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ORIGINAL ARTICLE

Arabidopsis thaliana WRKY25, WRKY26, and WRKY33 coordinate induction of plant thermotolerance

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Abstract Limited information is available regarding the exact function of specific WRKY transcription factors in plant responses to heat stress. We analyzed the roles of WRKY25, WRKY26, and WRKY33, three types of group I WRKY proteins, in the regulation of resistance to heat stress. Expression of *WRKY25* and *WRKY26* was induced upon treatment with high temperature, whereas *WRKY33* expression was repressed. Heat-treated WRKY single mutants exhibited small responses, while *wrky25wrky26* and *wrky25wrky33* double mutants and the *wrky25wrky26wrky33* triple mutants showed substantially increased susceptibility to heat stress, showing reduced germination, decreased survival, and elevated electrolyte leakage, compared with wild-type plants. In contrast, constitutive expression of WRKY25, WRKY26, or WRKY33 enhanced resistance to heat stress. Expression studies of selected heat-defense genes in single, double, and triple mutants, as well as in over-expressing lines, were correlated with their thermotolerance phenotypes and demonstrated that the three WRKY transcription factors modulate transcriptional changes of heat-inducible genes in response to heat treatment. In addition, our findings provided evidence that WRKY25, WRKY26, and WRKY33 were involved in regulation of the heat-induced ethylene-dependent response and demonstrated positive cross-regulation within these three genes. Together, these results indicate that WRKY25, WRKY26, and WRKY33 positively regulate the cooperation between the ethylene-activated and heat shock proteins-related signaling pathways that mediate responses to heat stress; and that these three proteins interact functionally and play overlapping and synergetic roles in plant thermotolerance.

Keywords Ethylene · Heat stress · Thermotolerance · *WRKY25* · *WRKY26* · *WRKY33*

Abbreviations

ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
APX	Cytosolic ascorbate peroxidase
EL	Electrolyte leakage
HS	Heat shock
Hsf	Heat shock transcription factor
Hsp	Heat shock protein
MBF1c	Coactivator multiprotein bridging factor 1c
SA	Salicylic acid

Introduction

Temperature levels beyond an organism's optimal tolerance range are regarded as a major abiotic stress. Extreme high temperature disturbs cellular homeostasis and

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severely impairs plant growth and development. Worldwide, extensive agricultural losses are attributed to heat, often in combination with drought or other stresses (Mittler 2006). Thus, finding an effective strategy for protecting plant cells from damage caused by rapid and/or drastic changes in temperature is of particular importance for agricultural production.

The induction of classical heat shock proteins (Hsps) through heat shock transcription factors (Hsfs) is important in plant thermotolerance (Mishra et al. 2002; Panchuk et al. 2002; Baniwal et al. 2004; von Koskull-Döring et al. 2007). Abscisic acid (ABA), salicylic acid (SA), hydrogen peroxide, ethylene, and calcium signaling pathways have also been reported to be involved in the heat stress response in different plant species, some of which control Hsps whereas others control the production or activation of diverse effector components (Larkindale and Knight 2002, Larkindale and Huang 2005, Larkindale et al. 2005; Liu et al. 2006, 2008). Moreover, recent studies have implicated many novel transcription factors and genes as components in thermotolerance in *Arabidopsis thaliana*. Examples include the coactivator multiprotein bridging factor MBF1c (Suzuki et al. 2005; Suzuki et al. 2008), cytosolic ascorbate peroxidase APX1 and APX2 (Miller et al. 2007; Larkindale and Vierling 2008), several zinc finger proteins (Zats) (Rizhsky et al. 2004; Miller et al. 2008), dehydration-responsive element-binding proteins DREB2A and DREB2C (Sakuma et al. 2006; Lim et al. 2007; Yoshida et al. 2008; Chen et al. 2010a, b), and the genes coding for enzymes involved in sugar metabolism (Busch et al. 2005). Thus, thermotolerance is affected by a complex network of pathways in plants, only one of which is the production of Hsps (Larkindale and Vierling 2008).

Previous studies have shown that the ethylene-mediated pathway protects creeping bentgrass and *A. thaliana* from heat stress (Larkindale and Knight 2002; Larkindale and Huang 2005, Larkindale et al. 2005). In *A. thaliana*, heat shock (HS) was found to induce ethylene production (Clarke et al. 2009), and ethylene receptor mutants, such as *ethylene resistant 1* (*etr1*) and *ethylene insensitive 2* (*ein2*), were sensitive to heat stress, particularly under high-light conditions (Larkindale et al. 2005; Suzuki et al. 2008). In addition, exogenous application of 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor to ethylene, can result in a degree of enhanced resistance to heat-induced oxidative stress (Larkindale and Knight 2002), indicating that ethylene might act as a signal to activate oxidative defenses during heat stress (Larkindale et al. 2005). These findings demonstrate that ethylene, first demonstrated to be involved in the senescence response, can also promote heat tolerance in *A. thaliana*. A recent study revealed that MBF1c is a key positive regulator of thermotolerance in *A. thaliana* that functions upstream of ethylene during heat stress (Suzuki et al. 2008). In addition, *MBF1c*- and ethylene-mediated thermotolerance are independent of *Hsps* expression (Larkindale and Knight 2002; Larkindale and Huang 2005; Larkindale et al. 2005b; Suzuki et al. 2008).

The family of WRKY transcription factors contains over 74 members in *A. thaliana*, and is subdivided into three groups based on the number of WRKY domains (the conserved amino acid sequence WRKYGQK) and the features of their zinc finger-like motif (Eulgem et al. 2000). The WRKY domain shows a high binding affinity to the TTGACC/T W-box sequence (Ülker and Somssich 2004). A large body of evidence indicates that many WRKY proteins are involved in regulating plant responses to biotic stress. A majority of studies on WRKY proteins have investigated their involvement in disease responses and SA-mediated defense (Dellagi et al. 2000; Eulgem et al. 2000; Asai et al. 2002; Zheng et al. 2007; Lai et al. 2008; Chen et al. 2010a, b). In addition, previous reports have demonstrated that a number of WRKY proteins are key regulators in wounding responses (Hara et al. 2000; Chen et al. 2010a, b) and certain developmental programs (Johnson et al. 2002; Gadjev et al. 2006; Jiang and Yu 2009; Jing et al. 2009). Although most WRKY proteins studied thus far have been implicated in regulating biotic stress responses, some WRKY proteins regulate plant responses to freezing (Huang and Duman 2002), oxidative stress (Rizhsky et al. 2004), cold, salinity, and drought (Seki et al. 2002; Jiang and Deyholos 2009; Qiu et al. 2009).

There is increasing evidence that WRKY proteins are involved in responses to heat. Our recent studies have shown that *A. thaliana* WRKY25 and WRKY39 are positive regulators in thermotolerance (Li et al. 2009, 2010). A WRKY transcription factor in tobacco (*Nicotiana tabacum* L.) responds to a combination of drought and HS (Rizhsky et al. 2002). OsWRKY11 in rice (*Oryza sativa* L.) has also been reported to play a role in heat and drought stress response and tolerance (Wu et al. 2009). The expression of *A. thaliana WRKY18*, *-33*, *-40*, and *-46* is elevated in *MBF1c* over-expressing plants, which possess enhanced thermotolerance compared with wild-type plants (Suzuki et al. 2005). Microarray analysis of *A. thaliana hsf1a/hsf1b* double knockout mutants has revealed that 9 of 60 analyzed *WRKY* genes are regulated by HS and, among these 9 genes, *WRKY7* is a HsfA1a/1b-dependent HS gene (Busch et al. 2005). Previously, we identified 18 *WRKY* genes, including *WRKY25*, *WRKY26*, and *WRKY33*, that were heat-responsive through microarray analysis in *A. thaliana* (data not shown) and also found that the promoters of a number of heat defense-related genes including *Hsp* and *Hsf* genes contain W-box sequences that are recognized by WRKY proteins (Li et al. 2009). These

observations emphasize a potentially important role for *WRKY* genes in mediating the heat stress response.

Arabidopsis thaliana WRKY25, *WRKY26*, and *WRKY33* encode three structurally related group I WRKY proteins (Dong et al. 2003). WRKY25 and WRKY33 have been experimentally localized in the nucleus (Zheng et al. 2006, 2007). Various biotic and abiotic stresses can activate the expression of *WRKY25* or *WRKY33* (Rizhsky et al. 2004; Andreasson et al. 2005; Zheng et al. 2006, 2007; Jiang and Deyholos 2009). Earlier studies have characterized the role of WRKY25 and WRKY33 in pathogen responses (Lippok et al. 2007; Zheng et al. 2006, 2007) and NaCl stress responses (Jiang and Deyholos 2009), and a MAPK cascade regulates WRKY25 and WRKY33 activity (Andreasson et al. 2005). However, reports on the roles of WRKY26 in response to stresses are comparatively rare. Apparent functional redundancy has severely hampered attempts to genetically define the roles of individual WRKY transcription factors in the regulation of plant defense. Xu et al. (2006) demonstrated that the closely related WRKY18, WRKY40, and WRKY60 had partially redundant roles in response to pathogens. Jiang and Deyholos (2009) showed that WRKY25 and WRKY33 function redundantly as positive regulators in salinity stress, as illustrated by our failure to observe stronger thermosensitive phenotypes in *wrky25* single mutants during heat stress (Li et al. 2009).

In the present study, we further characterized the function of WRKY25, WRKY26, and WRKY33 as positive regulators of the heat stress response by analyzing both potential upstream and downstream targets of these genes, and their loss-of-function and over-expression phenotypes. We suggest that these three regulators show partial redundancy in thermotolerance and regulate the cooperation between MBF1c (ethylene)-activated and Hsps-related signaling pathways that mediate responses to heat stress. In addition, our results reveal that WRKY25, WRKY26, and WRKY33 positively regulate each other's expression during heat stress and play synergetic roles in thermotolerance in *A. thaliana*.

Materials and methods

Plant materials and growth conditions

The *A. thaliana* mutants and wild-type plants used in this study are from the Columbia genetic background. T-DNA insertion mutants, including *wrky25-1* (SALK_136966), *wrky26-1* (SALK_137675), *wrky33-1* (SALK_006603), *hot1* (SALK_036423c), and *mbf1c* (SALK_083813c), were obtained from the Arabidopsis Biological Resource Center (ABRC) (Alonso et al. 2003). Semiquantitative RT-PCR and Northern blot analyses were performed to further confirm the T-DNA insertion mutants. The mutant lines *aba1*, *coi1*, *ein2*, *sid2*, *wrky33-2* (GABI_324B11), and *wrky25-1wrky26-2wrky33-1* (*wrky26-2*, SALK_063386) were obtained from Prof. Zhixiang Chen (Purdue University, USA).

Seeds were surface-sterilized in 20% (v/v) bleach for 15 min, sown on 1/2 Murashige and Skoog (MS) medium containing 1.5% (w/v) sucrose, stratified in the dark at 4°C for 2–3 days and grown in a growth chamber under a 14 h light (100 μE m^{-2} s^{-1})/10 h dark cycle at 22°C. The seedlings were transplanted into soil and grown in a growth chamber (22°C with a 14 h photoperiod) 7 days after germination on 1/2 MS medium.

Identification of *wrky25-1*, *wrky26-1*, *wrky33-1*, *wrky25-1wrky26-1*, *wrky25-1wrky33-1*, and *wrky25-1wrky26-1wrky33-1* mutant alleles

Confirmation of the T-DNA insertion was performed by PCR analysis using a combination of a T-DNA border primer (5′-AAACGTCCGCAATGTGTTAT-3′) and a gene-specific primer (*W25*: 5′-AACCAAGAAGGTCGGT GAAA-3′; *W26*: 5′-AGAAGGGAAATGGACAAATC A-3′; *W33*: 5′-TCCACTTTCTTCTTCGTT-GGA-3′). A further PCR was performed to identify plants homozygous for the insertions using the above gene-specific primers and respective reverse primers (*W25*R: 5′-AGTACTGGAC CAACCAAACCT-3′; *W26*R: 5′-AACCCATCAAA-AAA TAGCTGAGT-3′; *W33*R: 5′-ATGGACAATAGCAGAA CCAGACA-3′). Double and triple mutants were generated from crosses of single mutants and identified through PCR genotyping.

Generation of transgenic lines and plant transformation

The transgenic line constitutively expressing *WRKY25* has been described previously (Li et al. 2009) and was used in this study. The *WRKY26* full cDNA linked to the pUNI vector was obtained from ABRC and the coding region of *WRKY33* was cloned by RT-PCR. To generate the *35S:WRKY26* and *35S:WRKY33* constructs, the cDNA of *WRKY26* and *WRKY33* were subcloned by ligating the SmaI-BamHI and BamHI-SalI fragment, respectively, into the SmaI/BamHI and BamHI/SalI sites of pOCA30 (Chen and Chen 2002) behind the *CaMV 35S* promoter. The recombinant plasmids were introduced into *Agrobacterium tumefaciens* GV3101 and used to transform *A. thaliana* by the floral dip method (Clough and Bent 1998). Seeds were collected from the infiltrated plants and selected on 1/2 MS medium containing 50 μg ml^{-1} kanamycin. Kanamycin-resistant plants were transferred to soil 9 days later and grown in a growth chamber. Northern blot analyses were

performed to select the transgenic plants. Homozygous T_3 lines were obtained for phenotypic scoring under heat stress.

Measurement of chlorophyll content

Chlorophyll was extracted with 80% acetone from leaves of 25 days old seedlings. Chlorophyll content was determined at 663 and 645 nm according to Lichtenthaler (1987).

Heat and ACC treatment of plants

For germination assay, seeds were sown on water-saturated filter paper and treated at 45°C for 4 h immediately after removal from 4°C refrigeration. Thereafter they were incubated in a growth chamber at 22°C. Germination was recorded daily until no further germination was observed. The results for mutant and transgenic seeds were compared with the wild-type seeds on the same plate.

To examine the impact of heat stress on *WRKY25*, *WRKY26*, and *WRKY33* expression, vernalized seeds of wild-type were geminated on 1/2 MS medium. After 7 days, seedlings were transplanted into soil in pots. 21-day-old plants were incubated at 42, 45, and 48°C, respectively, for 0, 60, and 120 min. Then, the aerial parts of the plant were harvested and immediately frozen in liquid nitrogen.

To assess heat tolerance, plants were grown as described above for 25 days. Plants were then exposed to heat stress at 48°C for 6 h and returned to 22°C. The survival of the plants was scored and the photographs were taken after an additional 9 days. Plants with newly emerging leaves were scored as viable.

In all cases, seed and seedlings were heated in a BOXUN SPX-400 IC environment test chamber with 70% relative humidity. The heat treatments were applied in the dark to ensure that cell death was a result of increased temperature and not photooxidative stress.

ACC was dissolved in water as a 10 mM stock solution. For induction treatment, aerial parts of 3-week-old *A. thaliana* soil-grown plants were incubated with water (the control) or 100 μM ACC. To examine the effect of ACC on thermotolerance, 25-day-old plants cultured in soil were sprayed with 10 μM ACC, placed in the growth room for 1 h, then treated at 48°C for 6 h before returning to 22°C to continue to grow for 9 days. The survival rate was recorded.

Electrolyte leakage measurement

Electolyte leakage (EL) represents a rapid, sensitive and quantitative method to assess the effects of stress on plant cells (Howarth et al. 1997; Hong et al. 2003; Clarke et al. 2004, 2009). Measurement of leaf EL was performed essentially as described by Clarke et al. (2004). For experiments where EL was measured during heat treatment at 42°C, the leaves from the same whorl of each 3-week-old plant were placed in glass tubes containing 5 ml water and incubated in a waterbath at 42°C in the dark. The conductance of the water was measured at intervals during the heat treatment and calculated per milligram fresh weight.

RNA extraction, probe making, and Northern blot analysis

RNA was isolated from aerial parts of *A. thaliana* plants by standard protocols (Sambrook et al. 2001). [α-^{32}P] dATP (>3,000 Ci/mmol) was obtained from the Beijing FuRui Biological Technology Company. Total RNA (10 or 20 μg) was separated on agarose (1.5%)-formaldehyde gels and transferred onto nylon membranes, which were then hybridized with α-^{32}P-dATP-labeled gene-specific probes corresponding to the following genes: *AtWRKY25*, *WRKY26*, *WRKY33*, *HsfA2*, *HsfB1*, *Hsp70*, *Hsp101*, *MBF1c*, *APX1*, and *Zat10*. *A. thaliana HsfA2*, *HsfB1*, *Hsp70*, and *Hsp101* are well-known HS-induced genes that play important roles in thermotolerance (Kotak et al. 2007). *A. thaliana* APX1 is a key H_2O_2 removal enzyme (Panchuk et al. 2002; Pnueli et al. 2003), and the transcriptional regulator *Zat10* is a key regulator of reactive oxygen species signaling (Miller et al. 2008) and is also expressed specifically in response to 12-oxo-phytodienoic acid, a precursor of jasmonate biosynthesis (Taki et al. 2005). The probes were generated by PCR amplification with gene-specific primers. Transcripts for *WRKY25*, *WRKY26*, and *WRKY33* were detected with respective full-length cDNA as the probe. Hybridization was performed overnight in PerfectHyb plus hybridization buffer (Sigma-Aldrich) at 68°C. The membranes were then washed following standard procedures (Sambrook et al. 2001).

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed as described in Li et al. (2010). The primer sequences of *Actin2* were 5′-TGTGCCAATCT ACGAGGGTTT-3′ and 5′-TTTCCCGCTC-TGCTGTT GT-3′. The following primers were used for target gene amplification: *WRKY25*: 5′-ACCTCTTCCGATTTCACA-3′ and 5′-GTTCCATTAAAGCCTT-GC-3′; *WRKY26*: 5′-TCA TCCCAAGCCCCAATC-3′ and 5′-GCCTCTGTTT-CATC TTCACCAA-3′; *WRKY33*: 5′-TTGTGGGAGTGAACCT G-3′ and 5′-TAACCGTCGTCAAGAAT-3′; *EIN2*: 5′-GTA TGGTGCGTCTTATGGTCG-3′ and 5′-ACTGCTCAAAG GGCTGTCTGG-3′; 3′ end primers of *WRKY33* for detecting

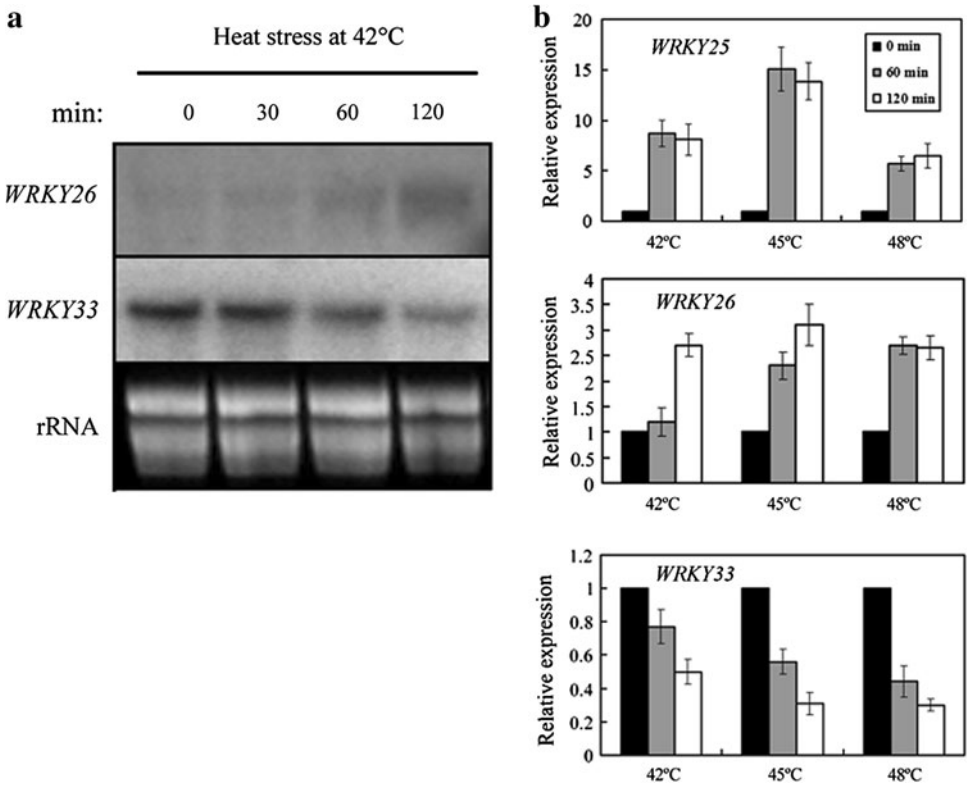

Fig. 1 Heat-responsive expression of *WRKY25*, *WRKY26*, and *WRKY33*. *Arabidopsis thaliana* plants (3 or 4 weeks old) were treated at 42, 45, or 48°C. Total RNA was isolated from heat-treated leaves harvested at the indicated time points after treatment. **a** Northern blot analysis. The gene-specific DNA fragments were used as probes, and ribosomal 18S RNA was used as the control for RNA loading. **b** qRT-PCR analysis. *Actin2* was used as an internal control and gene expression was normalized to the wild-type unstressed expression level, which was assigned a value of 1. Data represent the average of three independent experiments ±SD. All the experiments described above were repeated three times with similar results

endogenous *WRKY33*: 5′-AAAAGGAACCAGTTGTTC CTTTTT-3′ and 5′-TCCGTGTTCTAGTTCTATGGTACA AA-3′.

Accession numbers

Arabidopsis genome initiative numbers for the genes discussed in this article are as follows: *WRKY25*, At2g30250; *WRKY26*, At5g07100; *WRKY33*, At2g38470; *HsfA2*, At2g26150; *HsfB1*, At4g36990; *Hsp70*, At3g12580; *Hsp101*, At1g74310; *MBF1c*, At3g24500; *APX1*, At1g07890; *Zat10*, At1g27730; *Actin2*, At3g18780; and *EIN2*, At5g03280.

Results

Expression of *WRKY25*, *WRKY26*, and *WRKY33* during heat stress

To analyze the involvement of WRKY25, WRKY26, and WRKY33 in plant thermotolerance, we analyzed their expression in response to high temperature. According to microarray experiments (data not shown), both *WRKY25* and *WRKY26* transcripts were abundant in heat-treated wild-type plants. After 1 h heat stress at 42°C, the transcript levels of *WRKY25* increased by an average of 4.9-fold over the control, and *WRKY26* was up-regulated by 2-fold. In contrast, *WRKY33* was down-regulated by nearly 2.3-fold. To confirm the microarray results, we examined the transcript levels of *WRKY25*, *WRKY26*, and *WRKY33* in heat-treated plants using RNA blots. We observed that *WRKY25* transcription was induced transiently within 30 min and reached a maximum level after about 1 h of heat stress (Li et al. 2009). *WRKY26* expression was also induced following heat stress, but exhibited a slower rate and lower level of induction than *WRKY25* (Fig. 1a). *WRKY33* was expressed at higher levels than *WRKY25* and *WRKY26* in healthy, non-stressed wild-type plants and the transcript level of *WRKY33* decreased significantly after heat treatment (Fig. 1a).

Moreover, qRT-PCR was used to compare expression patterns of *WRKY25*, *WRKY26*, and *WRKY33* following 42, 45, and 48°C treatment. Just like expression profile during 42°C, *WRKY25* and *WRKY26* were induced after 45 and 48°C, however, the fold-induction of *WRKY26* in wild-type was apparently lower than that of *WRKY25* at each time-point after transferred to various high temperatures (Fig. 1b). On the other hand, *WRKY33* expression reduced continuously under each of the three heat treatments (Fig. 1b).

Multiple alignments of the amino acid sequences of WRKY25, WRKY26, and WRKY33 indicate that they share an overall 30–40% sequence identity (Supplementary Fig. S1). Besides the conserved WRKY domains, the three proteins share a certain level of homology at their N

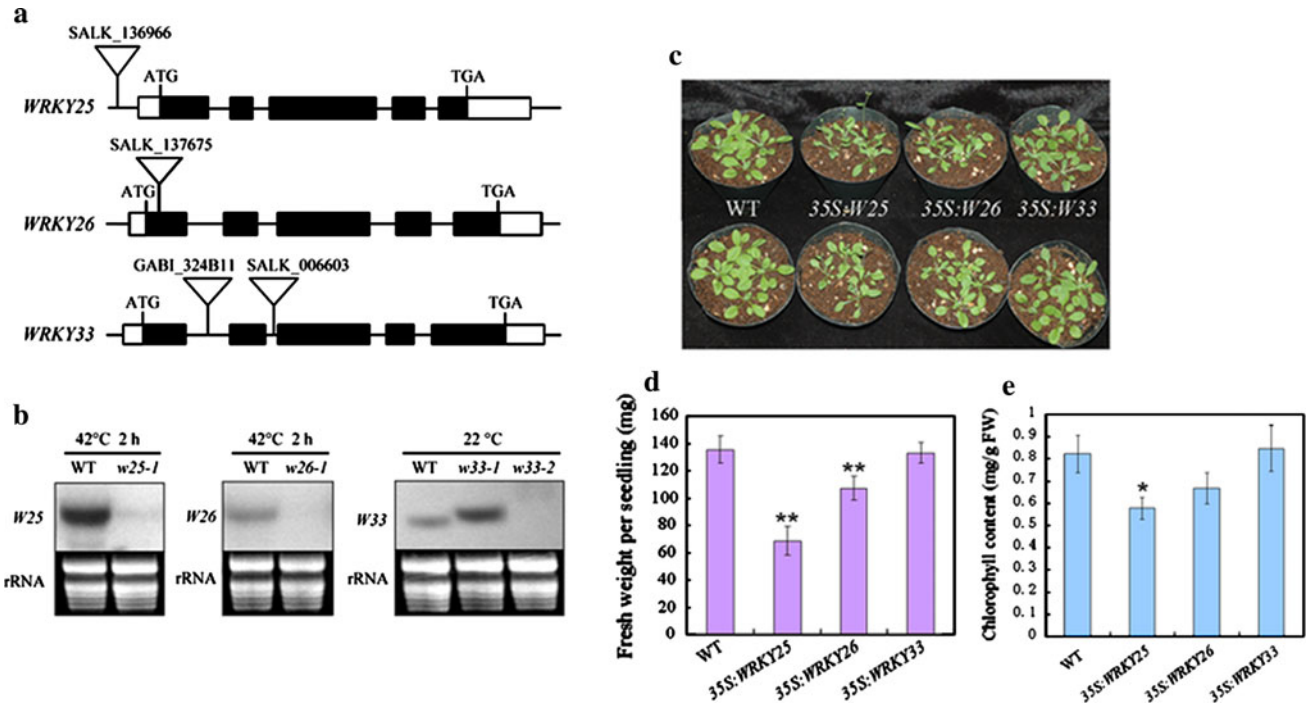

Fig. 2 Loss-of-function mutants for *WRKY25*, *WRKY26*, and *WRKY33* and construction of over-expression lines. **a** Diagram of *WRKY25*, *WRKY26*, and *WRKY33* and their T-DNA insertion mutants. **b** Northern blot analysis of *wrky25-1*, *wrky26-1*, *wrky33-1*, and *wrky33-2* mutants. Leaves from *wrky25-1*, *wrky26-1*, and their corresponding control (wild-type plant) were harvested after treatment at 42°C for 2 h, while non-stressed leaves of *wrky33-1*, *wrky33-2*, and the wild-type were harvested. After separation on an agarose-formaldehyde gel and blotting onto a nylon membrane, the blot was probed with gene-specific DNA fragments. Northern blotting was repeated at least three times with independently isolated RNA and similar results were obtained. **c** Morphology of 5-week-old wild-type and transgenic plants over-expressing *WRKY25*, *WRKY26* or *WRKY33*. **d** The fresh weight of aerial part of 25-day-old wild-type and transgenic seedlings. Each seedling was weighed separately. *Bars* indicate SD ($n = 30$). **e** Chlorophyll content in non-stressed wild-type and transgenic plants was measured as described in "Materials and methods". *Histograms* are the average of triplicate assays and the *bars* indicate SD. **Differences for the transgenic plants compared with wild-type are highly significant ($P < 0.01$). *Differences for the transgenic plants compared with wild-type are significant ($P < 0.05$)

termini, but the sequence similarity at their C termini is relatively low (Supplementary Fig. S1).

The finding that *WRKY33* expression decreased concurrently with increased expression of *WRKY25* and *WRKY26* during three different heat treatments suggests that *WRKY25*, *WRKY26*, and *WRKY33* were simultaneously involved in the heat stress response.

Identification of the mutants and generation of over-expressed transgenic *A. thaliana* plants

To analyze the role of *WRKY25*, *WRKY26*, and *WRKY33* in thermotolerance, we obtained one T-DNA insertion mutant for *WRKY25*, designated *wrky25-1* (SALK_136966); one for *WRKY26*, designated *wrky26-1* (SALK_137675); and two for *WRKY33*, designated *wrky33-1* (SALK_006603) and *wrky33-2* (GABI_324B11) (Fig. 2a). *wrky25-1* and *wrky26-1* contained a T-DNA insertion in the promoter region and the first exon of their respective genes (Fig. 2a). The *wrky33-1* mutant carried a T-DNA insertion in the second intron, while *wrky33-2* carried a T-DNA insertion in the first intron of the *WRKY33* gene (Fig. 2a). Homozygous mutant plants were identified by PCR with *WRKY25*-, *WRKY26*-, or *WRKY33*-specific primers. RNA gel blot analysis almost could not detect *WRKY25* and totally failed to detect *WRKY26* transcripts of the expected sizes in the respective homozygous mutants after heat treatment (Fig. 2b). *WRKY33* transcripts of the expected size were observed in non-stressed wild-type plants but were absent in plants carrying the *wrky33* mutant alleles (Fig. 2b). Northern blot analysis showed that *wrky33-1* produced a transcript larger than the wild-type *WRKY33* transcript (Fig. 2b) as previously described by Zheng et al. (2006). To determine possible functional redundancies between these three closely related transcription factors, we also generated the *wrky25-1wrky26-1* (*wrky25wrky26*) and *wrky25-1wrky33-1* (*wrky25wrky33*) double mutants and *wrky25-1wrky26-1wrky33-1* (*wrky25wrky26wrky33*) triple mutants through genetic crossing, and confirmed the progenies' homozygosity by Northern blotting or RT-PCR (Supplementary Fig. S2). In addition, another homozygous triple mutant, designated *wrky25-1wrky26-2wrky33-1*

(*wrky25wrky26-2wrky33*), was obtained. *wrky26-2* (SALK_063386) contained a T-DNA insertion in the 5′ untranslated region. When grown under normal conditions, the single, double, and triple mutants showed no differences in growth, development, or morphology from wild-type plants (data not shown).

To further examine the role of *WRKY25*, *WRKY26*, and *WRKY33*, we over-expressed the *WRKY* genes in transgenic *A. thaliana* plants. Constructs containing a full-length *WRKY25*, *WRKY26*, or *WRKY33* cDNA driven by the *CaMV 35S* promoter were transformed into *A. thaliana*. RNA gel blotting identified several transgenic plants that contained elevated levels of *WRKY25* (Li et al. 2009), *WRKY26*, or *WRKY33* transcripts in the absence of heat stress (Supplementary Fig. S3). Transgenic lines that constitutively expressed *WRKY25* (lines 2 and 7) (Li et al. 2009), *WRKY26* (lines 3 and 5), or *WRKY33* (lines 4 and 6) at elevated levels were chosen for further study (Supplementary Fig. S3). Constitutive over-expression of *WRKY25* resulted in reduced growth (Fig. 2c), advanced flowering time (Fig. 2c), decreased fresh weight (Fig. 2d), and decreased chlorophyll content (Fig. 2e). Analysis of F_3 homozygous plants revealed that over-expression of *WRKY26* also exhibited a weaker phenotype, smaller plant size, and lighter fresh weight than wild-type plants (Fig. 2c, d), while transgenic *35S:WRKY33* plants exhibited no such altered phenotype (Fig. 2c–e).

Altered response to heat stress in mutant and over-expressed lines

We first examined the germination of mutant and over-expressed transgenic seeds. At 3 days after heat treatment, transgenic *35S:WRKY25*, *35S:WRKY26*, and *35S:WRKY33* seeds already exhibited a 1.6–1.7-fold increase in germination frequency than wild-type seeds, while the germination rates of *wrky26-1* and *wrky33-1* were approximately one-half than that of the wild-type (Fig. 3a). At the same time, no germination was observed among *wrky25-1*, *wrky25wrky26*, *wrky25wrky33*, and *wrky25wrky26wrky33* seeds (Fig. 3a). By 5 days after heat treatment, transgenic seeds over-expressing *WRKY25*, *WRKY26*, and *WRKY33* showed 81–88% germination, whereas wild-type seeds showed 73% germination (Fig. 3a). Reduction in germination frequency was observed in the homozygous *wrky25-1* (37%), *wrky26-1* (44%), and *wrky33-1* (48%) mutants. An even greater reduction in germination frequency was observed in the homozygous *wrky25wrky26* (33%) and *wrky25wrky33* (30%) double mutants and *wrky25wrky26wrky33* (27%) triple mutants (Fig. 3a). Eight days later, when no further germination of over-expressed seeds was observed, the wild-type and *wrky25wrky26wrky33* triple mutants showed nearly 87 and 42% germination, respectively (Fig. 3a). Although some of the single-mutant seeds eventually germinated, wild-type seeds displayed a significantly higher germination frequency over time (Fig. 3a; wild-type and *wrky25-1*, $P < 0.01$; wild-type and *wrky26-1*, $P < 0.01$; wild-type and *wrky33-1*, $P < 0.01$). Double mutants caused a greater reduction in germination frequency than single mutants and *wrky25wrky26wrky33* triple mutants displayed the lowest germination percentage at each time-point after transfer to 22°C. The over-expressed seeds showed tolerance to HS (Fig. 3a; wild-type and *35S:WRKY25*, $P < 0.05$; wild-type and *35S:WRKY26*, $P < 0.05$; wild-type and *35S:WRKY33*, $P < 0.05$).

We used 25-day-old plants to compare the degree of thermotolerance between mutants and wild-type plants. Nine days after high-temperature treatment, surviving plants were clearly identified by the presence of newly emerging leaves, while non-surviving plants exhibited complete etiolation, loss of turgor, and arrested leaf formation. As shown in Fig. 3b, the homozygous *wrky25wrky26* and *wrky25wrky33* double mutants showed an approximately 1.7–1.9-fold reduction in survival rate compared to wild-type plants, while their parental single mutants displayed less significant reduction (Fig. 3b; wild-type and *wrky25-1*, $P = 0.063$; wild-type and *wrky26-1*, $P = 0.037$; wild-type and *wrky33-1*, $P = 0.081$; wild-type and *wrky25wrky26*, $P < 0.01$; wild-type and *wrky25wrky33*, $P < 0.01$). The *wrky25wrky26wrky33* triple mutants exhibited the largest reduction in survival rate with two to threefold lower survival than wild-type plants (Fig. 3b; wild-type and *wrky25wrky26wrky33*, $P < 0.01$). Thus, the degree of thermotolerance was correlated with the level of expression of *WRKY25*, *WRKY26*, and *WRKY33*, as the wild-type plants showed normal levels of expression and greater thermotolerance than mutant plants (Fig. 3b).

We extended our assay to measure EL in the 21-day-old mutants, wild-type plants, and transgenic lines. Modestly enhanced EL was detected for *wrky25-1* compared with the wild-type during heat stress after 4 h, whereas *wrky26-1* and *wrky33-1* showed little difference in EL from that in wild-type plants (Fig. 3c; wild-type and *wrky25-1*, $P < 0.05$; wild-type and *wrky26-1*, $P = 0.148$; wild-type and *wrky33-1*, $P = 0.097$). The EL after heat stress for 4 h in the *wrky25wrky26* and *wrky25wrky33* double mutants and *wrky25wrky26wrky33* triple mutants was significantly higher than that of wild-type plants (Fig. 3c; wild-type and *wrky25wrky26*, $P < 0.01$; wild-type and *wrky25wrky33*, $P < 0.05$; wild-type and *wrky25wrky26wrky33*, $P < 0.01$). Treatment of *35S:WRKY26* and *35S:WRKY33* at 42°C for 6 h resulted in nearly 1.3 and 1.7-fold less EL than that in the wild-type, respectively (Fig. 3c). The EL of *35S:WRKY25* was not significantly different from that of wild-type plants after 6 h at 42°C (Fig. 3c), which was consistent with previous results showing that seedlings of

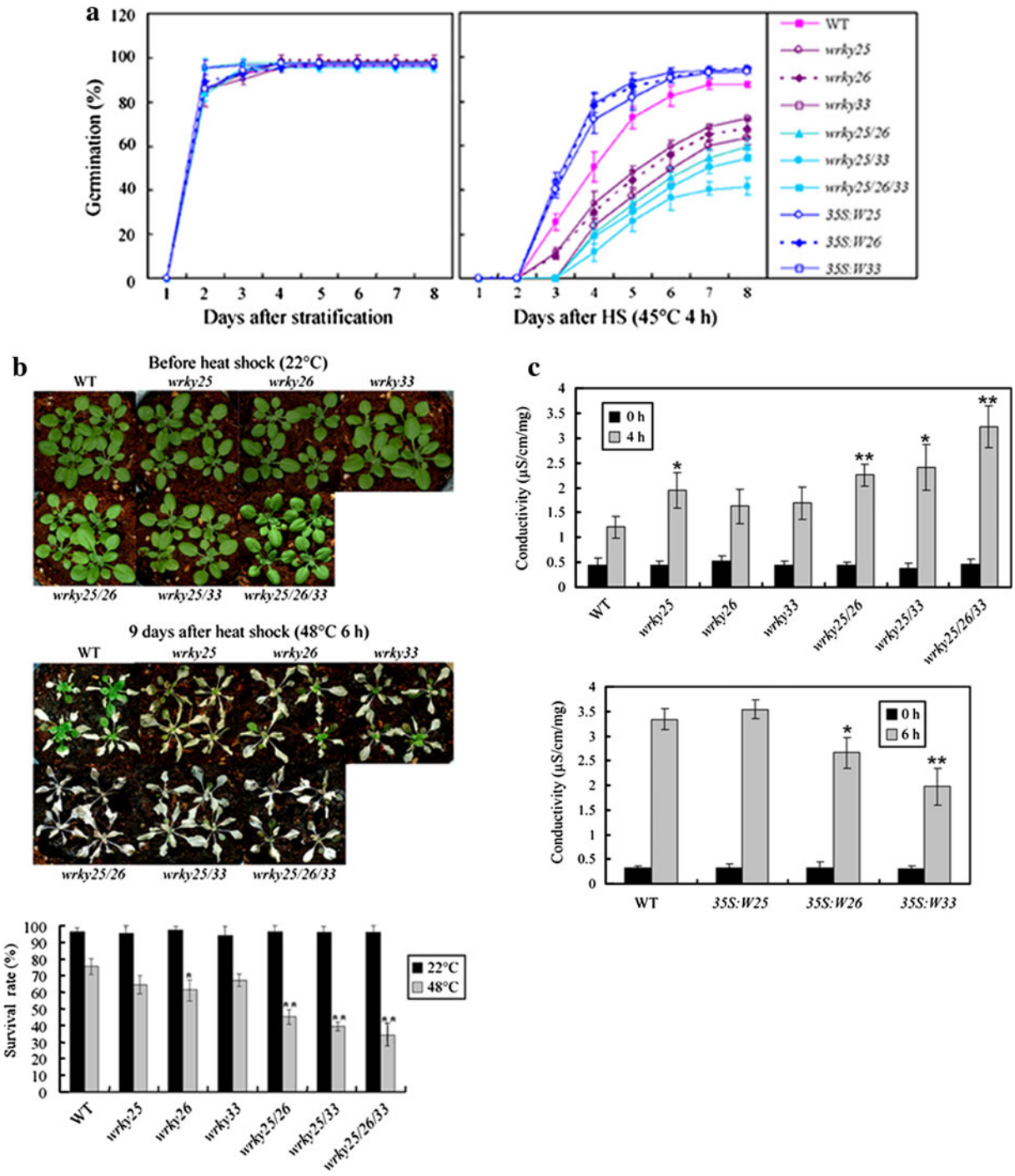

Fig. 3 The *WRKY25*, *WRKY26*, and *WRKY33* genes are required for resistance to heat stress. **a** Wild-type, single, double and triple mutant, and transgenic seeds were sown on water-saturated filter paper. Seeds were treated at 45°C for 4 h or incubated at 22°C (control) immediately after transfer from 4°C (for 3 days in the dark), and then incubated at 22°C. The percentage of radicle emergence was recorded daily until no further germination of over-expressing lines was observed. The data are shown as the mean ± SD ($n = 4$). Each replicate consisted of 50–70 seeds. **b** 25-day-old plants of the wild-type and mutant were treated at 48°C for 6 h or maintained at 22°C (control). Survival rate was recorded after a 9-day recovery period at 22°C. Data are shown as the mean ± SD ($n = 4$). Each replicate consisted of 12–15 seedlings. Photographs of representative results were taken before heat treatment and on the ninth day after treatment at 48°C. **c** The electrolyte leakage assay was performed with 21-day-old seedlings grown in soil. The aerial parts of mutant, wild-type, and transgenic plants were incubated at 42°C in a waterbath, transferred to 22°C at the indicated time point and the conductivity of the bathing solution was monitored. The data are presented as the means from three experiments, each with ten plants per treatment. *Bars* represent the SD ($n = 3$). The data was analyzed with Student's t test; significant at $^{*}P < 0.05$ and $^{**}P < 0.01$

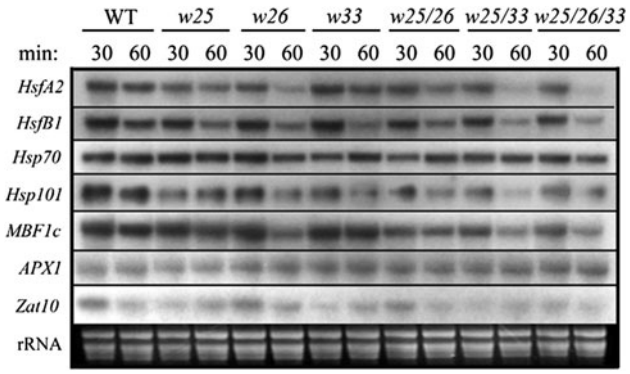

Fig. 4 Expression of heat-inducible and oxidative stress-responsive genes. Three-week-old plants of the wild-type and single, double, and triple mutants were subjected to heat treatment at 42°C. Total RNA was isolated from leaves harvested at 30 and 60 min after heat stress. Ribosomal 18S RNA was used as the control for RNA loading. Experiments were performed twice and similar results were obtained

transgenic plants expressing *WRKY25* were not more thermotolerant than wild-type seedlings (Li et al. 2009).

It should be mentioned that the thermosensitive phenotype of the *wrky25wrky26-2wrky33* triple mutants was similar to that of the *wrky25wrky26wrky33* triple mutants (Supplementary Fig. S4a–c). On the basis of the assays described above, constitutive over-expression of *WRKY25*, *WRKY26*, or *WRKY33* in *A. thaliana* enhances thermotolerance, while absence of *WRKY25*, *WRKY26*, and/or *WRKY33* leads to various degrees of thermosensitivity, indicating that WRKY25, WRKY26, and WRKY33 function redundantly with a positive role in resistance to heat stress.

Expression of heat stress defense genes in mutants and over-expressing plants

To investigate how WRKY25, WRKY26, and WRKY33 enhance plant thermotolerance, we examined expression of a number of heat stress-related genes in both mutants and over-expression lines following 42°C treatment. The expression patterns of well-characterized heat stress-responsive marker genes were analyzed by Northern blotting. As basal expression levels of most selected heat-defense genes could not be detected by RNA gel blotting (Supplementary Figs. S5 and S6; Li et al. 2009), we only present the results after treatment at 42°C for 30 and 60 min.

The *wrky25-1*, *wrky26-1*, *wrky33-1*, and *wrky33-2* single mutants exhibited little difference in the expression of *HsfA2*, *HsfB1*, *Hsp70*, *Hsp101*, *APX1*, and *Zat10* from that in wild-type plants (Fig. 4, Supplementary Figs. S5 and S6; Li et al. 2009). The *wrky25wrky26* and *wrky25wrky33* double mutants showed modest reduction in thermotolerance and had lower transcript levels of *HsfA2*, *HsfB1*, *Hsp101*, and *Zat10* than their parental single mutants, while the *wrky25wrky26wrky33* triple mutants had the lowest transcript levels of *HsfA2*, *HsfB1*, *Hsp101*, and *Zat10* that correlated with the most thermosensitive phenotype (Fig. 4). However, the expression of *Hsp70* and *APX1* was not altered in double and triple mutants compared to their parental signal mutants following heat treatment. Recent studies have shown that *A. thaliana MBF1c* is required for thermotolerance independent of Hsfs and Hsps, and functions upstream from SA and ethylene during heat stress (Suzuki et al. 2008). Figure 4 shows that the *wrky25-1*, *wrky26-1*, and *wrky33-1* single mutants had lower levels of heat-induced *MBF1c* transcripts than wild-type plants at 30 and 60 min after HS, in agreement with another set of experiments (Supplementary Figs. S5 and S6). The *wrky25wrky26* and *wrky25wrky33* double mutants and the *wrky25wrky26wrky33* triple mutants had substantially lower levels of *MBF1c* transcripts than wild-type plants at both time points. Likewise, induced expression of *Hsp101* and *MBF1c* was obviously reduced in *wrky25wrky26-2wrky33* triple mutants compared with wild-type plants (Supplementary Fig. S4d).

Enhanced resistance to heat stress in transgenic lines constitutively expressing *WRKY25*, *WRKY26*, or *WRKY33* is associated with slightly or moderately increased expression of these heat-inducible genes (Supplementary Figs. S5 and S6; Li et al. 2009). The *WRKY25* over-expressing lines had slightly higher levels of *HsfA2*, *HsfB1*, *HsfB2a*, and *Hsp101* transcripts than wild-type plants after heat treatment (Li et al. 2009). Over-expression of *WRKY26* showed slightly enhanced expression of *HsfA2*, *HsfB1*, *Hsp101*, *MBF1c*, and *APX1* (Supplementary Fig. S6). At the same time, the expression of *HsfA2*, *HsfB1*, *Hsp101*, *MBF1c*, *APX1*, and *Zat10* in *35S:WRKY33* plants was modestly increased compared with that in wild-type plants (Supplementary Fig. S5).

Taken together, these results indicate that *WRKY25*, *WRKY26*, and *WRKY33* function redundantly as positive regulators of these heat stress defense genes.

Expression of *WRKY25*, *WRKY26*, and *WRKY33*

To further investigate whether expression of *WRKY25*, *WRKY26*, or *WRKY33* is influenced by altered expressions of *Hsp101* and *MBF1c* during heat stress, expression of *WRKY25*, *WRKY26*, and *WRKY33* was checked in *hot1* (SALK_036423c) and *mbf1c* (SALK_083813c) mutants by qRT-PCR. Figure 5a shows that the expression of *WRKY25* and *WRKY26* was induced by heat stress in *hot1* and *mbf1c* mutants, with similar trends to that observed in wild-type plants. Likewise, *WRKY33* expression patterns in both mutants were the same as that in wild-type plants (Fig. 5a). Basal expression of *WRKY25*, *WRKY26* and *WRKY33* was slightly reduced in *hot1* and *mbf1c* mutants compared with wild-type plants, while the fold-induction of *WRKY25* and

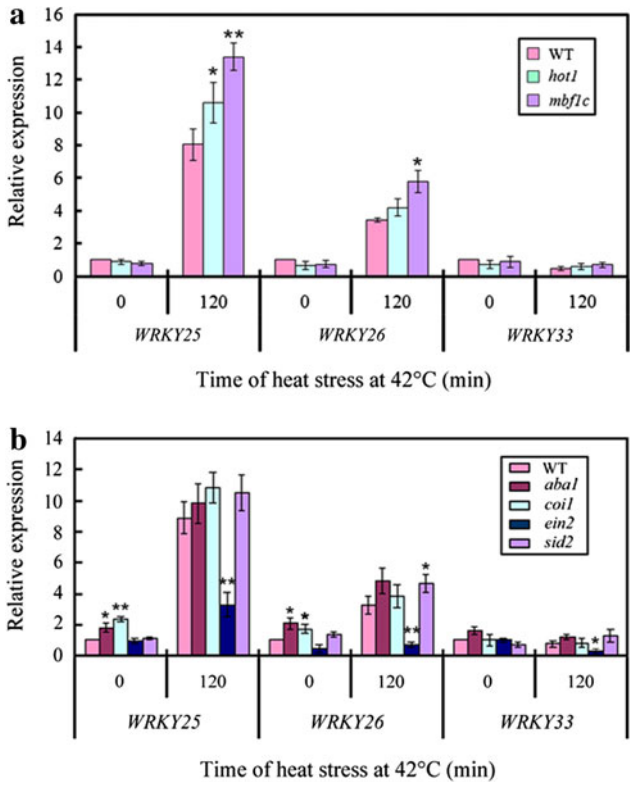

Fig. 5 qRT-PCR analysis of *WRKY25*, *WRKY26*, and *WRKY33* expression. **a** Expression of *WRKY25*, *WRKY26*, and *WRKY33* in wild-type, *hot1*, and *mbf1c* mutants before and after heat treatment at 42°C for 120 min. **b** Expression of *WRKY25*, *WRKY26*, and *WRKY33* in wild-type, *aba1*, *coi1*, *ein2*, and *sid2* plants exposed to heat stress at 42°C for 0 or 120 min. In **a** and **b**, *Actin2* was used as an internal control and gene expression was normalized to the wild-type unstressed expression level, which was assigned a value of 1. Data represent the average of three independent experiments ±SD ($n = 3$). **Differences for the mutant plants compared with corresponding wild-type are highly significant ($P < 0.01$). *Differences for the mutant plants compared with corresponding wild-type are significant ($P < 0.05$). The *A. thaliana* mutants and wild-type plants used in the experiments were 3 weeks old

WRKY26 expression in *hot1* and *mbf1c* mutants was slightly to modestly higher than that in wild-type plants at 120 min after transfer to 42°C (Fig. 5a). As shown in Fig. 5a, *WRKY25* expression in *hot1* and *mbf1c* mutants was increased by nearly 1.3- and 1.7-fold, respectively, compared with wild-type plants (wild-type and *hot1*, $P < 0.05$; wild-type and *mbf1c*, $P < 0.01$), while *WRKY26* was elevated by 1.2- and 1.7-fold (wild-type and *hot1*, $P = 0.069$; wild-type and *mbf1c*, $P < 0.05$). At the same time, the expression of *WRKY33* was slightly, but not significantly, enhanced in *hot1* or *mbf1c* mutants compared to the wild-type (Fig. 5a, wild-type and *hot1*, $P = 0.397$; wild-type and *mbf1c*, $P = 0.13$). It appears that slightly or moderately increased expression of *WRKY25*, *WRKY26*, and *WRKY33* in *hot1* and *mbf1c* mutants compensates for the absence of Hsp101 and MBF1c during heat stress.

To determine which signaling pathways are involved in the heat-responsive expression of *WRKY25*, *WRKY26*, and *WRKY33*, their expression was studied in a set of mutants that are defective in various defense response pathways, including ABA biosynthesis (*aba1*), jasmonate signaling (*coi1*), ethylene response (*ein2*), and SA biosynthesis (*sid2*). All of these mutants exhibit thermosensitivity (Larkindale et al. 2005; Clarke et al. 2009). Before and after heat stress, the expression of *WRKY25*, *WRKY26*, and *WRKY33* was enhanced slightly or moderately in *aba1*, *coi1*, and *sid2* compared with the wild-type (Fig. 5b). However, significantly reduced expression of *WRKY25* (nearly 2.7-fold; wild-type and *ein2*, $P < 0.01$), *WRKY26* (nearly 4.5-fold; wild-type and *ein2*, $P < 0.01$), and *WRKY33* (nearly 2.5-fold; wild-type and *ein2*, $P < 0.05$) occurred in heat-treated *ein2* mutant plants (Fig. 5b), indicating that *EIN2*, which is required for ethylene response and resistance to heat stress (Larkindale et al. 2005), was also required for heat-responsive expression of *WRKY25*, *WRKY26*, and *WRKY33*.

WRKY25, WRKY26, and WRKY33 function as positive regulators of plant thermotolerance by partially participating in ethylene-response signal transduction pathway

Because expression of *WRKY25*, *WRKY26*, and *WRKY33* during heat stress was mainly dependent on *EIN2* (Fig. 5b), we analyzed induction of *WRKY25*, *WRKY26*, and *WRKY33* in wild-type plants treated with ACC, the immediate precursor of ethylene. Expression of *WRKY25*, *WRKY26*, and *WRKY33* was induced by ACC with transcript levels increased by an average of 3.6-, 2.1-, and 3.4-fold, respectively, over the controls (Fig. 6a), which is consistent with previous reports by Zheng et al. (2006, 2007).

We also analyzed whether loss-of-function mutants for WRKY25, WRKY26, and/or WRKY33 or their respective over-expressing lines have an effect on the expression of *EIN2*. In the absence of heat stress, the expression of *EIN2* in the mutants and transgenic lines was nearly the same as that of wild-type plants (Fig. 6b). Heat treatment led to a 1.6-fold decrease in accumulation of *EIN2* in wild-type plants, 1.3–1.5-fold decrease in the over-expressing plants, and a 1.9–3-fold decrease in the mutants (Fig. 6b). Thus, loss-of-function mutants for WRKY25, WRKY26, and/or WRKY33 showed slightly or moderately decreased expression of *EIN2* compared to wild-type plants (a 1.3–1.7-fold decrease) after HS and *EIN2* transcript levels in the over-expressing lines were comparable to those in wild-type plants, indicating that, compared to the significance of *EIN2* in heat-responsive *WRKY25*, *WRKY26*, and *WRKY33* expression (Fig. 5b), *WRKY25*, *WRKY26*, and *WRKY33* are relatively less important in heat-influenced *EIN2* expression (Fig. 6b).

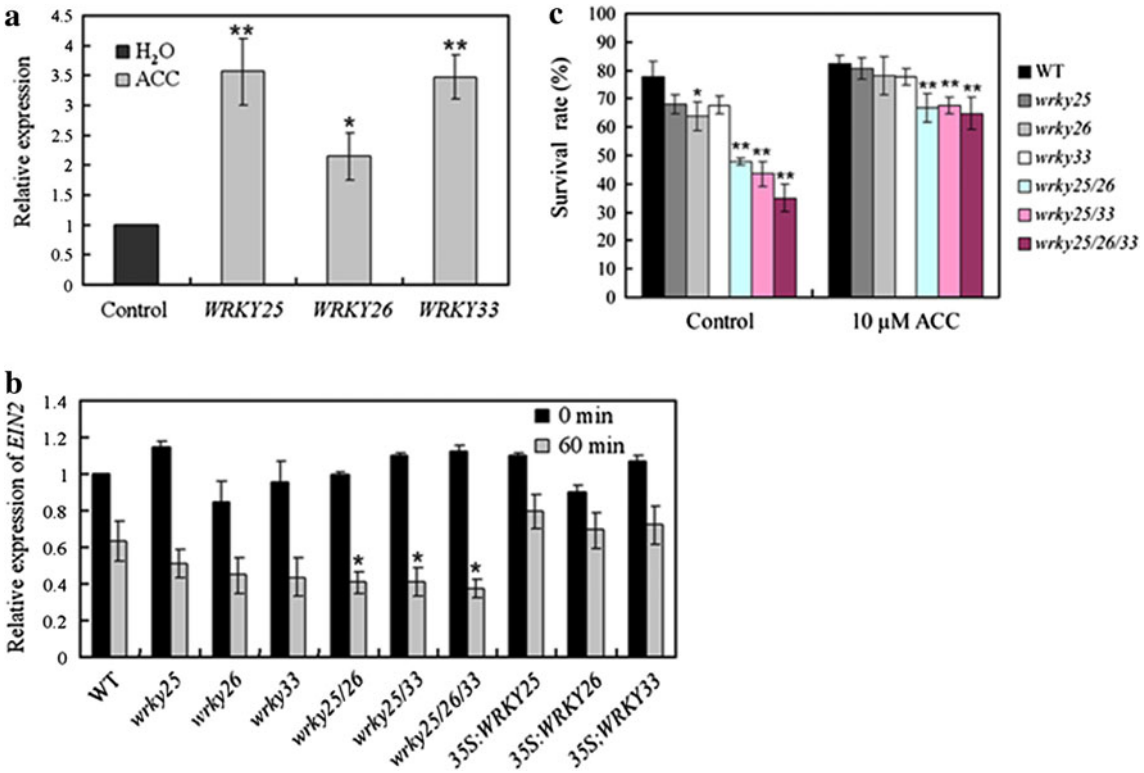

Fig. 6 Partial involvement of WRKY25, WRKY26, and WRKY33 in the ethylene-activated pathway. **a** Induced *WRKY25*, *WRKY26*, and *WRKY33* expression. qRT-PCR was performed on cDNA synthesized from total RNA extracted from aerial parts of 3-week-old wild-type plants incubated with H_2O or 100 μM ACC for 4 h. **b** *EIN2* expression in 21-day-old wild-type, mutant, and over-expressing plants. Total RNA was isolated from plants growing at 22°C that were exposed to either 42 or 22°C for 60 min. *EIN2* mRNA was determined by qRT-PCR. In **a** and **b**, *Actin2* was used as an internal control and gene expression was normalized to the wild-type unstressed expression level, which was assigned a value of 1. *Error bars* indicate the SD ($n = 3$). **c** Partial rescue of single, double, and triple mutants during heat treatment by 10 μM ACC. 25-day-old plants were subjected to heat stress (48°C for 6 h) in the presence or absence of ACC and scored for survival at the ninth day after heat stress. *Histograms* are the average of triplicate assays and the *bars* indicate SD. The data was analyzed with Student's *t* test; significant at $*P < 0.05$ and $**P < 0.01$

To further test whether *WRKY25*, *WRKY26*, and *WRKY33* function as positive regulators of plant thermotolerance through ethylene signaling, we tested the effects of ACC on wild-type plants and mutants subjected to heat stress. Without ACC, the survival rate of wild-type plants, single mutants, and double and triple mutants was 78, 64–68, and 35–48%, respectively, at 9 days after heat treatment (Fig. 6c). In the presence of 10 μM ACC, wild-type plants exhibited 83% survival, while single mutants and double and triple mutants showed 78–81 and 65–68% survival, respectively (Fig. 6c). These results indicate that ACC was able to partially rescue *wrky25-1*, *wrky26-1*, and *wrky33-1* single mutants, as well as *wrky25wrky26* and *wrky26wrky33* double mutants and *wrky25wrky26wrky33* triple mutants, from heat stress.

Cross-regulation among WRKY25, WRKY26, and WRKY33 during heat stress

Previous studies have shown that the promoters of multiple WRKY transcription factors are statistically enriched for W boxes and they can interact with the promoters of their own and other WRKY genes (Eulgem and Somssich 2007). Thus, the observation that *WRKY25*, *WRKY26*, and *WRKY33* contain 4, 4, and 5 W boxes (TTGAC) in their respective 1.5 kb of promoter regions (Dong et al. 2003) provides a possibility of autoregulation or mutual regulation within these three genes. To study this possibility, we first examined *WRKY25* expression in *wrky26-1* and *wrky33-1* single mutants, WRKY26 expression in *wrky25-1* and *wrky33-1* single mutants, and *WRKY33* expression in *wrky25-1* and *wrky26-1* single mutants before and after heat treatment.

As shown in Fig. 7a, non-stressed *wrky26-1* and *wrky33-1* mutants showed almost the same *WRKY25* expression, while heat-induced expression of *WRKY25* in *wrky26-1* and *wrky33-1* mutants showed a 1.9–2.1-fold reduction compared to that of the wild-type. Likewise, *wrky25-1* and *wrky33-1* mutants exhibited little difference in basal expression of *WRKY26* from that in wild-type plants, and *WRKY26* expression in heat-treated *wrky25-1*

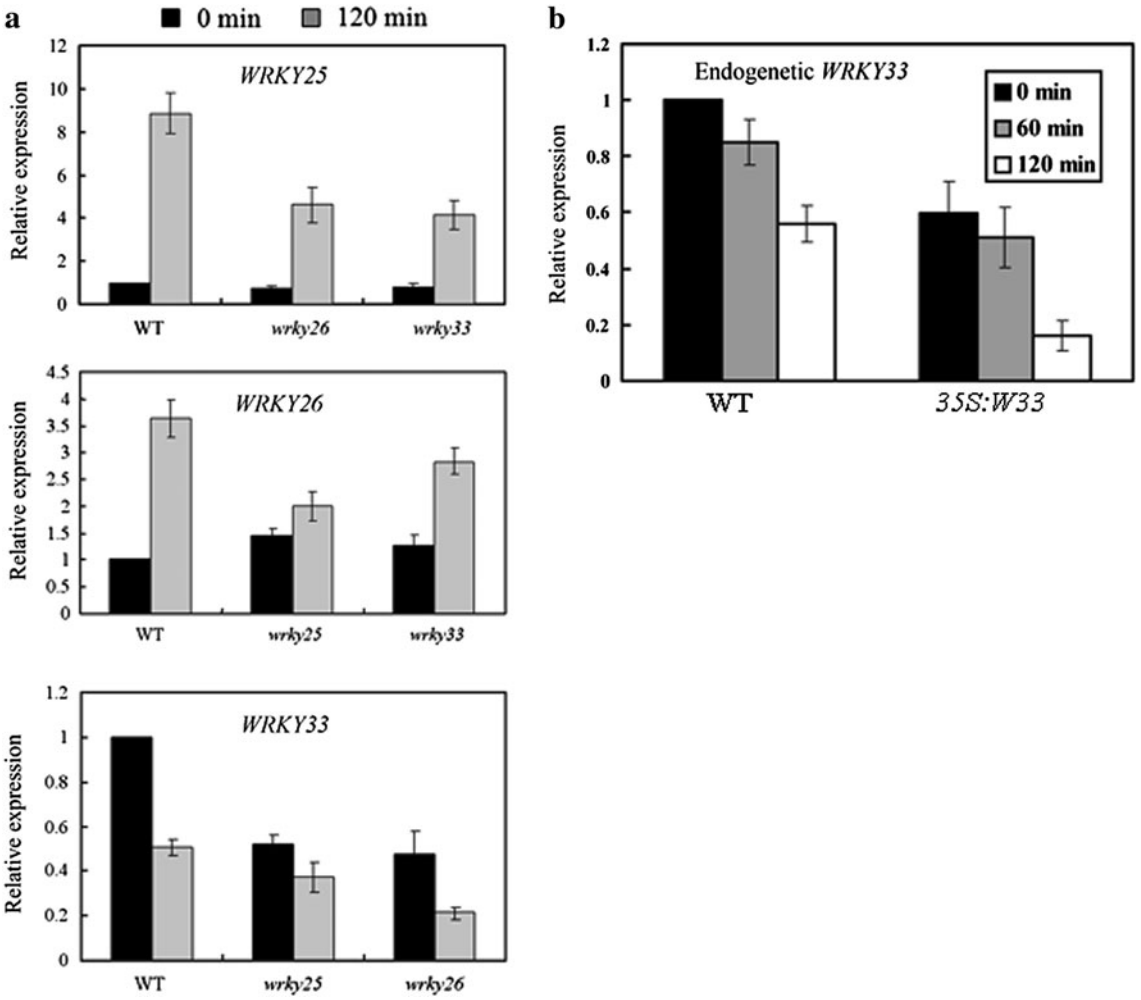

Fig. 7 Cross-regulation among WRKY25, WRKY26, and WRKY33 and autoregulation of WRKY33. **a** *WRKY25* expression was first checked in wild-type, *wrky26-1*, and *wrky33-1* single mutant plants, and then *WRKY26* expression was detected in wild-type, *wrky25-1*, and *wrky33-1* single mutant plants. At last, *WRKY33* expression in wild-type, *wrky25-1*, and *wrky26-1* single mutant plants was tested. The transcript level of *WRKY25*, *WRKY26*, and *WRKY33* was determined by qRT-PCR using cDNA generated from leaves of 21-day-old wild-type and single mutant plants treated at 42°C for 0 or 120 min. **b** qRT-PCR analysis of endogenous *WRKY33* expression in 21-day-old *35S:WRKY33* and wild-type plants during heat treatment at 42°C for 0, 60, and 120 min. In **a** and **b** *Actin2* was used as an internal control and gene expression was normalized to the wild-type unstressed expression level, which was assigned a value of 1. Data represent the average of three independent experiments ±SD

and *wrky33-1* mutants was 1.8- and 1.3-fold lower, respectively, than that in wild-type plants (Fig. 7a). The *WRKY33* transcript levels in *wrky25-1* and *wrky26-1* mutants were apparently lower than that in wild-type plants, especially in the absence of heat stress. Without heat stress, *WRKY33* expression in *wrky25* and *wrky26* mutants was nearly 1.9- and 2.1-fold reduced, respectively, compared with wild-type plants (Fig. 7a). After heat treatment, *WRKY33* expression in wild-type plants was 1.4- and 2.4-fold higher than that in *wrky25-1* and *wrky26-1* mutants, respectively (Fig. 7a).

Then, we compared the expression of endogenous *WRKY33* gene between wild-type and *35S:WRKY33* plants. Figure 7b shows that the endogenous *WRKY33* expression was clearly reduced in wild-type plants subjected to heat stress, in agreement with our previous reports (Fig. 1). Meanwhile, it was also decreased by heat stress in *35S:WRKY33* plants, with similar kinetics to that observed in wild-type plants (Fig. 7b). However, either the basal or heat-repressed expression of endogenous *WRKY33* in transgenic plants was lower than in wild-type plants. Particularly after 42°C for 120 min, the expression of endogenous *WRKY33* in *35S:WRKY33* plants was nearly 3.5-fold reduced compared with that in wild-type plants (Fig. 7b). In non-stressed plants, *35S:WRKY33* lines showed 1.7-fold lower endogenous *WRKY33* expression than wild-type plants (Fig. 7b).

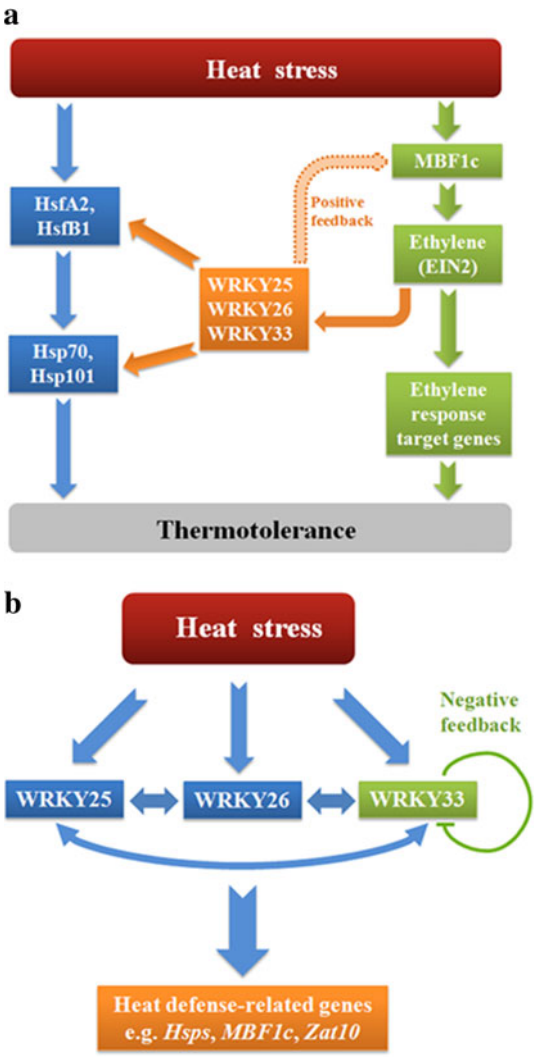

Fig. 8 Hypothetical model for WRKY25, WRKY26, and WRKY33 action. **a** WRKY25, WRKY26, and WRKY33 acts as regulator nodes linking heat (*blue arrowheads*, von Koskull-Döring et al. 2007) and ethylene signaling (*green arrowheads*, Suzuki et al. 2008) subnetworks. WRKY25, WRKY26, and WRKY33 are involved in heat stress by positively and redundantly regulating HSPS-relevant and ethylene signaling-related genes (*orange arrowheads*). **b** Heat defense-related genes are regulated in a positive manner by WRKY25, WRKY26, and WRKY33. Any one of these three genes is positively regulated by the other two during heat stress, reflecting a synergistic interaction among WRKY25, WRKY26, and WRKY33. WRKY33 is under negative autoregulation as previous reports (Turck et al. 2004; Lippok et al. 2007), leading to balanced amplification and diversification of heat defense signals

Taken together, expression of *WRKY25*, *WRKY26*, or *WRKY33* during heat stress was partially dependent on expression of the other two closely related genes, indicating that WRKY25 acts with WRKY26 and WRKY33 to confer thermotolerance in *A. thaliana*. Moreover, there was a self-negative feedback on *WRKY33* expression and the effect of self-inhibition increased during heat stress.

Discussion

Positive roles of WRKY25, WRKY26, and WRKY33 in plant thermotolerance

Arabidopsis thaliana WRKY25, *WRKY26*, *WRKY33* are heat-responsive genes (Fig. 1) that encode three structurally related WRKY proteins (Dong et al. 2003). Their positive roles in plant defense against heat stress were confirmed by analyzing the response of both their mutants and constitutive over-expression lines to high temperature (Fig. 3). In transgenic over-expression plants, constitutive expression of *WRKY25* or *WRKY26* resulted in a weak and pale phenotype (Fig. 2c–e; Li et al. 2009), as illustrated by the failure to observe more thermotolerant phenotypes in constitutive *WRKY25* or *WRKY26* over-expressing plants during heat stress (Fig. 3c).

The positive roles of *WRKY25*, *WRKY26*, and *WRKY33* in plant thermotolerance can also be deduced from patterns of heat stress-related marker gene expression (Fig. 4). Based on the finding that WRKY25, WRKY26, and WRKY33 redundantly regulated expression of *HsfA2*, *HsfB1*, *Hsp101*, and *MBF1c* (Fig. 4, Supplementary Fig. S4d) and a recent report that *MBF1c*-mediated thermotolerance is independent of *Hsps* expression, we speculate that *WRKY25*, *WRKY26* and *WRKY33* are likely to positively regulate the cooperation between the Hsps and MBF1c pathways that mediate responses to heat stress (Fig. 8a).

It should be noted that the basal expression of *WRKY25*, *WRKY26*, and *WRKY33* was apparently increased in *35S:WRKY25*, *35S:WRKY26*, and *35S:WRKY33* lines, respectively, but was relatively low in the wild-type (Li et al. 2009; Supplementary Fig. S3). However, expression of the putative target genes (*Hsp101*, *Hsp70*, *HsfA2*, *HsfB1*, *MBF1c*, *APX1*, and *Zat10*) was not significantly induced in *WRKY25*, *WRKY26*, or *WRKY33* over-expression lines (Supplementary Figs. S5 and S6; Li et al. 2009). This indicates that *WRKY25*, *WRKY26*, or *WRKY33* alone may not be sufficient to alter the expression of these heat-related genes. Considering that *wrky25wrky33* double mutants displayed reduced expression of heat-inducible genes to a level close to that in *wrky25wrky26wrky33* triple mutants (Fig. 4), while *wrky25wrky26* double mutants exhibited a lower reduction in marker gene expression than both *wrky25wrky33* double mutants and *wrky25wrky26wrky33* triple mutants after HS, particularly after 42°C for 60 min (Fig. 4), it is possible that *WRKY25* and *WRKY33* may be the major genes among *WRKY25*, *WRKY26*, and *WRKY33* in positively regulating these putative target genes.

Positioning of *WRKY25*, *WRKY26*, and *WRKY33* genes in heat stress defense

Arabidopsis thaliana resistance to heat stress depends on ethylene-signaling pathways, as mutations that block ethylene signaling result in enhanced thermosensitivity (Larkindale and Knight 2002; Larkindale et al. 2005; Suzuki et al. 2008). Furthermore, MBF1c has recently been reported to function upstream of ethylene during heat stress in *A. thaliana* (Suzuki et al. 2008). We observed that, after heat treatment, the *ein2* mutants showed significantly reduced expression of *WRKY25*, *WRKY26* and *WRKY33* (Fig. 5b), however, absence or overexpression of *WRKY25*, *WRKY26*, and *WRKY33* did not significantly alter the expression of *EIN2* (Fig. 6b). Moreover, *WRKY25*, *WRKY26*, and *WRKY33* are transcriptionally induced by ACC (Fig. 6a). These observations suggest that *WRKY25*, *WRKY26*, and *WRKY33* may function in thermotolerance downstream of the ethylene signal. Thus, a simplified model depicting the proposed role of WRKY25, WRKY26, and WRKY33 in regulating thermotolerance in *A. thaliana* is represented as MBF1c → ethylene (EIN2) → WRKY25, WRKY26, WRKY33 (Fig. 8a). In addition, our experiments suggested that WRKY25, WRKY26, and WRKY33 can amplify expression of *MBF1c* expression via a positive feedback loop, thereby amplifying the ethylene signal (Figs. 4, 6b, 8a, Supplementary Fig. S4d). Although this study and a previous report (Suzuki et al. 2008) provide data to support a model in which MBF1c and ethylene function upstream of WRKY25, WRKY26, and WRKY33, our findings that the expression of *WRKY25*, *WRKY26*, and *WRKY33* was more or less increased in heat-treated *mbf1c* mutants (Fig. 5a) and ACC was able to partially rescue *wrky25wrky26wrky33* triple mutants from heat stress (Fig. 6c) indicate that this model is not linear. Exogenous ACC might activate EIN2 (Qiao et al. 2009) which, in turn, induces the expression of unknown regulatory genes and heat resistance to compensate for the loss of WRKY25, WRKY26, and WRKY33 under high temperature stress (Fig. 8a).

A recent study reported that 3-week-old *ein2* mutants were less susceptible to heat stress compared with wild-type plants after stress at 38°C for 16 h (Clarke et al. 2009). This observation suggests that the ethylene-mediated response to heat stress may be conditional, which raises the possibility that *WRKY25*, *WRKY26* and *WRKY33* genes may contribute to thermosensitivity under certain conditions. We did not observe a thermotolerant phenotype in the *wrky25-1*, *wrky26-1*, and *wrky33-1* single mutants, which might be attributed to the different heat treatment conditions used in our assays. Further studies are required to elucidate the position of these three genes in Hsps pathway.

Functional interaction among WRKY25, WRKY26, and WRKY33

It is interesting that heat-repressed *WRKY33* acted with heat-induced *WRKY25* and *WRKY26* to positively regulate heat stress resistance in *A. thaliana* and all of these genes were required to activate heat defense gene expression and partly involved in the induction of ethylene-dependent resistance (Figs. 4, 5b, 6). Recent studies reported that the WRKY33 promoter is enriched for W-boxes (Dong et al. 2003) and WRKY33 might be under negative autoregulation as its ortholog parsley *Pc*WRKY1 (Turck et al. 2004; Lippok et al. 2007). Our data further illustrated that the effect of self-inhibition on *WRKY33* had a tendency to increase when heat treatment time was prolonged (Fig. 7b), suggesting that WRKY33 protein content or activity was enhanced by heat stress. We cannot completely exclude a contribution of other unknown factors or WRKY proteins in modulating heat-repressed expression of *WRKY33*. Nevertheless, our data strongly favor the hypothesis that the repressed *WRKY33* expression in heat-treated wild-type plants might result from feedback inhibition by heat-activated WRKY33 protein, which explained the role of WRKY33 as a positive regulator of the heat stress response (Fig. 8b). In addition, *Arabidopsis* MAP kinase 4 (MPK4), a repressor of SA-dependent resistance to pathogen responses, was recently found to interact with an MPK4 substrate (MKS1) that in turn interacts with *Arabidopsis* WRKY25 and WRKY33 (Andreasson et al. 2005). However, WRKY25 and WRKY33 appear to be involved in different defense mechanisms against a variety of stresses (Zheng et al. 2006, 2007; Jiang and Deyholos 2009; Li et al. 2009). Thus, further experiments are now being conducted that are aimed at finding the putative protein interacting with WRKY25, WRKY26, and/or WRKY33 during heat stress in *A. thaliana*, which will help to further establish the role of *WRKY25*, *WRKY26*, and *WRKY33* in mediating the response to heat stress.

As the promoters of *WRKY* genes are highly enriched for W-boxes (Dong et al. 2003), the complex pattern of autoregulation and cross-regulation seems to be a general characteristic of the WRKY family. Several examples of such interactions have been reported in the literature. Co-transfection experiments suggest that *At*WRKY22 and *At*WRKY29 can amplify expression of their own genes via a positive feedback loop (Asai et al. 2002), and *A. thaliana* WRKY11 and WRKY17 positively regulate *WRKY11* expression in a partly redundant manner, whereas WRKY11 negatively regulates *WRKY17* expression (Journot-Catalino et al. 2006). Furthermore, the group III WRKY protein WRKY54 has been shown to influence expression of other group III WRKY genes in response to different pathogens and pathogen-related treatments in a

complex manner (Kalde et al. 2003). Our results that positive cross-regulation of WRKY25, WRKY26, and WRKY33 reflects a synergistic interaction among these three genes during heat stress (Figs. 7a, 8b). In addition, the autoregulation of WRKY33 (Fig. 7b; Turck et al. 2004; Lippok et al. 2007) or cross-regulation by these three WRKY factors in thermotolerance (Fig. 7a) appears to consist of positive and negative control elements possibly allowing for an efficient yet balanced amplification and diversification of heat defense signals (Fig. 8b). Thus, the emerging picture is likely to be that of a highly interconnected network of WRKY transcription factors modulating *WRKY* gene expression and thereby fine-tuning the plant defense system (Journot-Catalino et al. 2006).

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