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Regulatory functions of evolutionarily conserved AN1/A20-like Zinc finger family proteins in *Arabidopsis* stress responses under high temperature

Geun-Don Kim [1], Young-Hee Cho [1], Sang-Dong Yoo *

Department of Life Sciences, Korea University, Seoul 136-713, Republic of Korea

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ABSTRACT

AN1/A20-like Zinc finger family proteins are evolutionarily conserved regulatory components in eukaryotic signaling circuits. In *Arabidopsis thaliana*, the AN1/A20 Zinc finger family is encoded as 14 members in the genome and collectively referred to as stress-associated proteins (SAPs). Here we described AtSAP5 localized to the nucleus, and played a role in heat-responsive gene regulation together with MBF1c. Seedling survival assay of *sap5* and *mbf1c* demonstrated consistent effects of AtSAP5 and MBF1C in response to two-step heat treatment, supporting their function in heat stress tolerance. Our findings yield an insight in A20/AN1-like Zinc finger protein AtSAP5 functions in plant adaptability under high temperature.

1. Introduction

A20 and/or AN1 Zinc finger (ZnF) family proteins have been identified and characterized in various eukaryotes [1,2]. In mammals, the ZnF family proteins (e.g., HsA20, ZNF216, Rabex-5, and A20) have been well-studied for their regulatory roles in immune responses [3–11]. Plant stress-associated proteins (SAPs) have been isolated based on their sequence homology to the A20/AN1-ZnF domains [12,13]. Among 14 *Arabidopsis* SAP members, AtSAP1 through AtSAP10 have both A20 and AN1 ZnF domains; AtSAP11 through AtSAP13 contain an AN1 and a C2H2 ZnF domains; and AtSAP14 has only one AN1-ZnF domain [12].

In addition, a small gene family that encodes A20/AN1-ZnF family proteins has been found in many plant species, such as rice (18 genes), maize (11 genes), sorghum (18 genes), poplar (19 genes), and grape (10 genes) [13]. The physiological functions of plant A20/AN1-ZnF family proteins have been implicated in various abiotic and biotic stress responses [12,14]. In general, transcript levels of SAP rapidly increase under various abiotic stressors that include cold, salt, dehydration, osmosis, metals [12,15–17]. The overexpression of rice OsSAP11 increases plant tolerance to salt and mannitol through its interaction with receptor-like cytoplasmic kinase 253 (OsRLCK253), via the A20 domain [18]. AtSAP5 is induced by various abiotic stressors and hormone responses, such as cold, mannitol, and abscisic acid (ABA), and the over-expression of AtSAP5 increases seedling tolerance to mannitol and salt [19]. Although the physiological functions of plant SAP are well-documented, the molecular and cellular mechanisms that underlie the physiological responses to stress remain unclear.

To investigate molecular mechanisms underlying SAP-dependent plant stress responses, we conducted a single cell-based functional screen, whole-genome gene chip analysis of global transcriptome response, molecular interaction assay using bimolecular fluorescence complementation (BiFC), and phenome analysis with two independent T-DNA insertional mutation lines of *AtSAP5*. The results consistently demonstrated that AtSAP5 has a regulatory role in the establishment of heat stress tolerance together with the AtSAP5-inducible transcription coregulator, MULTIPROTEIN BINDING FACTOR 1c (MBF1c, AT3G24500).

2. Materials and methods

2.1. Plant materials and growth conditions

Plants were grown in soil for 23–25 d under a photoperiod of 13 h light/11 h dark (60 μmol/m^2/s) at 25 °C. Colombia-0 (Col-0) plants were used as wild-type (WT) *Arabidopsis*. Two T-DNA *sap5* insertional mutants (SALK_073783 and SALK_062360C), and *mbf1c* (SALK_083813C) were used.

For the heat treatment assay, all the seedlings were grown on the same plate to ensure the same growth conditions. After 5 d, WT and two *sap5* and *mbf1c* T-DNA insertional mutants were

* Corresponding author. Fax: +82 2 927 9028.
E-mail address: sangdong@korea.ac.kr (S.-D. Yoo).
[1] These authors contributed equally to this work.

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exposed to high temperatures: plants were placed in the dark at 38 °C for 1 h 30 min, and then at 45 °C for 2 h 30 min.

2.2. Arabidopsis thaliana mesophyll protoplast transient expression assay

Protoplast isolation and transient expression assays were carried out as previously described [20]. Detailed primer sequences are listed in Supplementary Table S2. RD29A (EF090409), ARR6 (EF090414), GH3 (EF09410), and HSP18.2 (EF090413) promoter-fused firefly luciferase constructs were used as signal-specific reporters.

2.3. Interaction analysis using a bimolecular fluorescence complementation (BiFC) assay

To confirm the interaction between SAP5 and MBF1c, genes were inserted into a pUC SPYNE vector containing an N-terminal YFP fragment (YFP^N), and a pUC SPYCE vector containing a C-terminal YFP fragment (YFP^C), respectively [21].

2.4. RNA isolation and gene expression assay

Total RNA was extracted using Trizol (Invitrogen) for gene expression analysis, and 1 μg of RNA was used as the template for cDNA synthesis, using M-MLV reverse transcriptase (Promega). Real-time PCR (CFX Connect Real-Time System with a C1000 Thermal Cycler; Bio-Rad (Hercules, CA 94547, USA) was conducted using an iQ SYBR Green PCR mix (Bio-Rad). TUBULIN 4 (TUB4, AT1G04820) or ELONGATION INITIATION FACTOR 4a (ELF4a, AT3G13920) were used as internal controls. The gene-specific primers used for qRT-PCR are listed in Supplementary Table S2.

For the global gene expression analysis, an ATH1 whole-genome gene chip assay was conducted. Microarray expression data were analyzed with CEL files using the FlexArray open-source program (www.gqinnovationcenter.com). The expression values were normalized using MAS 5.0 and transformed to a log2 scale. The probes were selected with a cutoff of $p < 0.04$ to ensure data reliability.

3. Results and discussion

3.1. AtSAP5 functions in the nucleus

To determine the evolutionary relationship among AtSAPs, we grouped and showed the 14 members in a root-less phylogenetic tree based on their amino acid sequences, using the Cluster X program (Fig. 1A). In the group that contained both A20 and AN1 ZnF domains (AtSAP1–AtSAP10), some members seemed to be redundant in the genome, as they were grouped together in the same clade, but others were not. For example, AtSAP5 diverged from the closest clade (containing AtSAP8 and AtSAP10) and also from the clade containing AtSAP2, which suggested that AtSAP5 is distinct from other SAP members and may have a unique function in plant growth and development.

All the *AtSAP* members were expressed in *Arabidopsis* leaf mesophyll cells, except for *AtSAP8* and *AtSAP14* (Supplementary Fig. S1A). For protein functional analysis of AtSAPs, 12 AtSAPs (excluding AtSAP8 and AtSAP14) were cloned into a plant cell expression vector and the clones were confirmed by DNA sequencing. AtSAP protein expression was assured by protein blot analysis using anti-epitope antibody, and then these clones were used for further experiments.

To analyze AtSAP functions based on protein subcellular localization, GFP-tagged AtSAPs were expressed in leaf mesophyll protoplasts (LMPs) and observed under fluorescence microscopy. Transfected cells were incubated less than 6 h prior to image analysis to avoid mis-localization of AtSAP-GFPs by extremely high expression. AtSAP3-GFP and AtSAP5-GFP were all most exclusively observed in the nucleus, whereas other AtSAP-GFPs were observed in many different locales including both the cytosol and the nucleus (Fig. 1B). AtSAP3 and AtSAP5 have conserved bipartite nuclear localization signals in their protein sequence according to the data analysis using cNLS mapper (Supplementary Fig. S1B). The nuclear localization of AtSAP3 and AtSAP5 was further confirmed by superimposing their GFP images with a nuclear-localized red fluorescent protein (Fig. 1C). We then decided to investigate these two nuclear-localized proteins, AtSAP3 and AtSAP5, since they may have regulatory functions in nuclear events, such as gene expression regulation.

With AtSAP3 and AtSAP5, we screened several plant hormone signaling reporters to examine any possibility that these two AtSAPs may have a role in transcription regulation of hormonal responses. As previously reported [20], RD29A, ARR6, and GH3 promoters are differentially activated in response to ABA, t-zeatin, and IAA (auxin), respectively. AtSAP5, but not AtSAP3 (data not shown), further enhanced the activation of all three promoters (Supplementary Fig. S2). This result suggests that AtSAP5 may have a role in gene expression as a transcription co-regulator or mediator, and enhance transcription once committed. These hormone signals, however, did not affect AtSAP5 protein localization and accumulation patterns (Fig. 1D).

3.2. AtSAP5 regulates heat stress-responsive gene expression

The analysis of global transcriptome response was carried out to have a clue about AtSAP5-dependent gene regulation using ATH1 whole-genome chip. Total RNA was isolated from LMPs transfected with AtSAP5, or control plasmids, which has been incubated for 6 h after transfection, to identify early gene responses. After normalization (see Supplemental materials and methods for details), 41 early genes were upregulated by more than twofold upon a fivefold induction of AtSAP5 expression (Supplementary Table S1). Annotation of the differentially expressed genes revealed that at least 14 out of 41 genes were related to heat stress response. Among those genes activated by AtSAP5, we selected genes related to heat stress response, *HEAT SHOCK PROTEIN 18.2* (*HSP18.2*; AT5G59720), *HSP70T-2* (AT2G32120), and *HEAT STRESS ASSOCIATED 32* (*HSA32*; AT4G21320), and reconfirmed the induction of these marker gene expression by AtSAP5 using qRT-PCR (Supplementary Fig. S3).

To examine whether the AtSAP5-activated gene response is linked with any specific abiotic signal response, they were compared with various stress-inducible genes obtained from a public database (TAIR). The metagenome analysis of the gene lists revealed that AtSAP5-induced early genes indeed co-regulated with those induced by heat stress (Fig. 2A), but not as much as those induced by drought, osmotic balance, salt, or H_2O_2 (Supplementary Fig. S4). Based on results obtained from AtSAP5-inducible early gene expression (Fig. 2A), we decided to investigate AtSAP5 function in heat-responsive gene regulation.

The induction of *MBF1c* upon *AtSAP5* expression could not be escaped from our attention (Supplementary Table S1). The induction of *MBF1c* expression by AtSAP5 was reconfirmed using a semi-quantitative RT-PCR (Fig. 2B). MBF1c is an evolutionarily conserved regulatory protein that is present in all eukaryotes, and plays a key role in general transcription through its interaction with transcriptional factors such as GCN4, TBP, and ATF1 at the TATA site in yeast [22–24]. The transcription co-regulatory function of MBF1c is consistent with our initial observation of AtSAP5 in gene regulation as a putative transcription co-regulator (Supplementary Fig. S2), suggesting that AtSAP5 activates *MBF1c* and modulates gene expression in the nucleus. Intuitively,

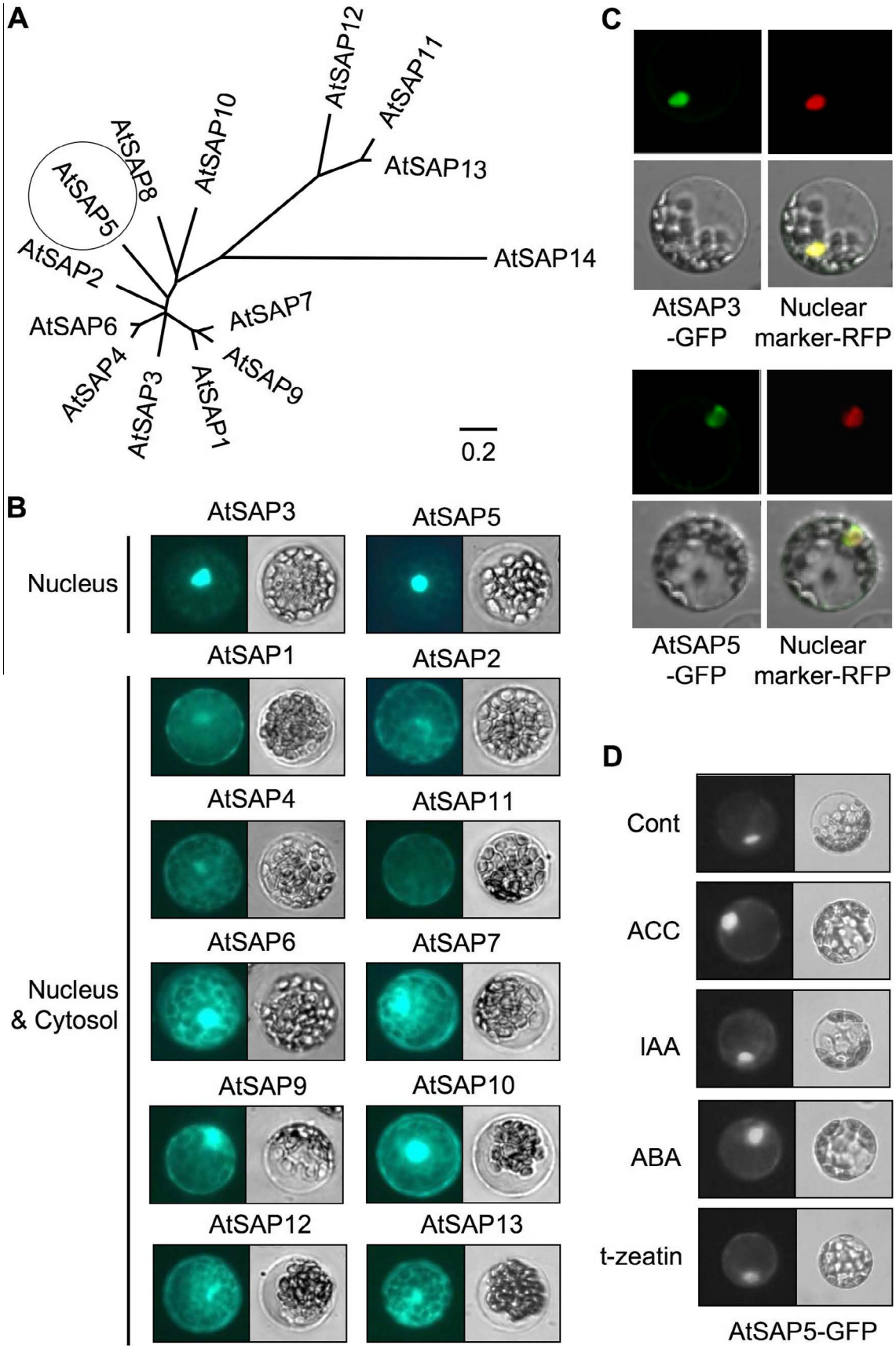

Fig. 1. AtSAP5 modulates general transcription in the nucleus. (A) Phylogenetic tree analysis of AtSAPs. (B) Distinct subcellular localization of AtSAP-GFPs. (C) AtSAP3-GFP and AtSAP5-GFP were localized in the nucleus. The nuclear red-fluorescence protein (RFP) served as a nuclear marker. (D) AtSAP5 was located in the nucleus after various hormone treatments. Protoplasts were transfected with an indicated GFP-tagged AtSAP construct, and observed using fluorescence microscopy, $n > 20$ (200×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

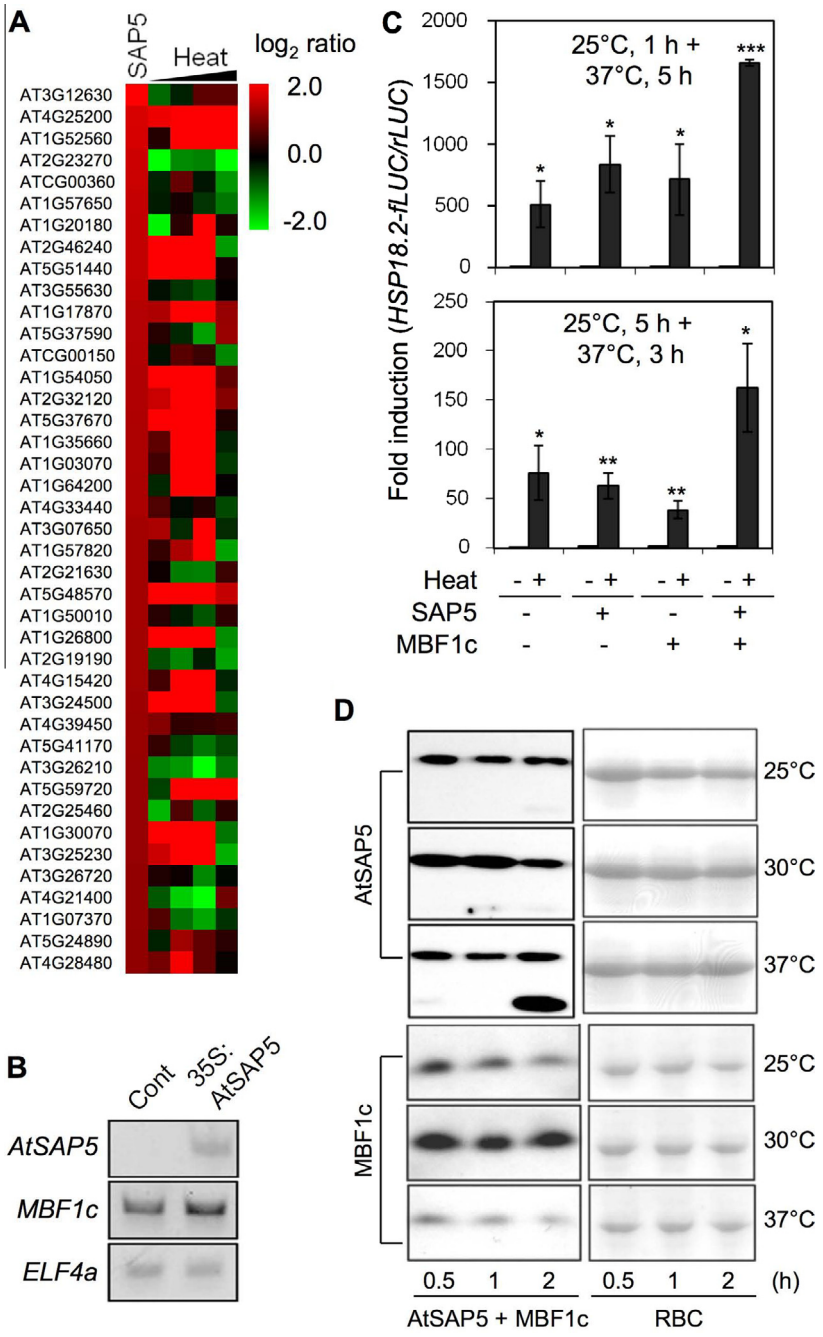

Fig. 2. The AtSAP5-mediated gene expression correlates with t heat responsive genes. (A) Transient *AtSAP5* expression inducible heat stress-response genes. (B) The relative gene expression of *MBF1c* was monitored in the presence of *AtSAP5*. *ELF4a* was used as an internal control. (C) AtSAP5 induces *HSP18.2* promoter activity, together with the transcriptional coactivator MBF1c. Transfected protoplasts were kept at: 25 °C for 1 h under light and then incubated in the dark at 37 °C for 5 h (upper); or 25 °C for 5 h under light and then incubated in the dark at 37 °C for 3 h (lower). The means of triplicate measurements were shown with standard error bars. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$. (D) Protein accumulation patterns of AtSAP5 and MBF1c upon different heat treatment conditions, determined by protein blot analysis using anti-HA antibody. Experiments were repeated and showed consistent results.

Arabidopsis MBF1c has been implicated in thermotolerance and other environmental stress responses [25–27].

Functional analysis of AtSAPs was previously carried out with genetically modified mutants or transgenic lines [19]. Here, we took an alternative approach and focused on the characterization of early gene response activated by AtSAP5. Unlike before, no obvious correlation was shown between AtSAP5-inducible genes and drought responsive genes in our metagenome analysis (Supplementary Fig. S4). AtSAP5 function in drought tolerance might result from a system-level complex responses of cross stress tolerance. For example, *HSPs* expression is induced by heat shock, and also under water, salt, oxidative stress, and a low temperature [27,28].

An AtSAP5-inducible early gene was *HSP18.2*, gene products of which act as a stress-inducible molecular chaperone, and is involved in both basal and acquired heat tolerance, as well as oxidative responses, in *Arabidopsis* [29]. To further examine AtSAP5 functions in heat responsive gene expression regulation, a single cell-based functional assay was conducted using an *HSP18.2* promoter-fused firefly luciferase (*HSP18.2-fLUC*) reporter. To confirm

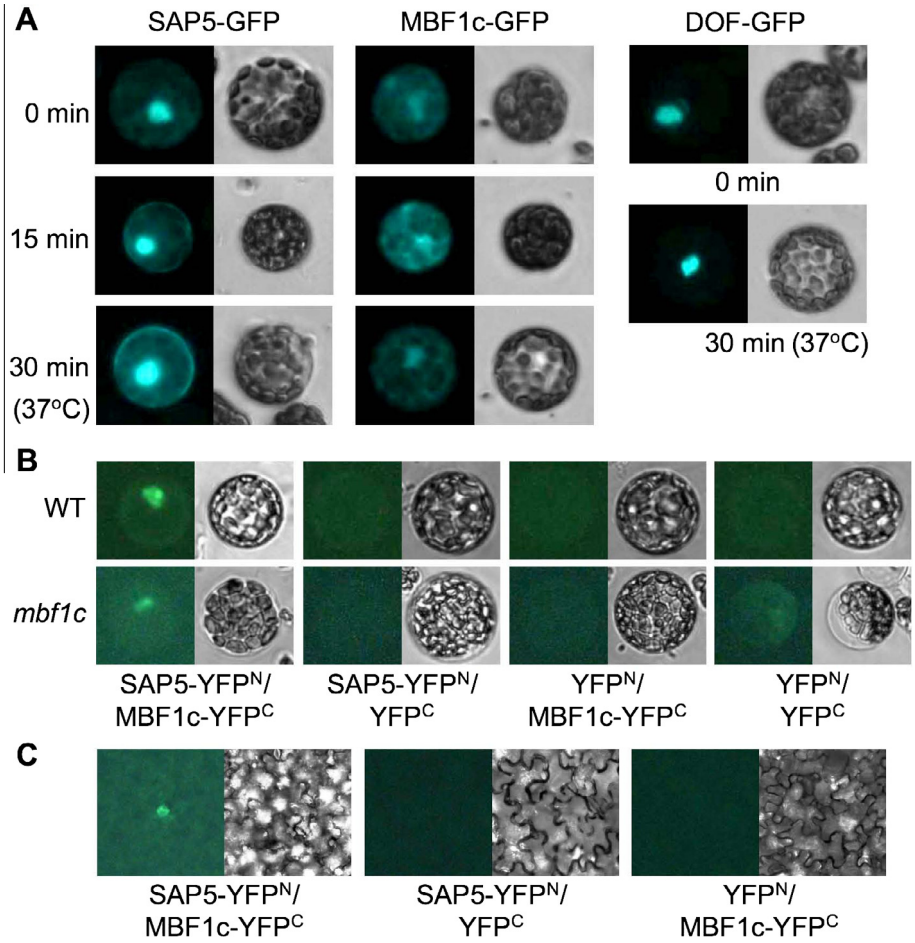

Fig. 3. AtSAP5 and MBF1c localization under heat treatment. (A) Localization of tSAP5-GFP, MBF1c-GFP and DOF-GFP before after heat treatment Protoplasts were incubated at 25 °C for 5 h, then at 37 °C for a designated time period, and subsequently observed using fluorescence microscopy, $n > 20$ (×200). (B) Bimolecular fluorescence complementation signal analysis for AtSAP5 and MBF1c interaction. Protoplasts were co-transfected with constructs encoding YFP^N or YFP^C fusion proteins. After transfection, protoplasts were incubated for 1 h under dim light and in the dark for another 10 h at room temperature, and then observed using fluorescence microscopy, $n > 15$ (200×). (C) *Agrobacterium*-mediated BiFc analysis. YFP was observed in tobacco epidermal leaf cells 5 d after infiltration, using fluorescence microscopy, $n = 5$ (200×).

its specific response to heat stress, the *HSP18.2-fLUC* activity was examined in response to various stress signals. The reporter activity was significantly induced by heat treatment (37 °C), weakly by oxidative stress (10 mM H_2O_2), but not by oxygen deficiency (hypoxia) (Supplementary Fig. S5). LMPs were then transfected with a combination of AtSAP5 and MBF1c, incubated at 25 °C for 1 h, and then placed in the dark for 5 h at either 25 °C or 37 °C (Fig. 2C, upper figure), since the reporter activity was significantly activated in 5 h after heat treatment (Supplementary Fig. S6). Neither of individual protein notably enhanced the reporter activity after heat treatment (Fig. 2C, upper figure). However, the co-expression of AtSAP5 and MBF1c resulted in a significant induction of the reporter activity, suggesting a cooperative function of AtSAP5 and MBF1c in heat stress response gene regulation.

To further investigate functions of AtSAP5 and MBF1c in heat response gene regulation, we adopted another heat treatment scheme to avoid cellular complications associated with extended heat treatment. LMPs were incubated at 25 °C for the first 5 h after effector construct transfection to ensure protein expression, and then subjected to heat treatment (Fig. 2C, lower figure). And then we measured the reporter activity in 3 h after the heat treatment, again the co-expression of AtSAP5 and MBF1c notably induced *HSP18.2-fLUC* activity (Fig. 2C, lower figure).

To examine protein behavior under heat stress conditions, HA epitope-tagged AtSAP5 and MBF1c were observed at 25 °C, 30 °C, and 37 °C for different time points after 5 h pre-incubation. Most of the accumulation patterns of these proteins were more or less similar to each other regardless different heat treatment conditions (Fig. 2D). A peculiar and interesting observation was that a protein species, which was approximately a half size of AtSAP5, was detected in 2 h after the heat treatment at 37 °C. Since the epitope was tagged at the C-terminus, this protein band may correspond to the C-terminal half of AtSAP5. It would be interesting to uncover whether this is a split-half of AtSAP5, and is involved in heat stress-inducible gene expression.

3.3. Interaction of AtSAP5 with MBF1c in the nucleus

In order to assess whether the locales of AtSAP5 and MBF1c were made any change by heat treatment, LMPs were individually transfected with *AtSAP5-GFP* or *MBF1c-GFP* construct, and incubated at 25 °C for 5 h and then heat treated at 37 °C for 15 or 30 min. Under the limited exposure of the transfected LMPs to high temperatures, MBF1c-GFP was confined to the nucleus (Fig. 3A) [25]. However, AtSAP5-GFP was dispersed to the cytosol, in a time-dependent manner. Although the dispersed protein pattern of AtSAP5-GFP was clear and universal in LMPs, the nuclear accumulation of this protein was unaffected. Interestingly, such protein pattern changes in localization were not observed for nuclear DOF1-GFP apart from heat treatment (Fig. 3A), indicating that the heat-inducible change of protein locales is specific to AtSAP5.

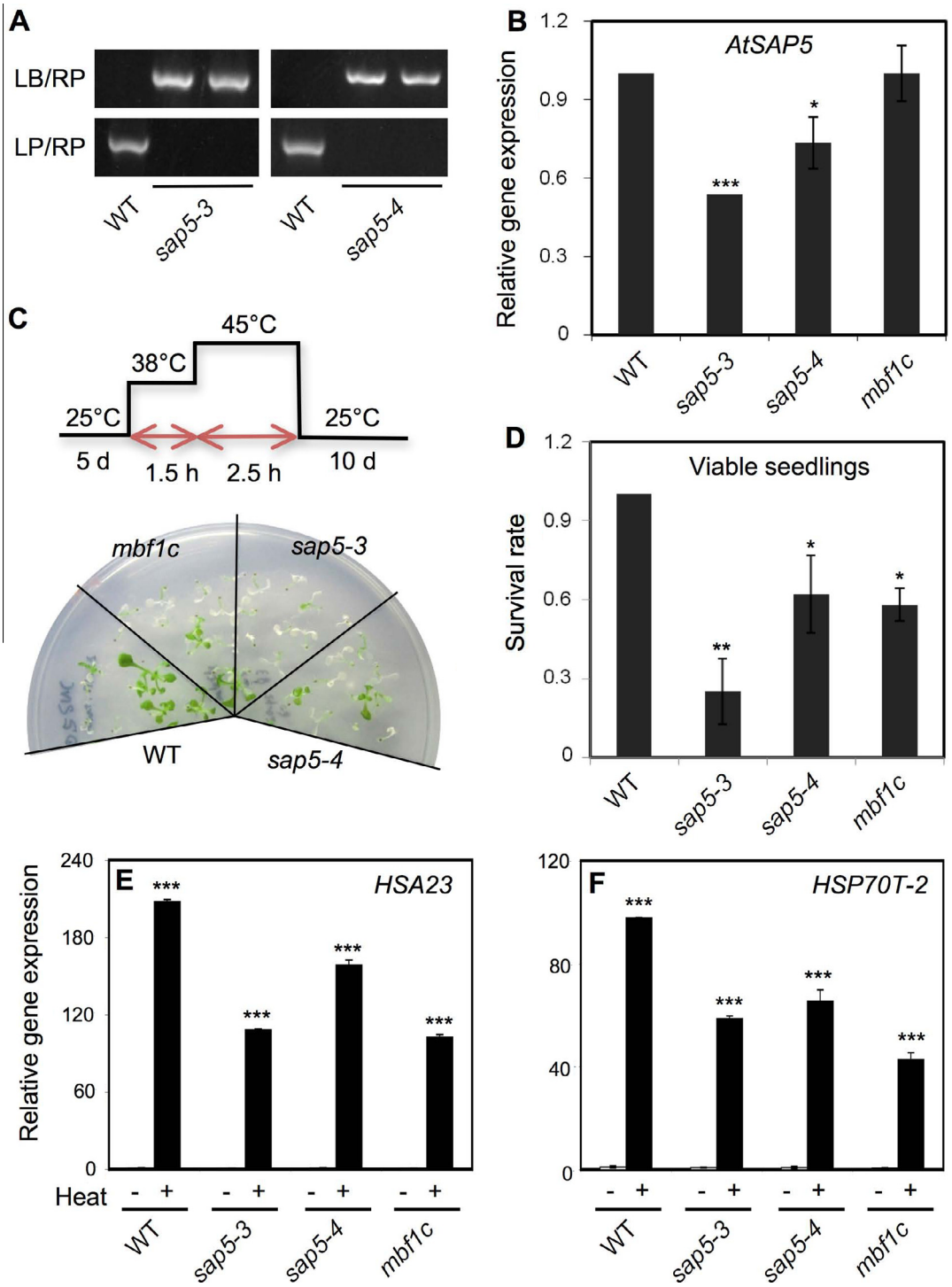

Fig. 4. AtSAP5 is involved in heat tolerance. (A) Molecular analysis of two independent *sap5* mutants. The T-DNA insertion sites and primer (LB1.3, LP, and RP) locations are indicated. (B) Levels of AtSAP5 expression were evaluated in *sap5-3*, *sap5-4*, and *mbf1c* mutants, together with WT. Total RNA was extracted from 7-d-old seedlings, and AtSAP5 was measured using qRT-PCR. ELF4a was used as an internal control for normalization. The means of triplicate measurements were shown with standard error bars. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$. (C) For heat treatment, 5-d-old seedlings were incubated in the dark at designated heat treatment schemes and then the seedling survival rate was calculated for the WT, *sap5-3*, *sap5-4*, and *mbf1c* mutants. (D) Seedling survival rates were normalized based on those of Col-0, $n > 57$. (E, F) Expression of *HSA32* and *HSP70T-2* was measured using qRT-PCR. The means of triplicate measurements were shown with standard error bars. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$.

Since both AtSAP5 and MBF1c observed in the nucleus, a possible protein–protein interaction was investigated using a BiFC assay in leaf mesophyll cells (Fig. 3B) [21]. YFP complementation signals between AtSAP5 and MBF1c were detected in the nucleus within 6 h after transfection, in both WT and *mbf1c* LMPs (Fig. 3B). Such signal was not detected with control plasmids, indicating YFP complementation occurred through AtSAP5 and MBF1c. Interaction between AtSAP5 and MBF1c was reconfirmed using an *Agrobacterium* infiltration assay as well. Again with the combination of AtSAP5 and MBF1c, a complementation signal was detected in the nucleus in tobacco leaf tissue (Fig. 3C), and this interaction signal was unaltered by heat treatment (data not shown). Results

from both single cells and leaf tissues indicate that AtSAP5 interacted with MBF1c and influenced heat stress-responsive gene expression.

3.4. Genetic analyses of AtSAP5 and MBF1c in heat stress tolerance

For analysis *in planta*, we obtained two independent lines of T-DNA insertional mutant of *AtSAP5* (*sap5-3*; SALK_073183 and *sap5-4*; SALK_062360C). T-DNA insertions and gene expression levels in these lines were monitored using PCR with genomic DNA (Fig. 4A) and qRT-PCR with total RNA (Fig. 4B), respectively. Even though both mutant lines were inserted with T-DNA in homozygous (Fig. 4A and Supplementary Fig. S7), *AtSAP5* expression was only reduced in *sap5-3* and *sap5-4* (Fig. 4B).

Five-day-old WT and mutant seedlings of *sap5-3*, *sap5-4*, and *mbf1c* were subjected to a two-step heat treatment to determine the effect of AtSAP5 on heat stress tolerance. For a two-step heat treatment, seedlings were incubated at 38 °C for 1.5 h and then immediately placed at 45 °C for another 2.5 h in the dark (Fig. 4C). Ten days later the seedling survival rate was determined and normalized based on that of WT. *sap5-3*, *sap5-4*, and *mbf1c* showed reduced tolerance under heat stress conditions compared to WT, which was seemingly acclimated by the two-step heat treatment. *sap5-3* with a lower level of endogenous *AtSAP5* expression (Fig. 4B) exhibited a relatively lower stress tolerance than others (Fig. 4C and D), and *atsap5-4* with moderate reduction of *AtSAP5* expression conferred tolerance to a moderate level in response to heat treatment. Likewise, the gene expression of two heat shock proteins *HSA32* and *HSP70T-2* activated by AtSAP5 as well as by heat treatment (Fig. 2A and Supplementary Fig. S3) was compromised in *sap5-3*, *sap5-4*, and *mbf1c* seedlings after 1 h incubation at 37 °C (Fig. 4E and F).

The heat stress tolerance and HSP gene expression levels in *mbf1c* were similarly compromised as the two *sap5* mutants (Fig. 4C–F), indicating AtSAP5 and MBF1c together contribute to the heat stress tolerance to some extent in *Arabidopsis*. Even though MBF1c is known to function in basic heat stress responses [25–27], our seedling assay further suggested that MBF1c may also function in the two-step heat stress response (Fig. 4C–F), which is distinct from acquired heat stress tolerance as it does not include an intervening cooling step between two heat treatment steps [30,31]. Further examination would be required with a *sap5 mbf1c* double mutant to conclusively determine whether these two regulatory proteins function in the same pathway for the heat stress.

Nuclear AtSAP5 (Fig. 1B and C) has previously been reported to influence cellular responses to abiotic stressors, including salt, osmotic imbalance, and water deficit [19]. AtSAP5 appeared to exhibit E3 ubiquitin ligase activity as well as polyubiquitin recognition capacity, but the way in which these activities contributes to stress resistance, and what the *Arabidopsis* E2 partners of AtSAP5 that may function as E3 ligases are, remains unknown. Recently, an AtSAP5 interaction with *Arabidopsis* c-myc binding protein (MBP-1)-like transcription factor was reported, with an ubiquitin-dependent destabilization [32]. It needs to be further examined whether MBP1 is a direct target of AtSAP5 *in vivo*.

Further information regarding the molecular mechanisms of AtSAP5 that underlie multiple stress response mechanisms is crucial in understanding the high level of plasticity and adaptability shown by plants in response to a wide range of environmental conditions.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Conflict of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.090.

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