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Molecules and Cells

Functional Characterization of *Arabidopsis thaliana* WRKY39 in Heat Stress

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Arabidopsis thaliana **WRKY39, a transcription factor that is induced by heat stress, is a member of the group II WRKY proteins and responds to both abiotic and biotic stress. Heat-treated seeds and plants of *WRKY39* knock-down mutants had increased susceptibility to heat stress, showing reduced germination, decreased survival, and elevated electrolyte leakage compared with wild-type plants. In contrast, *WRKY39* over-expressing plants exhibited enhanced thermotolerance compared with wild-type plants. RT-PCR and qRT-PCR analysis of *wrky39* mutants and *WRKY39* over-expressing plants identified putative genes regulated by WRKY39. Consistent with a role for WRKY39 in heat tolerance, the expression levels of salicylic acid (SA)-regulated *PR1* and SA-related *MBF1c* genes were down-regulated in *wrky39* mutants. In contrast, over-expression of *WRKY39* increased the expression of *PR1* and *MBF1c*. The *WRKY39* transcript was induced in response to treatment with SA or methyljasmonate. Analysis of heat stress-induced *WRKY39* in defense signaling mutants, including *coi1*, *ein2*, and *sid2*, further indicated that *WRKY39* was positively co-regulated by the SA and jasmonate (JA) signaling pathways. Together, these findings reveal that heat stress-induced WRKY39 positively regulates the cooperation between the SA- and JA-activated signaling pathways that mediate responses to heat stress.**

INTRODUCTION

Extreme temperature is an adverse environmental stress that severely impairs plant growth and development (Guy, 1999). Plants have a natural capacity to ameliorate the effects of heat shock (HS) (Hong and Vierling, 2000). Previous studies have shown that HS transcription factors (Hsfs) play an important role in thermotolerance in plants and other organisms by regulating HS proteins (Hsps) (Baniwal et al., 2004; Mishra et al., 2002; Panchuk et al., 2002; von Koskull-Döring et al., 2007). However, plants with mutations disrupting abscisic acid (ABA), salicylic acid (SA), hydrogen peroxide, ethylene (ET), or calcium signaling, which appear to accumulate similar Hsp101 and other Hsps to wild-type plants, displayed thermosensitivity (Larkindale and Huang, 2005; Larkindale et al., 2005). Exogenous application of these signaling agents to plants can also result in a degree of enhanced thermotolerance without an accompanying accumulation of Hsps (Larkindale and Knight, 2002). Thus, thermotolerance is affected by a complex network of changes in plants, only one of which is the production of Hsps (Larkindale and Vierling, 2008).

The SA-mediated pathway has been reported to protect potato, mustard, tobacco, tomato, bean, and *Arabidopsis thaliana* from heat stress (Dat et al., 1998; 2000; Larkindale and Knight, 2002; Lopez-Delgado et al., 1998; Senaratna et al., 2000). HS was found to induce SA-regulated pathogenesis-related 1 (*PR1*) transcripts, and the constitutive expression of PR1 protein mutant (*cpr1-5*) exhibited an enhanced thermotolerant phenotype (Clarke et al., 2004; 2009). This indicates that SA and PR1, originally defined as being involved in antipathogenic responses, can also promote heat tolerance in *A. thaliana*. The earlier study revealed that multiple bridging factor 1c (MBF1c) is a key regulator of thermotolerance in *A. thaliana* that functions upstream of SA and PR1 during heat stress. In addition, *MBF1c*-mediated thermotolerance is independent of *Hsps* expression (Suzuki et al., 2008).

Jasmonate (JA) has been implicated in signaling in response to both biotic and abiotic stresses (Balbi and Devoto, 2008; Wasternack, 2006; 2007). Wounding and pathogen infection, or exposure to ozone, cause endogenous JA accumulation (Howe, 2004; Kanna et al., 2003; Rao et al., 2000; Vijayan, et al., 1998; Wasternack, 2006). Heat stress also induces the JA signal transduction pathway (Clarke et al., 2009). The relationship between JA- and SA- signaling has often been shown to be antagonistic. In *A. thaliana*, pathogen-induced SA accumulation is associated with the suppression of JA signaling (Spoel et al., 2003). In contrast, a recent finding has demonstrated that JA acts with SA to confer thermotolerance in *A. thaliana,* and that Hsps apparently play no role in JA-conferred thermotolerance in this species (Clarke et al., 2009).

The WRKY transcription factor superfamily has been suggested to have a key role in biotic and abiotic stress (Eulgem and Somssich, 2007; Miller et al., 2008). The *A. thaliana* WRKY superfamily consists of over 74 members, and is sub-divided into three groups on the basis of the number of WRKY (WRKYGQK) domains and the features of their zinc finger-like motif (Eulgem et al., 2000). Earlier reports have demonstrated

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that a number of *WRKY* genes are involved in the response to diverse sources of biotic stress (Dong et al., 2003; Eulgem and Somssich, 2007; Yu et al., 2001) and senescence (Gadjev et al., 2006; Jing et al., 2009). There is increasing evidence that several *WRKY* genes are involved in the response to various abiotic stresses, including heat (Miller et al., 2008). A WRKY transcription factor in tobacco (*Nicotiana tabacum* L.) has been found to respond to a combination of drought and HS (Rizhsky et al., 2002). Our previous studies have shown that *A. thaliana* WRKY25 is a positive regulator in thermotolerance (Li et al., 2009). The expression of *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 knock-out mutants has revealed that nine of 60 analyzed *WRKY* genes are regulated by HS, and among these nine, *WRKY7* is a HsfA1a/1b-dependent HS gene (Busch et al., 2005). Although several *WRKY* genes in *A. thaliana* are known to be influenced by HS, the roles of WRKY genes in thermotolerance during heat stress have been rarely reported.

WRKY39, a group II WRKY protein, is composed of 330 amino acids. Pathogen infection and SA can activate the expression of *WRKY39* (Dong et al., 2003). Through microarray analysis, we found that WRKY39 was one of the most abundant *WRKY* transcripts in heat-treated wild-type plants. After 1 h heat stress at 42°C, the transcript levels of *WRKY39* increased by an average of 5.3-fold over non-treatment controls, and WRKY25 was up-regulated 4.9-fold (data not shown). Earlier findings that showed WRKY39 is a calmodulin (CaM)-binding transcript factor with a protein structure close to WRKY7 (Park et al., 2005) allowed us to explore its thermotolerance function in *A. thaliana*. In the present study, we characterized the positive role of the *A. thaliana* WRKY39 transcription factor in plant responses to high temperature, by analyzing its potential upstream and downstream targets, and its loss-of-function and over-expression phenotypes. We suggest that WRKY39 regulates the cooperation between the SA- and JA-activated signaling pathways that mediate responses to heat stress.

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. The mutant lines *coi1*, *ein2*, and *sid2* were from Prof. Zhixiang Chen (Purdue University, USA). T-DNA insertion mutants, including *wrky39* (SALK_073483c), *hot1* (SALK_036423c), and *mbf1c* (SALK_083813c), were obtained from 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. Procedures for growing plants were as previously described (Fu et al., 2009; Li et al., 2009).

Generation of transgenic lines and plant transformation

The *WRKY39* full cDNA linked to a vector pUNI was obtained from ABRC. To generate the *35S:WRKY39* construct, the cDNA was subcloned by ligating the SacI-SalI fragment into the SacI and SalI sites of pOCA30 (Chen and Chen, 2002) behind the *CaMV 35S* promoter. For *promoter-β-glucuronidase (GUS)* fusion, the promoter region of *WRKY39* gene was amplified by PCR from wild-type genomic DNA using the primers 5′-TTTGAGCTC-AACAATCGTGATTCGTGAT-3′ and 5′-TTTCCATGGTTTTTC-TTCTACCCAAGTTTCAGA-3′ (underlined sections are SacI and NcoI sites, respectively). The PCR product was restricted and inserted into pSJ131 upstream of the *GUS* gene. Then, a BamHI-SacI fragment of pSJ131 containing the promoter of *WRKY39*, the *GUS* gene, and poly(A) signal sequences was inserted into the BamHI and SacI sites of pOCA28 (Du and Chen, 2000). The recombinant plasmids were introduced into *Agrobacterium tumefaciens* GV3101 and used to transform *A. thaliana* by the floral dip method (Clough and Bent, 1998). Transformed lines were selected for resistance to kanamycin (50 μg/ml). PCR and Northern blot analyses were performed to select the transgenic plants. Homozygous T_3 lines were obtained for phenotypic scoring under heat stress or by GUS staining.

Histochemical GUS staining

Plants were placed into staining solution containing 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (first dissolved in dimethyl formamide at 5 mg ml^{-1}) in 50 mM sodium-phosphate buffer, pH 7.0, 0.5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, and 0.1% Triton X-100. The samples were then placed under vacuum three times for 1 min each time for infiltration and incubated at 37°C overnight (Weigel and Glazebrook, 2002). Chlorophyll was removed by dipping in 75% ethanol.

Heat stress treatments

To examine the heat stress test response in seed germination, 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 allowed to germinate in a growth cabinet at 22°C. Germination was recorded daily until no further germination was observed. To assess heat tolerance, seeds were geminated on 1/2 MS medium. After 7 days, seedlings were transplanted into soil pots. Twenty-five-day-old plants grown in soil were exposed to heat stress at 48°C for 6 h, returned to 22°C for 9 days, and then photographed. The 30-day assay was done by placing leaves from the same whorl of each 30-day-old plant into a 24-well plate, with 2.5 ml of water in each well. The leaves were then heat-treated at 45°C for 4 h in a waterbath and left to recover at 22°C for 4 days. In all cases, the heat treatment was given in the dark, and the results for mutant and transgenic plants were compared with the wild-type plants on the same plate.

Electrolyte leakage measurements

Measurement of leaf electrolyte leakage (EL) was carried out essentially as described by Clarke et al. (2004). For experiments where EL was measured during heat treatment at 42°C, the aerial parts of 3-week-old plants were placed into glass tubes containing 5 ml of 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 of fresh weight.

Chemical treatment of plants

SA was dissolved in water as a 100 mM stock solution and adjusted to pH 6.5 with KOH. Methyljasmonate (MeJA) was dissolved in 50% ethanol as a 10 mM stock solution and 1-aminocyclopropane-1-carboxylic acid (ACC) was dissolved in water as a 10 mM stock solution. Aerial parts of 3-week-old *A. thaliana* soil-grown plants were incubated with 1 mM SA, 100 μM MeJA, and 100 μM ACC solution diluted from the stock.

RNA extraction, cDNA synthesis, and semiquantitative RT-PCR analysis

RNA was isolated from aerial parts of *A. thaliana* plants by standard protocols (Sambrook et al., 2001). The first-strand cDNA was synthesized from 2 μg of DNase-treated RNA using

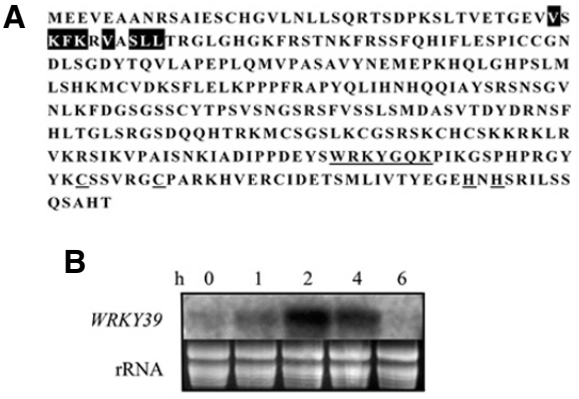

Fig. 1. Sequence of WRKY39 protein and accumulation of *WRKY39* transcripts during heat stress in *A. thaliana*. (A) Amino acid sequence of WRKY39. One WRKY motif is indicated with the highly conserved WRKYGQK sequence and the residues forming the C_2H_2 zinc finger are underlined. The deduced amino acid sequence of the C-region (conserved primary motif in WRKY group IId members, CaM-binding domain) of *A. thaliana* WRKY39 is shown in white on a black background. (B) Northern blot of *WRKY39* accumulation at 42°C for 0, 1, 2, 4, and 6 h in wild-type plants. The full length *WRKY39* cDNA was used as a probe, and ribosomal 18S RNA was used as the control for RNA loading.

M-MuLV reverse transcriptase (Fermentas, EU) with oligo$(dT)_{18}$ primer, and 1 μl of the cDNA was used in the subsequent PCR. The following gene-specific primers were used: *WRKY39* (At3g04670) 5′-TGACTCTGTATCATCAAGTGTGA-3′ and 5′-AGTATTCTTGGAGGAAGTATGGA-3′; *PR1* (At2g14610): 5′-TCTTCCCTCGAAAGCTCAAG-3′ and 5′-ACACCTCACTTTGGCACATC-3′; *MBF1c* (At3g24500): 5′-TCTCAATTCATCGACGATGC-3′ and 5′-CGACATCGTTTAATCATTTCCC-3′; *Hsp101* (At1g74310): 5′-GATTTGTTGAACGAAGTCGGTGT-3′ and 5′-ACCAGTTATGTAACGAGCAGACAGC-3′; *APX1* (At1g07890): 5′-AGAGCTTAGCTAAGATGACGAAGAA-3′ and 5′-TGATGGAAATCAGCAAAAGAGA-3′; *Zat10* (At1g27730): 5′-TTAACTTAATGGCGCTCGAG-3′ and 5′-ACAACTCTCAACAGTATACAAACGAA-3′; *Hsp70* (At3g12580): 5′-AGGTTATCAAAGGAAGAGATCGA-3′ and 5′-AGTAAACAAAAGCCAAAAGGCT-3′; *Actin2* (At3g18780): 5′-TGTGCCAATCTACGAGGGTTT-3′ and 5′-TTTCCCGCTCTGCTGTTGT-3′. The RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, EU) was used according to the manufacturer's protocol.

Primer design and quantitative RT-PCR (qRT-PCR)

Primers for qRT-PCR were designed and synthesized by TaKaRa. qRT-PCR was performed on the Roche LightCycler 480 machine using home-made 2 × SYBR Green I master mix as per manufacturer's instructions. Each 20 μl reaction contained 1 μl of cDNA and 10 μM of each gene-specific primer. *Actin2* (At3g18780) was used as the reference gene internal control for comparison of the target gene transcripts amplified per cDNA sample. The primer sequences of *Actin2* were as described above. The following primers were used for target gene amplification: *WRKY39* (At3g04670): 5′-TGCGGAAGTCGAAGCAAATGTCA-3′ and 5′-CGTGGATGCGGTGAACCCTTTAT-3′; *PR1* (At2g14610): 5′-AACTACAACTACGCTGCGAACA-3′ and 5′-GTTACACCTCACTTTGGCACAT-3′; *MBF1c* (At3g24500): 5′-ATGCCGAGCAGATACCCAGGAGC-3′ and 5′-TTAACCGTTTGAACCGCGACACC-3′. The LightCycler Software 480 (Roche Diagnostics) relative quantification analysis module was used to determine the relative fold expression changes between samples. All PCRs were performed under the following conditions: 4 min at 95°C followed by 50 cycles of 30 s at 95°C, 30 s at 58°C, and 20 s at 72°C, in LightCycler capillaries (Roche Diagnostics). The specificity of amplicons was verified by melting curve analysis (60-95°C) after 50 cycles. At least three biological replicates for each sample were used for qRT-PCR analysis and at least two technical replicates were analyzed for each biological replicate.

Northern blot analysis

[α-^{32}P] dATP (> 3000 Ci/mmol) was obtained from Beijing Fu-Rui Biological Technology Company. Total RNA (10 or 20 μg) was separated on agarose-formaldehyde gels and transferred onto nylon membranes, which were hybridized and washed following standard procedures (Sambrook et al., 2001). Transcripts for *WRKY39* were detected using full-length *WRKY39* cDNA as a probe, which was labeled by [α-^{32}P] dATP using the TaKaRa Random Primers DNA Labeling System.

RESULTS

***WRKY39* is induced by heat stress**

A. thaliana WRKY39 (At3g04670) encodes a protein of 330 amino acids with a molecular weight of 36.718 kD and an isoelectric point of 9.78 (Fig. 1A). Based on the presence of one WRKY domain (Fig. 1A) and additional short conserved structural motifs (Eulgem et al., 2000), WRKY39 is classified as a group IId WRKY protein. An earlier study has shown that WRKY39 is a calmodulin-binding protein (Fig. 1A; Park et al., 2005).

According to microarray experiments, the *WRKY39* gene is expressed at low levels in healthy, non-stressed wild-type plants. However, after 1 h heat stress at 42°C, the transcript levels of *WRKY39* increased by an average of 5.3-fold over non-treatment controls. To confirm the microarray results, we examined the transcript levels of *WRKY39* in heat-treated plants using RNA blots. The transcript levels of *WRKY39* increased rapidly and strongly within 1-2 h after heat treatment, and then returned to basal levels within 6 h (Fig. 1B). This expression pattern shows that HS might play a role in significantly and transiently up-regulating transcription factor activity toward the *WRKY39* promoter, indicating that WRKY39 is involved in responses to heat stress.

Promoter-*GUS* reporter fusion

To test the ability of the putative promoter region from *WRKY39* to direct heat stress-responsive *WRKY39* expression, we fused a genomic fragment from 1.5 kb upstream of the translational start codon to a *GUS* reporter gene.

Five independent T_3 transgenic *A. thaliana* lines carrying the *WRKY39* promoter-*GUS* fusion were examined, and representative consensus expression patterns are described in Fig. 2. In non-stressed plants harboring the P_{wrky39}:*GUS* fusion, we detected moderate expression in the roots and veins of cotyledons of 3-d seedlings (Fig. 2A), and in the veins of fresh leaves and roots in both 7-day seedlings and 21-day plants (Figs. 2B and 2C). Within the inflorescences, moderate to strong GUS expression was observed in stamens, petals, stems, and tips and nodes of siliques under normal growth conditions (Fig. 2D).

Having established the basal pattern of GUS expression for the P_{wrky39}:*GUS* fusion construct, we next examined GUS expression in 21-day plants heat-treated at 42°C for 4 h, or in the presence of SA, MeJA, or ACC, the immediate precursor of ethylene), compared with non-stressed or mock-treated controls

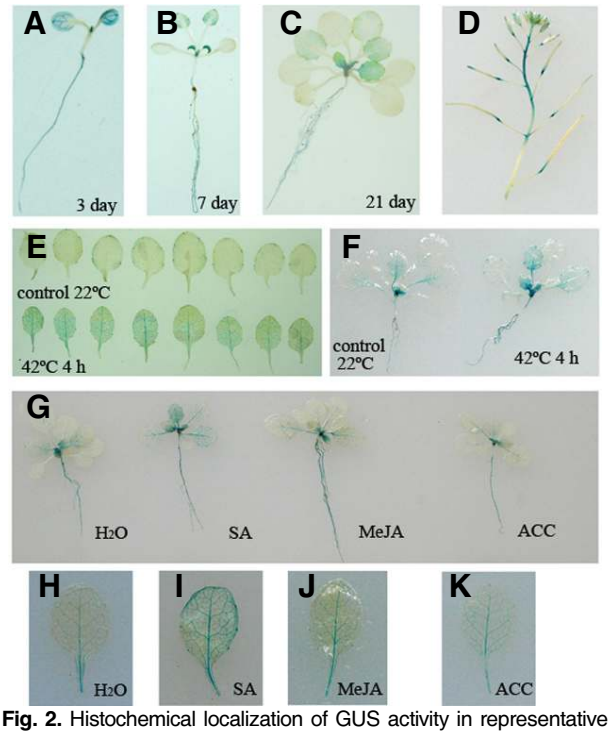

Fig. 2. Histochemical localization of GUS activity in representative T_3 transgenic *A. thaliana* plants harboring *WRKY39* promoter-*GUS* constructs. (A-D) *WRKY39-GUS* expression in non-stressed, representative transgenic plants. (A) 3-day-old seedlings; (B) 7-day-old seedlings; (C) 21-day-old plants; (D) inflorescence and siliques. (E-F) *WRKY39-GUS* expression during heat stress. (E) 21-day-old *WRKY39-GUS* plants were incubated at 42°C for 0 or 4 h, and leaves were stained; (F) 21-day-old *WRKY39-GUS* plants were incubated at 42°C for 0 or 4 h, and whole plants were stained. (G) GUS staining patterns in 21-day-old *WRKY39-GUS* plants treated with H_2O, 1 mM SA, 0.1 mM MeJA, or 0.l mM ACC for 4 h. (H–K) GUS staining pattern in 21-day-old leaves of *WRKY39-GUS* plants with different treatments for 4 h: (H) H_2O; (I) 1 mM SA; (J) 0.1 mM MeJA; (K) 0.1 mM ACC.

(Figs. 2E-2K). Although a basal level of GUS expression was detected throughout the plant, an increase in GUS expression was detected following 4 h of heat stress (Figs. 2E and 2F). We also observed an apparent increase in GUS staining intensity in leaf veins of P_{wrky39}:*GUS* lines treated with SA, and a slight increase in lines treated with MeJA. Water and ACC treatment did not induce GUS expression (Figs. 2G-2K).

Identification of *wrky39* knock-down mutants and generation of *WRKY39*-overexpressed transgenic *A. thaliana* plants

To analyze the role of WRKY39 in thermotolerance, we identified one T-DNA knock-down mutant for *WRKY39*. Homozygous *wrky39* (SALK_073483c) mutants contain a T-DNA insertion at the beginning of the 5′ untranslated region, 596 nucleotides upstream of the start codon (Fig. 3A). We performed RT-PCR to compare wild-type plants and *wrky39* mutants for induced accumulation of *WRKY39* transcripts during heat stress at 42°C. *WRKY39* transcripts of the expected induction level were observed in wild-type plants after heat treatment. In contrast, the basal level of *WRKY39* transcripts was apparently reduced, and heat stress did not induce *WRKY39* expression in *wrky39* mutants (Fig. 3B). When grown under normal conditions, both seedling and adult *wrky39* mutants showed no morphological or developmental difference compared with wild-type plants (data not shown).

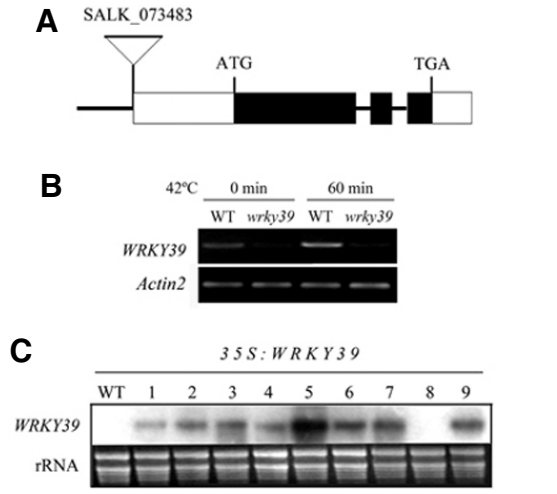

Fig. 3. Characterization of *wrky39* T-DNA knock-down mutants and *WRKY39* over-expressing plants. (A) Diagram of the *WRKY39* gene and its T-DNA insertion mutant. (B) RT-PCR analysis of the *wrky39* mutant line. Leaves from *wrky39* mutant and wild-type plant were harvested before heat stress or after treatment at 42°C for 60 min. Total RNA was isolated and *Actin2* amplification was used as constitutive control. (C) Northern blot analysis of *WRKY39* in wild-type and transgenic plants. Each lane was loaded with 10 μg of RNA prepared from 3-week-old plants. Ribosomal 18S RNA was used as the control for RNA loading.

To further investigate the function of WRKY39, we constructed and analyzed transgenic *A. thaliana* plants that constitutively over-express *WRKY39*. The full-length *WRKY39* cDNA was cloned behind the *CaMV 35S* promoter and used to transform *A. thaliana* plants. Northern blot analysis identified several transgenic plants that contained elevated levels of *WRKY39* transcript in the absence of heat stress (Fig. 3C). Two transgenic lines (lines 5 and 6 in Fig. 3C) were selected for further study. Line 5 showed a markedly higher level of expression of *WRKY39* compared with the wild-type, and line 6 also showed a higher level of expression than the wild-type (Fig. 3C). Analysis of T_3 homozygous plants from both lines revealed no differences in growth or development from that of wild-type plants (data not shown).

The *wrky39* mutants and over-expression of *WRKY39* result in altered responses to heat stress

We first examined the germination of *wrky39* and transgenic *35S:WRKY39* seeds. Seeds were exposed to a temperature of 45°C for 4 h after vernalization for three days at 4°C and then returned to 22°C for recovery. Radicle emergence was scored daily until no further germination of *35S:WRKY39* was observed. At 3 days after heat treatment, *35S:WRKY39* lines already showed 2.5-4-fold increase in germination than wild-type seeds, whereas germination of *wrky39* seeds was nearly one-half that of the wild-type (Fig. 4). By 5 days after heat treatment, *35S: WRKY39* seeds exhibited 84-95% germination, while wild-type and *wrky39* showed approximately 63% and 47% germination, respectively (Fig. 4). Although some of the *wrky39* seeds were finally able to germinate, wild-type seeds displayed higher germination over time (Fig. 4; wild-type and *wrky39*, $P < 0.05$). *35S:WRKY39* seeds showed tolerance to

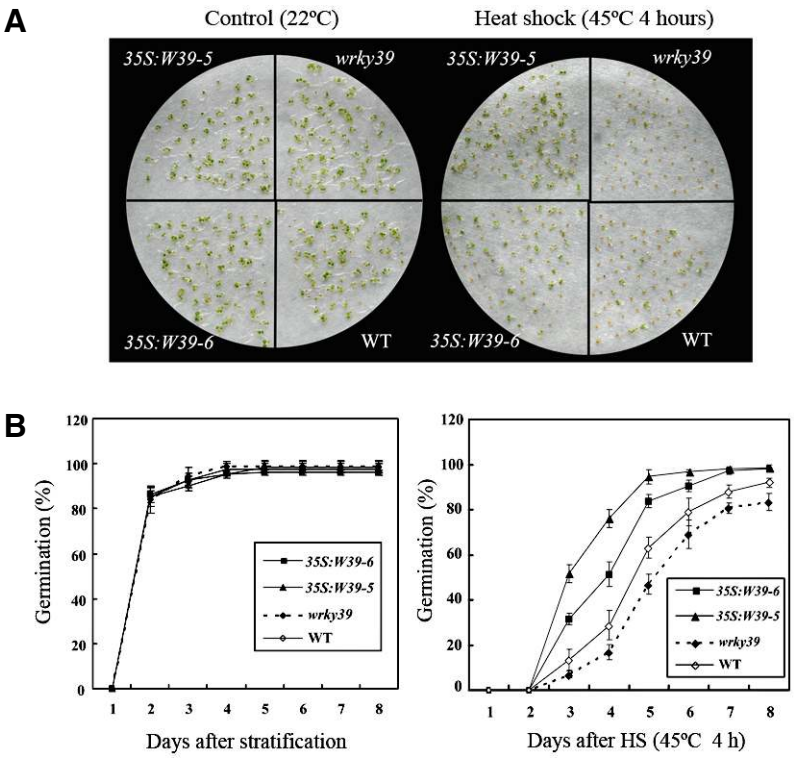

Fig. 4. Response of the *wrky39* mutants and *35S:WRKY39* lines to heat stress during seed germination. Wild-type, *wrky39*, *35S:WRKY39-5*, and *35S:WRKY39-6* seeds were sown on water-saturated filter paper. Seeds were treated at 45°C for 4 h or incubated at 22°C (control) immediately after removal from 4°C (3 days in the dark), and then maintained at 22°C. Germination was assessed at the indicated intervals. (A) Illustration of representative seeds/seedlings 4 days after heat treatment. (B) The percentage of radicle emergence was recorded daily until no further germination of *35S:WRKY39* was observed. The data are shown as mean ± SD (n = 4). Each replicate consisted of 50-70 seeds. The Mann-Whitney U-test was used for data analysis.

HS (Fig. 4; wild-type and *35S:W39-5*, $P < 0.01$; wild-type and *35S:W39-6*, $P < 0.05$). Moreover, *35S:W39-5* caused greater seed germination than *35S:W39-6* (Fig. 4; *35S:W39-5* and *35S:W39-6*, $P < 0.05$). Heat treatment did not significantly alter seedling viability after ger-mination (data not shown), but did impair development (Fig. 4).

We also used 25-day-old plants to compare the degree of thermotolerance among *wrky39* mutants, wild-type plants, and *35S:WRKY39* lines. Plants were directly subjected to heat stress (48°C) for 6 h, and then returned to 22°C for recovery. Nine days later, surviving plants could be clearly identified by the presence of newly emerging leaves, while non-surviving plants exhibited complete etiolation, loss of turgor, and arrested leaf formation (Fig. 5A). The degree of thermotolerance correlated with the level of *WRKY39* expression, in which the *35S:W39-5* and *35S:W39-6* plants showed higher levels of expression and greater thermotolerance than *wrky39* mutant and wild-type plants (Fig. 5A).

Thermosensitivity of *wrky39* mutants and enhanced thermotolerance of *35S:WRKY39* lines were also observed in a 30-d assay (Fig. 5B). Leaves of the same whorl were removed from 30-d plants and heated to 45°C for 4 h in a waterbath, and then left to recover at 22°C for 4 days. Figure 5B illustrates that reduction of leaf chlorophyll content by heat stress was aggravated significantly in the *wrky39* mutants in comparison to the wild-type and *35S:WRKY39* plants.

We extended our assay to measure EL in the *wrky39* mutants, wild-type plants, and *35S:WRKY39* lines. EL represents a rapid, sensitive and quantitative method to assess the effects of stress on plant cells (Clarke et al., 2004; 2009; Hong et al., 2003; Howarth et al., 1997). Significantly less EL was detected for *35S:WRKY39* compared with wild-type during heat stress after 3 h (Fig. 5C; wild-type and *35S:W39-5*, $P < 0.01$; wild-type and *35S:W39-6*, $P < 0.05$). In addition, heat treatment of *wrky39* at 42°C for 4 h resulted in nearly 1.3-fold greater EL than in the wild-type (Fig. 5C).

On the basis of the assays described above, constitutive over-expression of *WRKY39* in *A. thaliana* enhances thermotolerance, while reduced expression of *WRKY39* leads to thermosensitivity, indicating that this gene has a positive role in thermotolerance.

Expression of heat stress defense genes in *wrky39* mutants and *WRKY39* over-expressing plants

The expression of a number of heat stress-related genes was analyzed in *wrky39* mutants, wild-type plants, and *WRKY39* over-expressing lines (lines 5 and 6) to further confirm the role of WRKY39 during heat stress. The expression patterns of well-characterized heat stress-responsive marker genes were analyzed by semi-quantitative RT-PCR or qRT-PCR. Total RNA of *wrky39*, wild-type, and *35S:WRKY39* were isolated after heating at 42°C for 0, 30, 60, or 120 min.

A. thaliana Hsp70 and *Hsp101* are well-known HS-induced genes that play important roles in thermotolerance (Larkindale and Huang, 2005; Larkindale et al., 2005). *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). In our study we found that *Hsp70*, *Hsp101*, and *APX1* expression patterns were similar in *wrky39* mutants, wild-type plants, and *35S:WRKY39* lines after HS treatment at 42°C (Fig. 6A). Although the *35S:W39-5* line had higher levels of *Zat10* transcript than wild-type plants after heat treatment, the expression of *Zat10* was not significantly different in *wrky39* mutants, wild-type plants, or the *35S:W39-6* line (Fig. 6A).

Recent studies have shown that SA-mediated pathway promotes thermotolerance in plants (Clarke et al., 2004; 2009; Dat et al., 1998; 2000; Larkindale and Knight, 2002; Lopez-Delgado et al., 1998; Senaratna et al., 2000). To determine whether the altered responses of the *wrky39* mutants and the transgenic

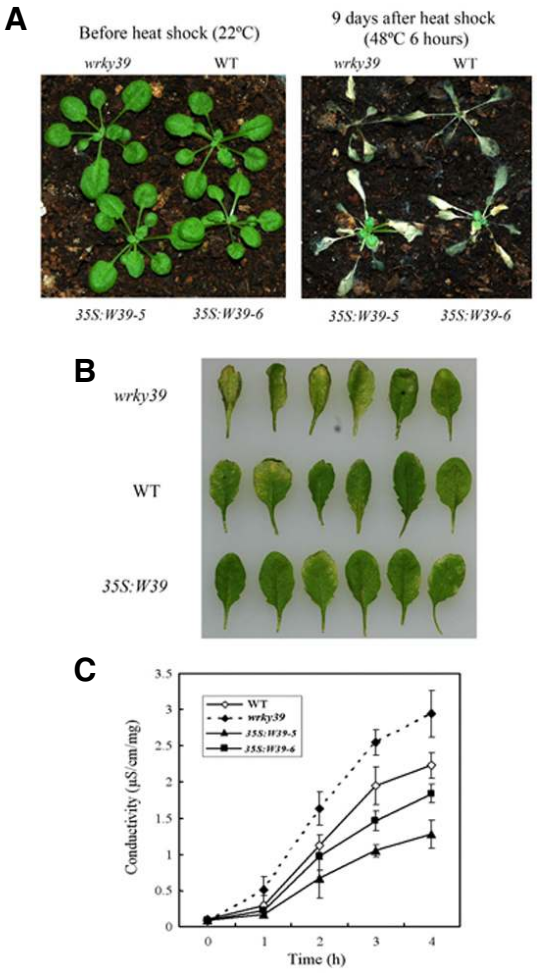

Fig. 5. Altered responses of *wrky39* mutants and *35S:WRKY39* plants to heat stress. (A) 25-day-old plants of the wild-type, *wrky39* mutant, *35S:WRKY39-5*, and *35S:WRKY39-6* were treated at 48°C for 6 h. Photographs were taken before heat treatment or after a 9 days recovery period at 22°C. The experiments were repeated four times with similar results and a representative result is shown. (B) Photograph of leaves removed from 30-day-old *wrky39* mutant, wild-type, *35S:WRKY39-5*, and *35S:WRKY39-6* plants, heated to 45°C for 4 h in a waterbath and left to recover for 4 days. The experiments were repeated four times with similar results and a representative result is shown. (C) The assay of EL measurement was done with 21-day-old seedlings grown in soil. The aerial parts of *wrky39* mutant, wild-type, *35S:WRKY39-5*, and *35S:WRKY39-6* 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 shown as means from three experiments, each with five plants per treatment. Bars are presented as SD ($n = 15$). The Mann-Whitney *U*-test was used for data analysis.

35S:WRKY39 plants to heat stress are related to altered SA-mediated mechanisms, we examined the expression of two SA-related genes after heat treatment. *A. thaliana MBF1c* and *PR1* are heat-inducible genes associated with the SA pathway in thermotolerance; *MBF1c* is also required for thermotolerance independent of Hsfs and Hsps, and functions upstream of SA and *PR1* (Suzuki et al., 2008). Figure 6 shows that the expression of *PR1* and *MBF1c* was clearly induced in wild-type plants subjected to heat stress, in agreement with previous reports (Clarke et al., 2004; 2009; Suzuki et al., 2008). The expression of *PR1* and *MBF1c* was also induced by heat stress in *wrky39* mutants, with similar kinetics to that observed in wild-type plants. However, the fold-induction of *PR1* and *MBF1c* expression in *wrky39* mutants was apparently lower than in wild-type plants at each time-point after being transferred to 42°C, particularly at 60 min. At this point, the expression of *PR1* and *MBF1c* in *wrky39* mutants was nearly 5- and 1.25-fold reduced, respectively, compared with wild-type plants (Fig. 6). Consistent with the thermotolerant phenotype, we consistently observed an increase in the inducible expression of *PR1* and *MBF1c* in transgenic *35S:WRKY39* plants relative to wild-type plants (Fig. 6). In non-stressed plants, *wrky39* mutants showed 2.8-fold lower *PR1* expression than wild-type plants, and the expression of *PR1* in *35S:WRKY39* lines was moderately increased compared with wild-type plants (Figs. 6A and 6B). No differences in *MBF1c* expression were observed among unstressed mutant, wild-type, and over-expressing plants, indicating that *WRKY39* alone is not sufficient for altered *MBF1c* expression (Figs. 6A and 6C).

Taken together, these results indicate that *WRKY39* is a positive regulator of these SA-related heat stress defense genes. The expression of *Hsp70*, *Hsp101*, *APX1*, and *Zat10* was the same in *wrky39* mutants, wild-type plants, and *35S:WRKY39* lines, suggesting that *WRKY39*-mediated thermotolerance is independent of *Hsps* and heat-induced oxidative stress pathways.

Expression of *WRKY39*

To further investigate whether *WRKY39* expression is influenced by altered expressions of *Hsp101* and *MBF1c* during heat stress, *WRKY39* expression was checked in *hot1* (SALK_036423c) and *mbf1c* (SALK_083813c) mutants. Figures 7A and 7D show that in wild-type plants, *hot1*, and *mbf1c* mutants, the expression of *WRKY39* was induced by heat stress, reaching peak expression after 60 min. At the same time, basal *WRKY39* expression was slightly reduced and induced *WRKY39* expression was modestly increased in *hot1* and *mbf1c* mutants compared with wild-type plants (Figs. 7A and 7D). It appears that moderately increased expression of *WRKY39* in *hot1* and *mbf1c* mutants compensates for the absence of Hsp101 and MBF1c during heat stress.

We also analyzed *WRKY39* induction in wild-type plants treated with SA, ACC, or MeJA. The *WRKY39* gene was induced rapidly and strongly by SA (Figs. 7B and 7E), consistent with a previous report by Dong et al. (2003). The transcript levels of *WRKY39* increased rapidly but modestly within 1–8 h after treatment with MeJA, and returned to basal levels within 24 h (Figs. 7B and 7E). ACC treatment did not induce *WRKY39* expression (Figs. 7B and 7E). SA was by far the most potent inducer of *WRKY39* transcript accumulation among the defense signal molecules we tested (Figs. 7B and 7E). These results agree with the GUS expression patterns (Figs. 2G-2K).

To determine which signaling pathways are involved in the heat-induced expression of *WRKY39*, its expression was studied in a set of mutants that are defective in various defense response pathways including JA signaling (*coi1*), ET response (*ein2*), and SA biosynthesis (*sid2*). All of these mutants exhibit thermosensitivity (Larkindale et al., 2005). During heat stress, *WRKY39* expression was reduced in all of these mutants compared with the wild-type (Figs. 7C and 7F). *WRKY39* expression was lower in *coi1* and *sid2* mutants than in *ein2* mutants, indicating that *COI1* and *SID2*, which are required for JA signaling and SA biosynthesis, respectively, induce expression of the *WRKY39* gene during heat stress.

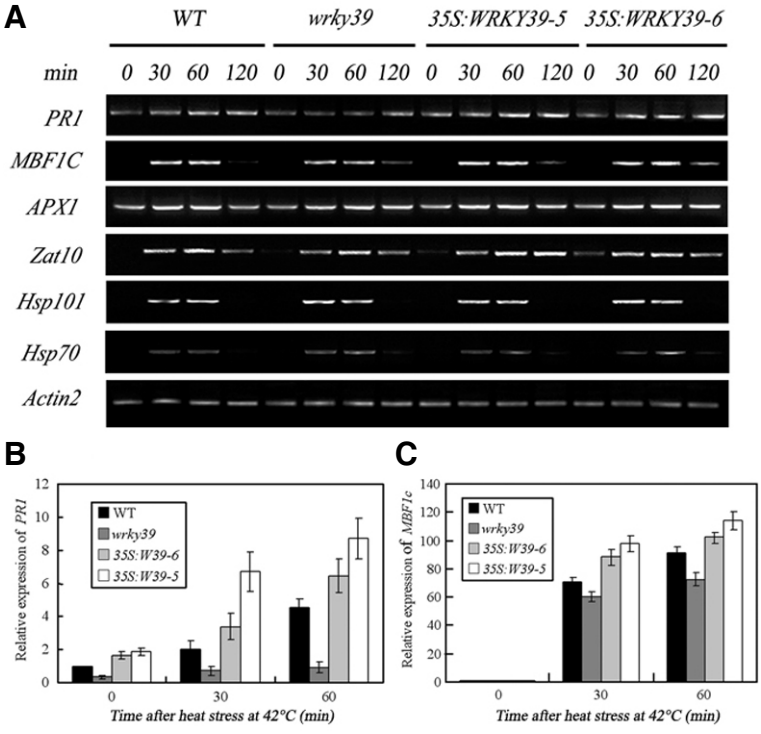

Fig. 6. Expression of heat-inducible and oxidative stress-responsive genes in *wrky39* mutants and *35S:WRKY39* plants. (A) Semi-quantitative RT-PCR analysis of *wrky39* mutant, wild-type, and *35S:WRKY39* plants. Total RNA was isolated from 21-day-old plants exposed to 42°C for 0, 30, 60, and 120 min. *Actin2* amplification was used as a constitutive control. RT-PCR was repeated three times using RNA extracted from three independent experiments and similar results were obtained. (B) qRT-PCR analysis of *PR1* expression. (C) qRT-PCR analysis of *MBF1c* expression. In (B) and (C), the expression of target genes (*PR1* and *MBF1c*) was detected in 21-day-old *wrky39* mutant, wild-type, and *35S:WRKY39* plants treated at 42°C for 0, 30, or 60 min. *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.

DISCUSSION

Here, we analyzed the roles of the *A. thaliana WRKY39* gene in response to heat stress, extending observations made in our previous study (Li et al., 2009). Evidence that the heat stress-induced *WRKY39* gene (Fig. 1) functions as a positive regulator in thermotolerance comes from analysis of transgenic over-expression lines and a T-DNA insertion mutant. The *wrky39* knock-down mutants exhibited enhanced thermosensitivity by reduced germination, decreased survival, and enhanced EL in heat-treated seeds or plants (Figs. 4 and 5). Transgenic plants constitutively expressing *WRKY39*, on the other hand, were more resistant to high temperature stress than wild-type plants (Figs. 4 and 5). Thus, mutations and constitutive *WRKY39* over-expressing lines had contrasting effects in response to heat stress. In addition, we observed that the enhanced thermosensitivity of *wrky39* mutants was associated with reduced expression of *PR1* and *MBF1c* (Fig. 6), two heat-related genes often associated with SA-mediated pathways (Suzuki et al., 2008). *PR1* and *MBF1c* were expressed at relatively higher levels in *WRKY39* over-expressing plants than in wild-type plants after heat treatment (Fig. 6). *A. thaliana* mutants *sid2* and *npr1*, defective in SA biosynthesis or signaling, display reduced heat tolerance (Clarke et al., 2004; 2009), indicating that SA-mediated signaling plays a vital role in thermotolerance in plants. Our data suggest that *WRKY39* might play a positive role in SA-mediated signaling pathways, and that over-expression of the gene could have a positive impact on SA-mediated heat stress defense mechanisms, increasing plant resistance to high temperatures. Moreover, because the expression of *PR1* was reduced more than that of *MBF1c* in *wrky39* mutants (Fig. 6) and a recently reported model (Suzuki et al., 2008), we speculate that *WRKY39* is likely to act upstream of *PR1* and downstream of *MBF1c*.

It should be noted that the basal expression of *WRKY39* was apparently increased in *35S:WRKY39* lines, but was very low in wild-type (Fig. 3C). However, expression of the putative target gene (*MBF1c*) was not significantly induced in *WRKY39* over-expression lines, although it was slightly increased compared with the wild-type (Fig. 6). This indicates that *WRKY39* alone may not be sufficient to alter the expression of *MBF1c*. Meanwhile, as a member of a large gene family, *WRKY39* may have functional overlap with other *WRKY* genes, as explained by the failure to observe stronger phenotypes in *wrky39* mutants during heat stress (Figs. 4 and 5). Indeed, phylogenetic analysis based on WRKY protein structures has revealed that WRKY39 and several other WRKY proteins, including WRKY74 and WRKY21, share very similar protein structures (Dong et al., 2003). Therefore, the expression of *WRKY74* and *WRKY21* might compensate for the absent function of WRKY39. Isolation of the loss-of-function mutants for these similar WRKY proteins and construction of composite mutants for multiple *WRKY* genes should reveal whether these structurally related WRKY proteins perform overlapping functions in thermotolerance (Zheng et al., 2006).

Using the P_{wrky39}:*GUS* fusion, we detected inducibility of GUS expression by heat (42°C), SA, and MeJA. The GUS expression patterns were generally consistent with the mRNA accumulation patterns (Figs. 2, 7B, and 7E). This suggests that regulatory elements exist within the promoter regions examined in our study, and that the sensitivity of the reporter system is sufficient to detect moderate changes in various treatments.

The proposed role of *WRKY39* as a positive regulator of SA-dependent heat stress defense pathways can also be deduced from the rapid and strong induction of *WRKY39* expression by SA (Figs. 7B, 7E, and 2G; Dong et al., 2003). Comparisons between the three established defense signal molecules showed that only SA induced *WRKY39* at high levels, and that JA or ET did not (Figs. 7B, 7E, and 2G). Moreover, SA was required for heat-induced *WRKY39* expression, as shown by the significantly reduced induction of *WRKY39* in *sid2* mutant plants (Figs. 7C and 7F). As *WRKY39* expression was also

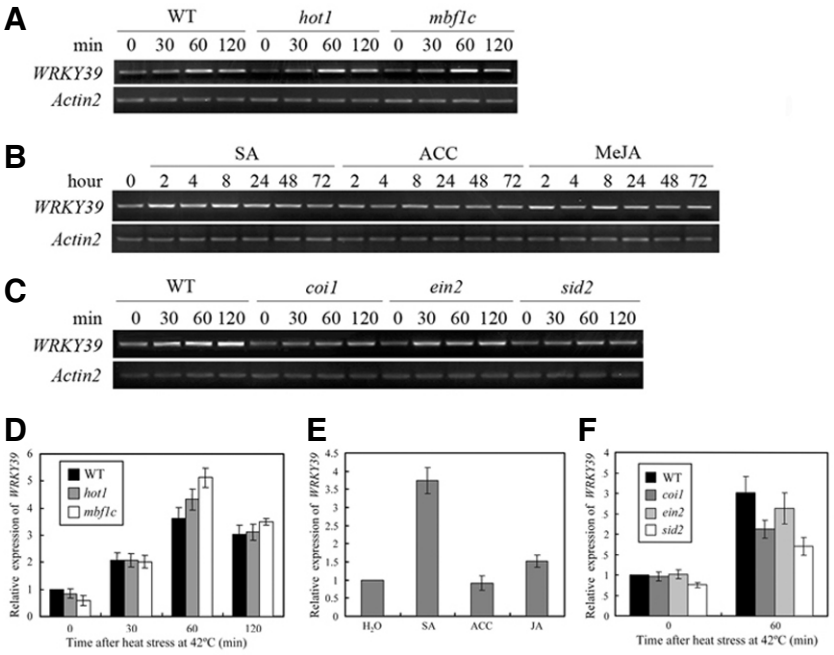

Fig. 7. Expression of *WRKY39*. (A) RT-PCR analysis of *WRKY39* expression in wild-type, *hot1*, and *mbf1c* mutants during heat treatment at 42°C for 0, 30, 60, and 120 min. (B) Induced *WRKY39* expression in wild-type plants incubated with 1 mM SA, 0.1 mM MeJA, or 0.1 mM ACC. Leaves were harvested at the indicated times after treatment and used for preparation of total RNA and RT-PCR. (C) Expression of *WRKY39* in wild-type, *coi1*, *ein2*, and *sid2* plants during heat treatment at 42°C for 0, 30, 60, and 120 min. In (A), (B), and (C), *Actin2* amplification was used as constitutive control. The experiments were repeated three times using RNA extracted from three independent experiments and similar results were obtained. (D-F) qRT-PCR analysis of *WRKY39* expression. (D) Expression of *WRKY39* in wild-type, *hot1*, and *mbf1c* mutants during heat treatment at 42°C for 0, 30, 60 and 120 min; (E) *WRKY39* expression in wild-type plants incubated with H_2O, 1 mM SA, 0.1 mM MeJA, or 0.1 mM ACC for 8 h; (F) Expression of *WRKY39* in wild-type, *coi1*, *ein2*, and *sid2* plants exposed heat stress at 42°C for 0 or 60 min. In (D), (E), and (F), *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. The *A. thaliana* mutants and wild-type plants used in the experiments above were 3 weeks old.

slightly induced by JA, and moderately decreased in *coi1* during heat stress, JA is a possible candidate for one of the signal molecules that mediate heat- and SA- induced expression of *WRKY39*. Because JA acts with SA to confer thermotolerance in *A. thaliana* (Clarke et al., 2009), the induction of *WRKY39* through JA-mediated signaling mechanisms is consistent with its demonstrated role in thermotolerance. Although *WRKY39* expression was unaffected by ACC treatment, heat-induced expression of this gene was slightly reduced in *ein2* mutants. Therefore, the role of ET in *WRKY39* expression remains unclear.

The role of *WRKY39* as a positive regulator in thermotolerance is also consistent with possible regulation of WRKY39 by calcium, a second messenger in *A. thaliana*. Calmodulin (CaM) interacts with the WRKY39 protein (Park et al., 2005) and calcium is involved in HS in plants through CaM (Liu et al., 2008; Zhang et al., 2009). Calcium does not appear to be involved in the classic HS signaling pathways, i.e. the induction of *Hsps* (Gong et al., 1997), which is similar to *WRKY39* in heat stress (Fig. 6). Thus, WRKY39 may function as a downstream component of the CaM-mediated calcium signaling pathway during heat stress. Given that the WRKY39 protein may act as a regulatory node in thermotolerance by linking the SA, JA, and calcium signaling pathways, establishing the mechanism(s) for WRKY39-conferred thermotolerance is an important future task.

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