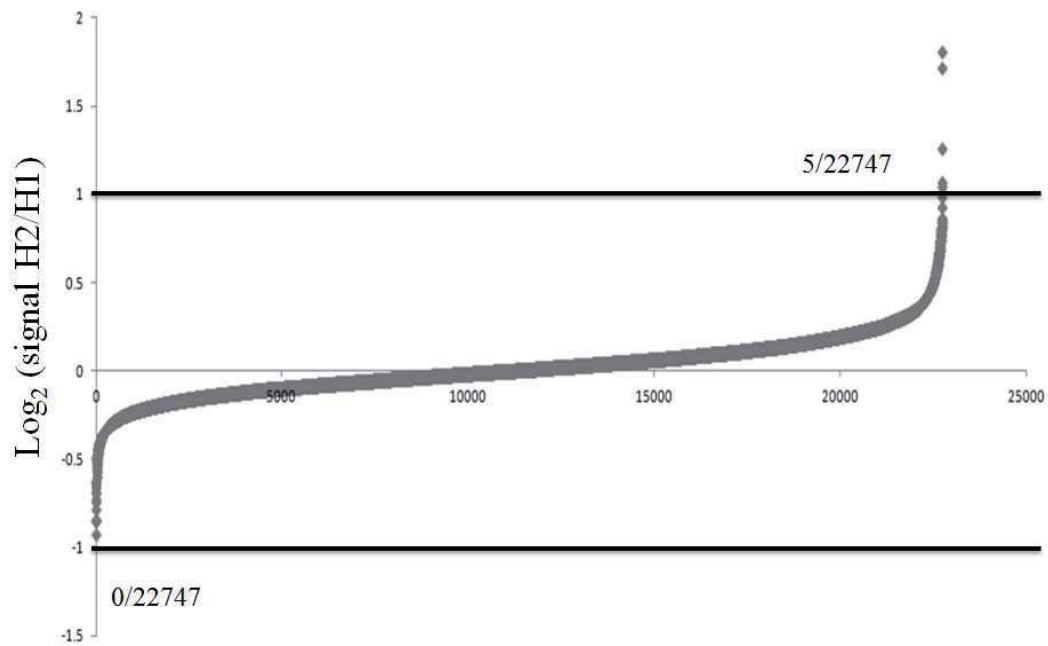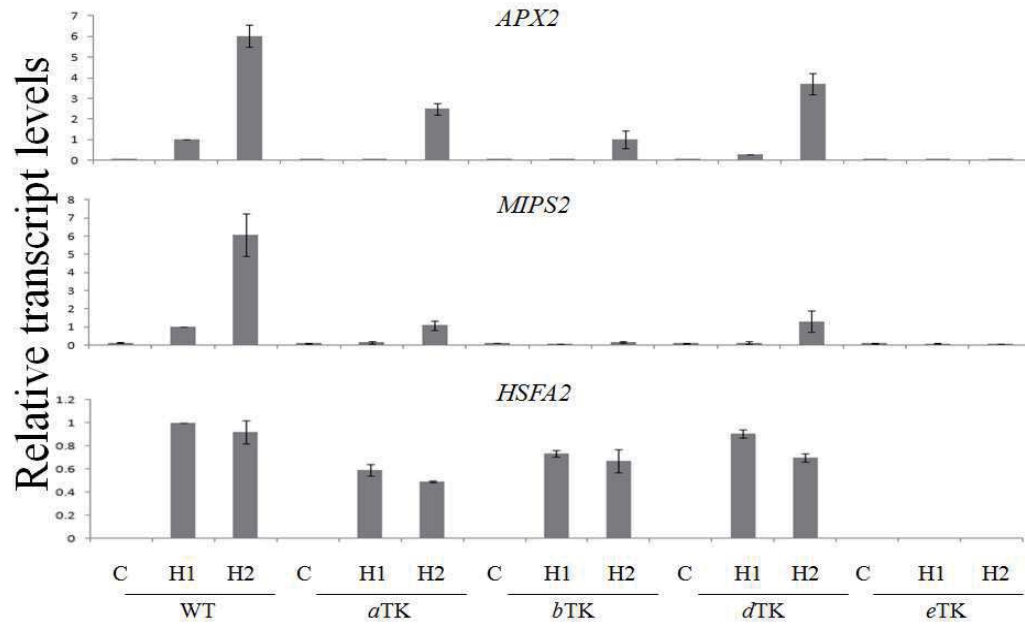
Fig. 4 Microarray analysis *HSFA2* KO line. 7-d-old seedlings were given H1
or H2 treatments. The vertical axis represents the ratio of microarray signal
intensities of the two heat treatments. The ratio of each gene was arranged from
the lowest to the highest along the horizontal axis for 22,747 gene probes on the
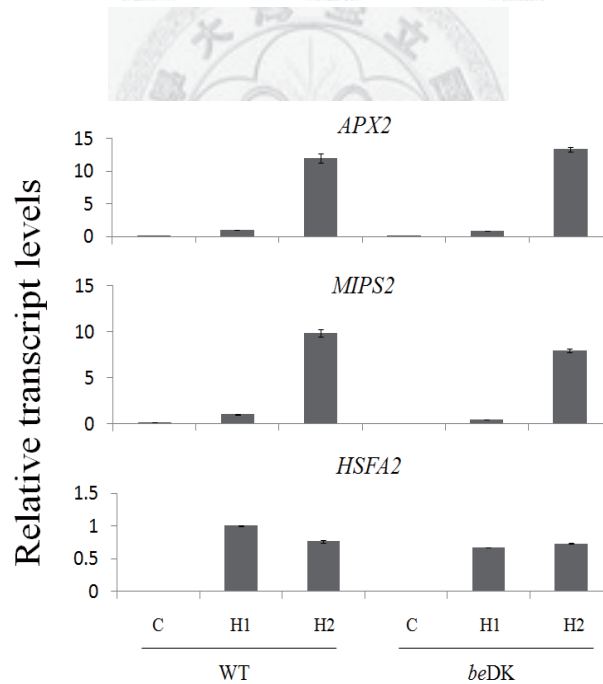ATH1 chip.

Fig. 5 Analysis the HSMR in *HSFA1s* multiple KO lines. 7-d-old seedling were

given C, H1, or H2 treatments. (A) Transcription level of *APX2*, *MIPS2* and *HSFA2*

in WT and triple KO lines, *aTK*, *bTK*, *dTK*, and *eTK* (the prefix letter indicated the

remaining intact gene of *HSFA1a/b/d/e*) (B) Transcription level of *APX2*, *MIPS2*,

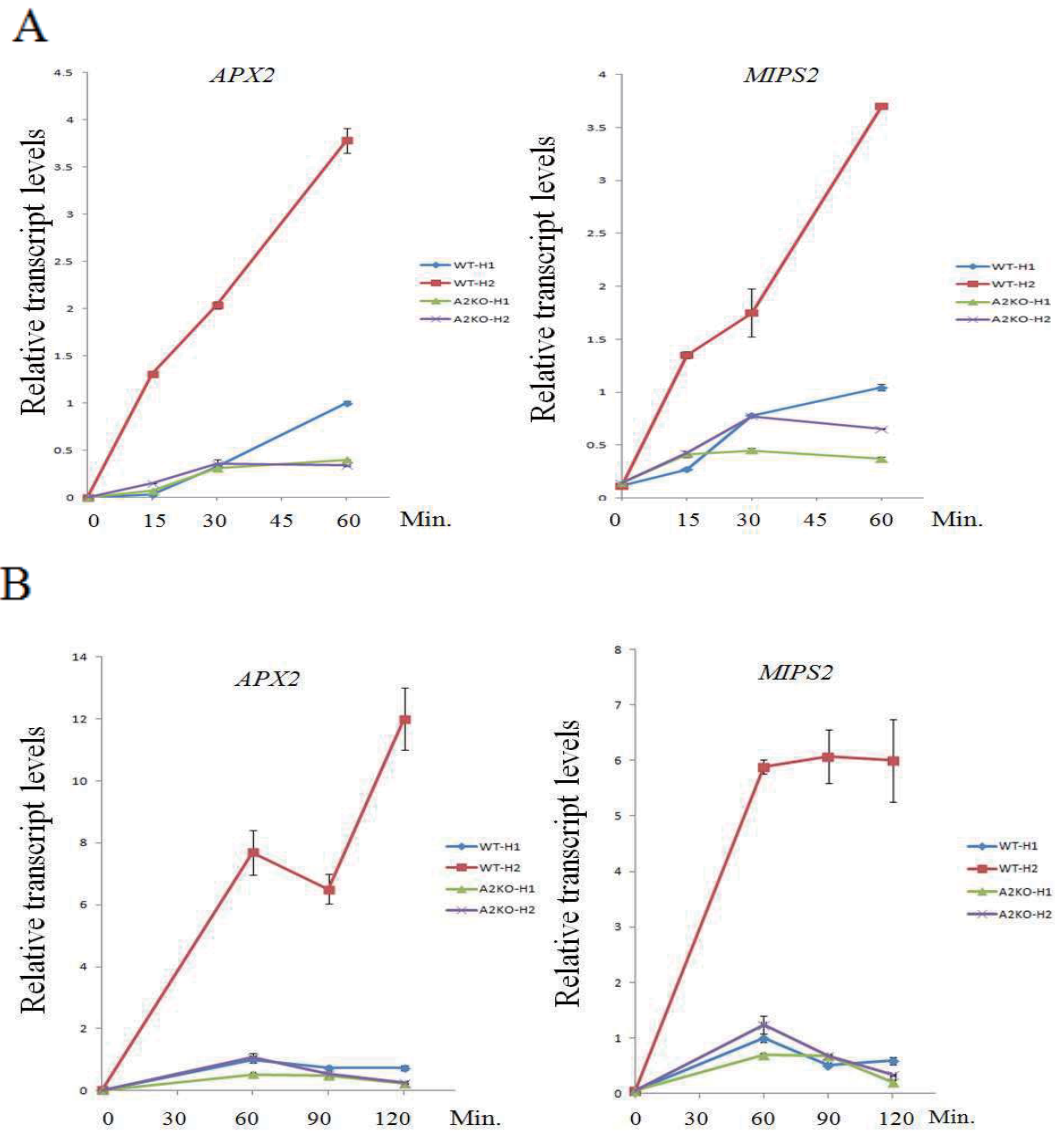and *HSFA2* in WT, *HSFA1b/e* double KO line, *beDK*.

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Fig. 6 Time-course analysis of *APX2* and *MIPS2* expression after once and twice HS in WT and *HSFA2* KO lines. (A) Transcript levels of *APX2* and *MIPS2* after 15, 30, and 60 min heat treatments with or without prior heat treatment. (B) Transcript levels of *APX2* and *MIPS2* after 60, 90, and 120 min heat treatments with or without prior heat treatment.
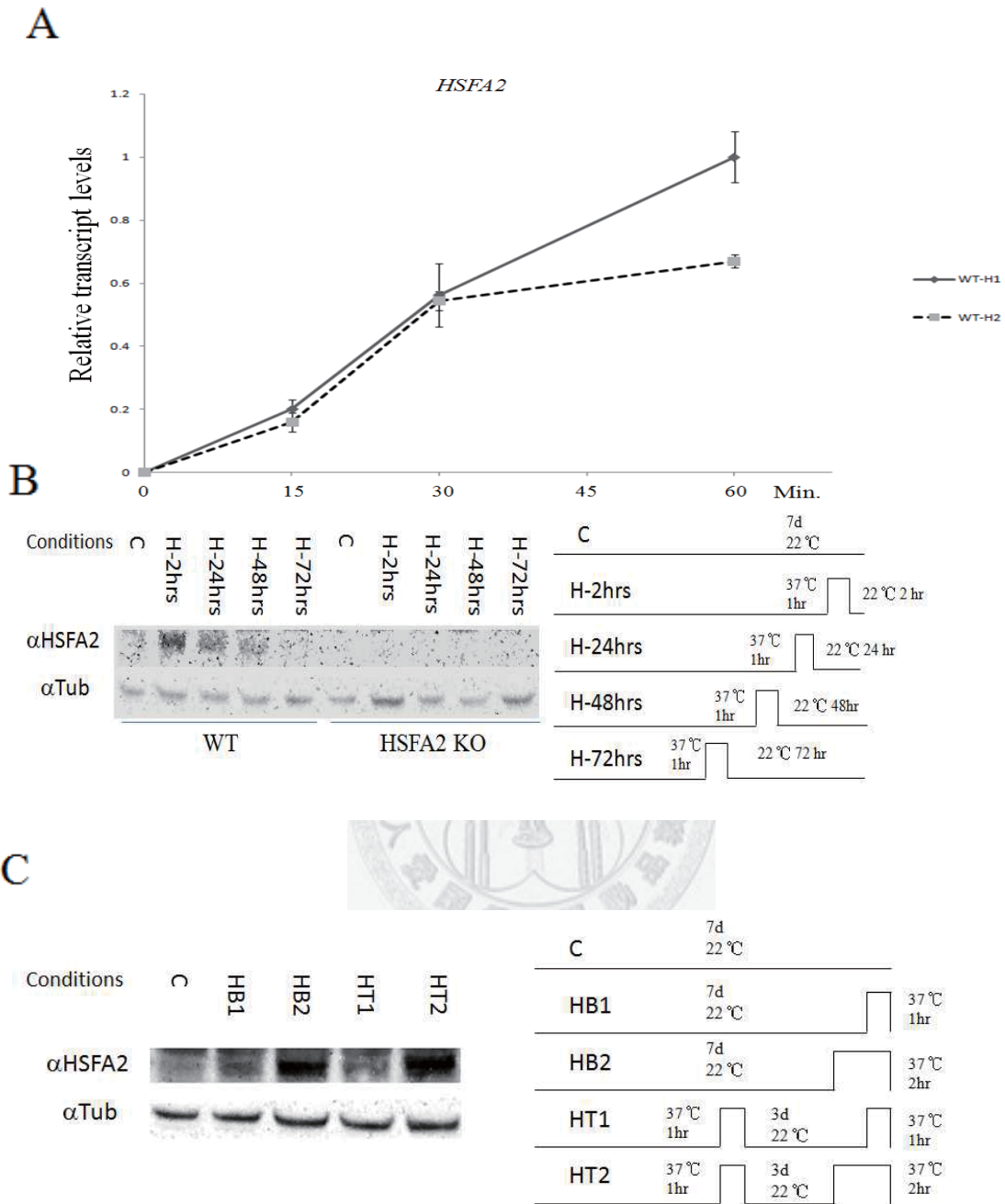
Fig. 7 Time course analyses of HSFA2 expression by RT-PCR and western blot. (A) Transcript of *HSFA2* after 15, 30, and 60 min heat treatments with (H2) or without (H1) prior heat treatment. (B) Protein level of HSFA2 at 2, 24, 48, and 72 hrs after heat treatment, with tubulin as loading control. (C) Western blot analysis protein levels of HSFA2 after one or hours heat treatments that given prior HS or not. Tubulin (Tub) is loading control, 50 μg of total protein was loaded in each lane.
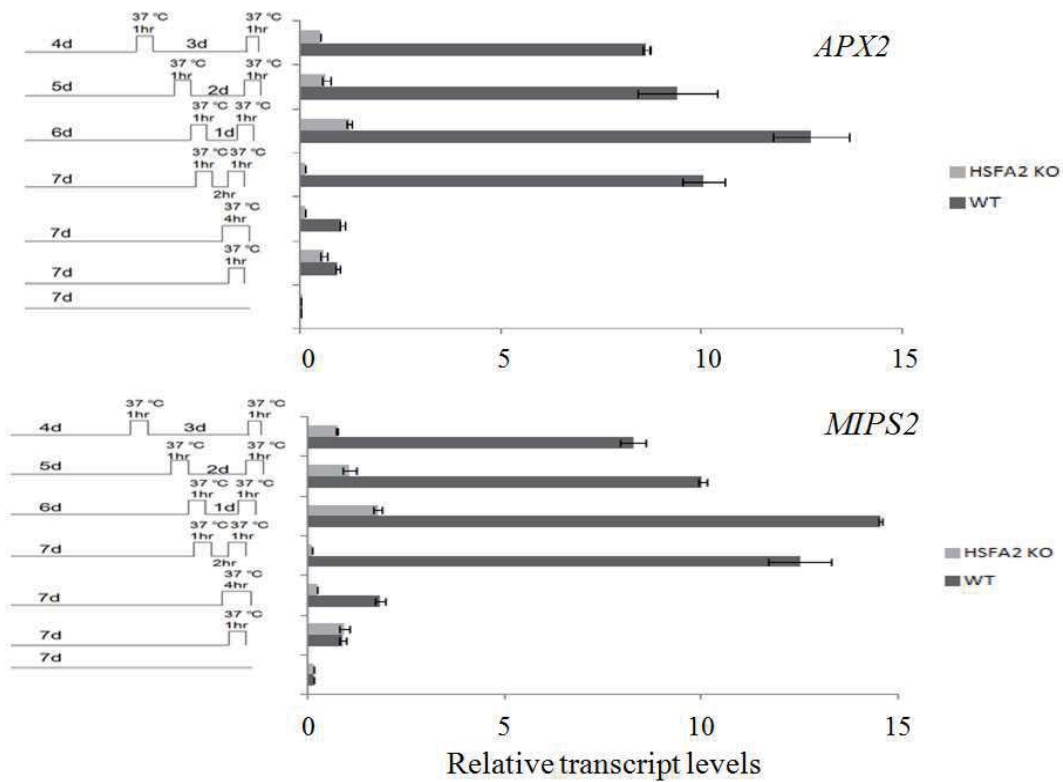
Fig. 8 The relationship between room temperature recovery and HSMR.

Transcript levels of *APX2* and *MIPS2* in WT and *HSFA2* KO line after different heat

treatments; schematic diagrams of HS regimes are shown on the left.
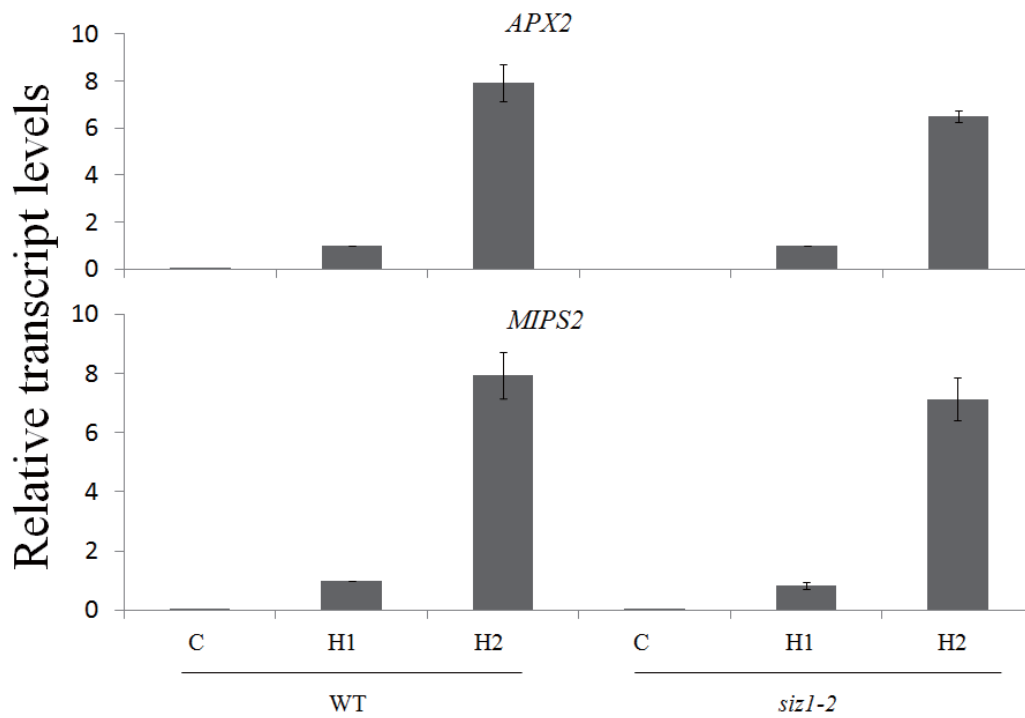
Fig. 9 The effect of disruption of E3 SUMO ligase *SIZ1* on HSMR. 7-d-old WT

or *siz1-2* seedlings were given C, H1, or H2 treatments. Transcript levels of *APX2*

and *MIPS2* were analyzed by RT-PCR, ACTIN2 was used to normalize signal.
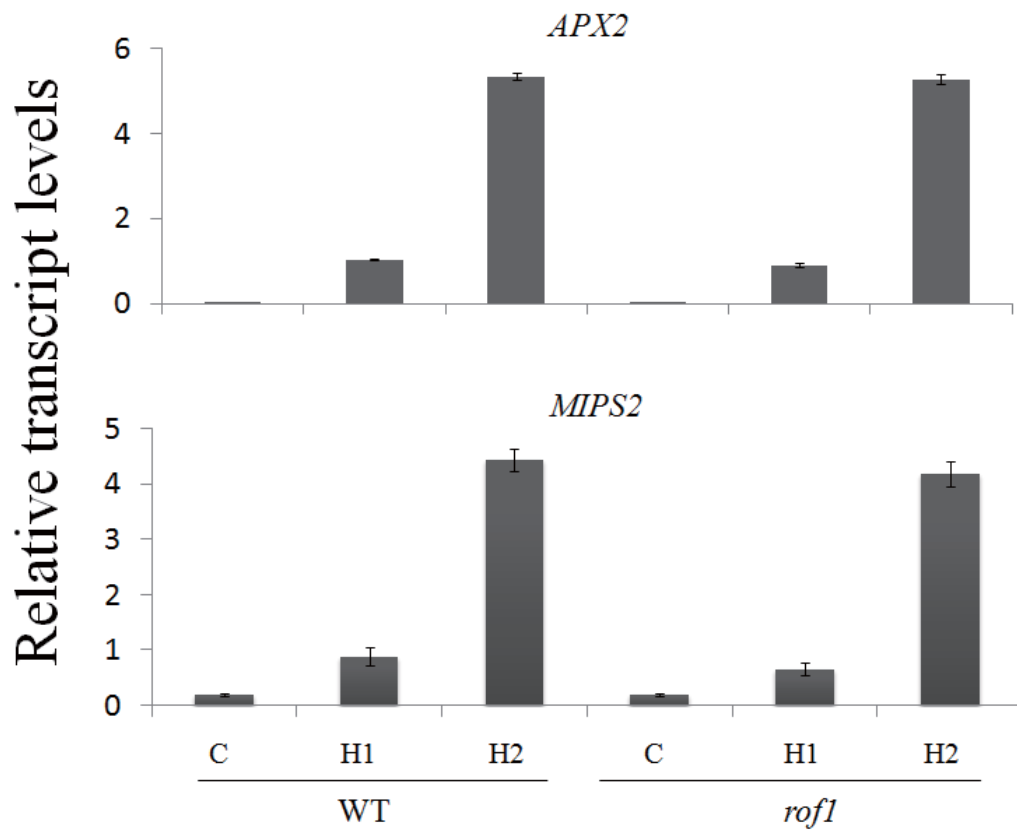
Fig. 10 The effect of disruption of *ROF1* on HSMR. 7-d-old WT and *rof1*

seedlings were given C, H1, or H2 treatments. Transcript levels of *APX2* and

MIPS2 were analyzed by RT-PCR; *ACTIN2* was used to normalize signal.
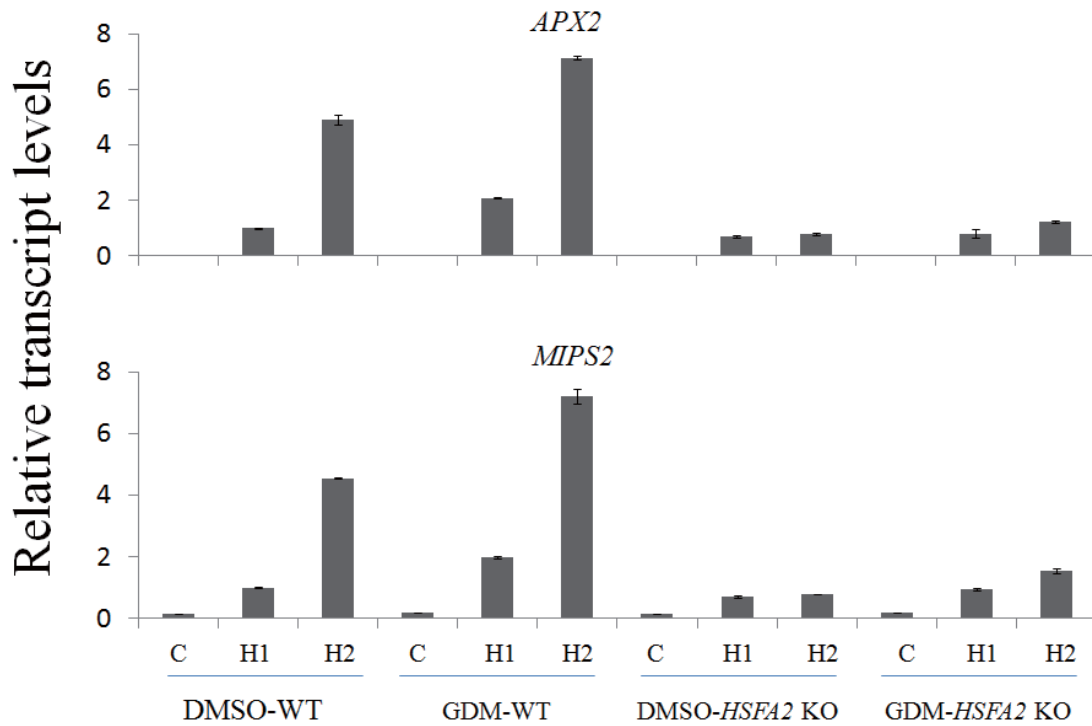
Fig. 11 The effect of HSP90 inhibitor on HSMR. Seeds were sown on 1/2

MS medium plate with 1.5μM geldanamycin (GDM) or DMSO control.

Three-d after germination, the seedlings were given C, H1, or H2 treatments.

Transcript levels of *APX2* and *MIPS2* were analyzed by RT-PCR; *ACTIN2*

was used to normalize signal.
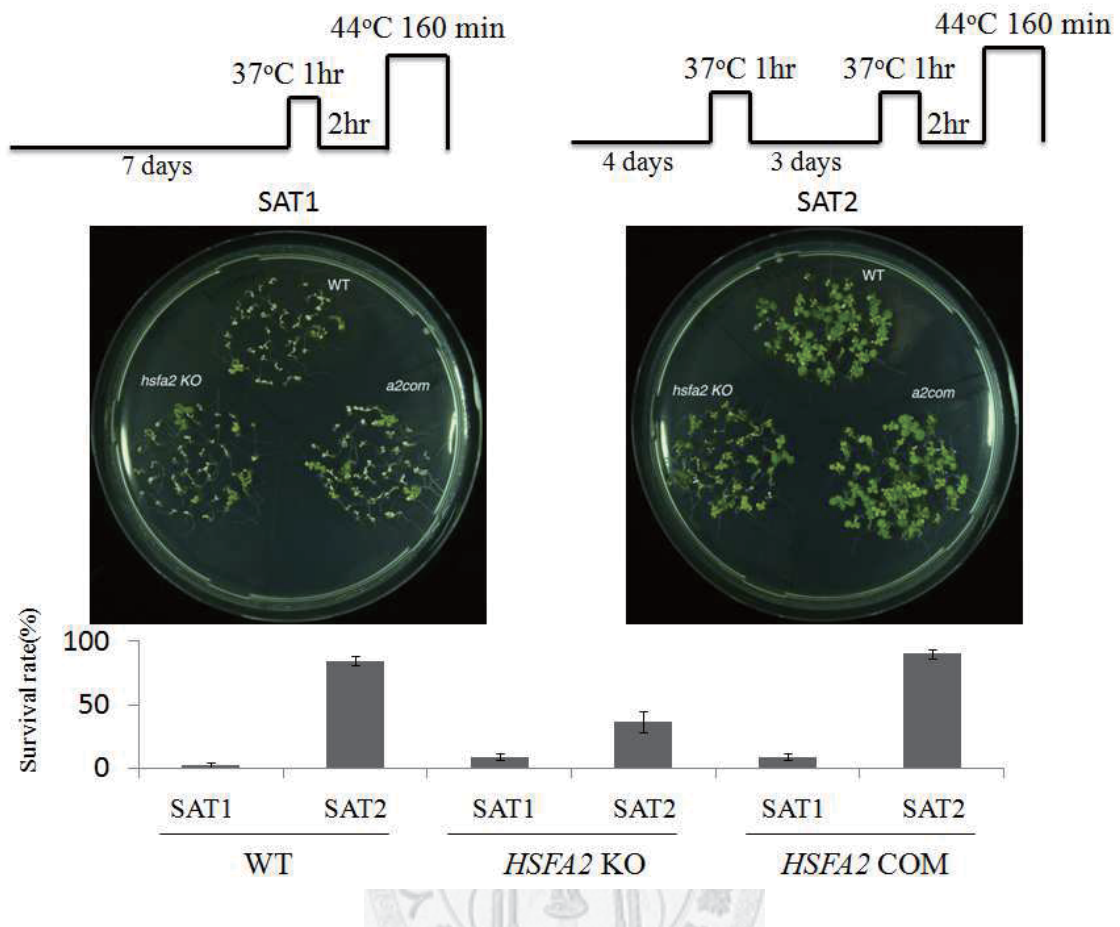
Fig. 12 Thermotolerance assays of *HSFA2* KO and complementation (COM) lines.

Seedlings were subjected to HS treatments as indicated by the schematic diagrams of HS regimes. The photographs were taken after 8 d of recovery. Viability was evaluated after 8d of recovery.

Table 1. Primers used for quantitative RT-PCR.

Target Genes	Primer name	Oligo Sequence(5' to 3')
ACT2	ACT-F	CGCTCTTTCTTTCCAAGCTCAT
	ACT-R	GCAAATCCAGCCTTCACCAT
APX2	Apx2-F	CGTGGTCTTATTGCCGAGAAG
	Apx2-R	CCCAAACGGTCCTCCTGTCT
MIPS2	At2g22240-1f	GTAGCTAGTAATGGCATCCTCTTTGA
	At2g22240-1r	ATCCGCAACATATGGCACATAC
HSFA1e	ATHSFA1E-7	TGAGCTCCATTCCGCCTTT
	ATHSFA1E-8	GTCCGTCAATGGATCGTCAAC
HSFA2	Hsfa2-f-abi	GGTTCTGTAGCGGCTTCTTCAT
	Hsfa2-r-abi	TGGTGGCCCTGTTTCGTTA
PD1	At2g27820-1f	AAGGACTAGCTCAATGCGAACAC
	At2g27820-1r	AGCTTCACGAGCTACGTTGAGA

Table 2. HS memory response up-regulated genes

AGI#	Gene name	Description	Fold change (H1/C)	Fold change (H2/H1)
AT1G02700	—	Unknown protein	4.36	3.61
AT1G11660	HSP70	Heat shock protein 70 (HSP 70) family protein	3.85	2.38
AT1G13340	—	Regulator of Vps4 activity in the MVB pathway	1.77	2.08
AT1G19020	—	Unknown protein	4.24	2.77
AT1G21140	—	Vacuolar iron transporter (VIT) family protein	1.06	2.71
AT1G23330	—	Alpha/beta-Hydrolases superfamily protein	0.93	2.43
AT1G33055	—	Unknown protein	1.98	2.35
AT1G47480	—	Glycine-rich protein family	0.57	3.73
AT1G52690	—	Late embryogenesis abundant protein (LEA) family protein	0.88	3.29
AT1G58170	—	Disease resistance-responsive (dirigent-like protein) family protein	6.54	2.17
AT1G60470	GOLS4	Galactinol synthase 4	1.98	3.48
AT1G62290	—	Saposin-like aspartyl protease family protein	0.91	2.83
AT1G64940	—	Unknown protein	1.19	2.75
AT1G66500	—	Pentatricopeptide repeat (PPR) superfamily protein	4.86	4.06
AT1G73040	—	Mannose-binding lectin superfamily protein	1.65	2.64
AT2G02220	ATPSKR1	Phytosulfokin receptor 1	1.51	2.36
AT2G19580	TET2	Tetraspanin2	1.1	2.48
AT2G20720	—	Pentatricopeptide repeat (PPR) superfamily protein	2.83	2.38
AT2G21640	—	Unknown Protein	1.48	2.14
AT2G21820	—	Unknown protein	6.15	6.68
AT2G22240	MIPS2	Myo-inositol-1-phosphate synthase 2	14.22	2.45
AT2G22500	DIC1	Dicarboxylate carriers	0.41	2.99
AT2G27820	PD1	Prephenate dehydratase 1	1.03	2.69
AT2G29740	—	UDP-glucosyl transferase 71C2	1.14	5.17
AT2G37870	—	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	0.68	3.12
AT2G41190	—	Transmembrane amino acid transporter family protein	1.06	2.22
AT2G43290	—	Calcium-binding EF-hand family protein	0.58	2.45
AT2G43900	5PTASE12	5-inositol-phosphate phosphatase	1.49	2.35
AT2G46720	KCS13	3-ketoacyl-CoA synthase	0.96	2.93
AT3G02480	—	Late embryogenesis abundant protein (LEA) family protein	0.62	2.09
AT3G02990	HSFA1e	Heat shock transcription factor A1E	5.38	5.5
AT3G03950	—	Evolutionarily conserved C-terminal region 1	2.13	2.14
AT3G09640	APX2	Ascorbate peroxidase 2	219.5	3.32
AT3G16670	—	Pollen Ole e 1 allergen and extensin family protein	0.39	2.46
AT3G18850	LPAT5	Lysophosphatidyl acyltransferase 5	1.1	4.41
AT3G19270	CYP707A4	Cytochrome P450	1.46	2.69
AT3G28270	—	Protein of unknown function	1.16	2.6
AT3G58550	—	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	0.52	2.04
AT3G60250	—	Casein kinase II beta chain 3	2.15	2.16
AT4G13180	—	NAD(P)-binding Rossmann-fold superfamily protein	1.66	2
AT4G18340	—	Glycosyl hydrolase superfamily protein	0.59	2.28
AT4G20350	—	Oxidoreductase	1.31	3.05

AT4G22340	CDS2	Cytidinediphosphate diacylglycerol synthase 2	3.06	2.04
AT4G25810	XTR6	xyloglucan endotransglycosylase-related protein	23.74	3.53
AT4G28390	AAC3	ADP/ATP carrier 3	5.11	2.57
AT4G30480	TPR1	Tetratricopeptide repeat (TPR)-like superfamily	1.37	2.64
AT4G33550	—	Bifunctional inhibitor/lipid-transfer protein	0.43	2.64
AT4G37410	CYP81F4	Cytochrome P450	0.17	2.13
AT5G01180	PTR5	Peptide transporter 5	59.52	2.31
AT5G04980	—	DNase I-like superfamily protein	0.79	2.1
AT5G05960	—	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	0.65	2.07
AT5G07000	ST2B	Sulfotransferase 2B	1.81	2.25
AT5G07330	—	Unknown protein	102.71	3.1
AT5G08380	AGAL2	Alpha-galactosidase 2	1.37	4
AT5G09930	—	ABC transporter family protein	2	6.28
AT5G11100	SYTD	Calcium-dependent lipid-binding (CaLB domain) family protein	4.73	5.82
AT5G12290	DGS1	Dgd1 suppressor 1	2.17	2.46
AT5G13170	SAG29	Senescence-associated gene 29	0.93	2.77
AT5G19110	—	Eukaryotic aspartyl protease family protein	1.14	2.75
AT5G26660	MYB86	Myb domain protein 86	0.99	3.25
AT5G38850	—	Disease resistance protein	1	2.14
AT5G40645	—	RPM1-interacting protein 4 (RIN4) family protein	1.18	5.03
AT5G43170	ZF3	Zinc-finger protein 3	28.28	3.78
AT5G53680	—	RNA-binding (RRM/RBD/RNP motifs) family protein	80.42	2.13
AT5G55050	—	GDSL-like Lipase/Acylhydrolase superfamily protein	1.28	3.29
AT5G59310	LTP4	Lipid transfer protein 4	0.4	3.92

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