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RESEARCH PAPER

CRISPR/Cas9 edited *HSFA6a and HSFA6b* of *Arabidopsis thaliana* offers ABA and osmotic stress insensitivity by modulation of ROS homeostasis

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ABSTRACT

The role of Heat Shock Transcription Factor 6 (*HSFA6a* & *HSFA6b*) in response to abiotic stresses such as ABA, drought, salinity, drought, and osmotic stress is individually well established. Unfortunately, the functional redundancy between the *HSFA6a* and *HSFA6b* as well as the consequences of simultaneous editing of both in response to aforementioned stresses remains elusive. Therefore, this study was designed with the aim of addressing whether there is any functional redundancy between *HSFA6a* and *HSFA6b* as well as to decipher their role in abiotic stresses tolerance in *Arabidopsis thaliana*, by using the CRISPR-Cas9. We have generated the single (*hsfa6a and hsfa6b*) as well as double mutants (*hsfa6a/hsfa6b-1* and *hsfa6a/hsfa6b-2*) of *HSFA6a* and *HSFA6b* with higher frequencies of deletion, insertion, and substitution. The phenotypic characterization of generated double and single mutants under abiotic stresses such as ABA, mannitol, and NaCl identified double mutants more tolerant to subjected abiotic stresses than those of their single mutants. It warrants mentioning that we have identified that *HSFA6a* and *HSFA6b* also involved in other major ABA responses, including ABA-inhibited seed germination, stomatal movement, and water loss. In addition to the above, the simultaneous editing of *HSFA6a* and *HSFA6b* lead to a reduced ROS accumulation, accompanied by increased expression of much abiotic stress and ABA-responsive genes, including involved in regulation of ROS level. In conclusion, these results suggest that *HSFA6a* and *HSFA6b* may offer abiotic stress tolerance by regulating the ROS homeostasis in plants.

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Abiotic stresses; *Arabidopsis*; genome editing; heat Shock Transcription Factor (HSF); ROS; stomata

Introduction

Plants spend their entire lives at a fixed spot and are frequently challenged with various environmental cues, among which heat stress and drought are of significant concern because of global warming.[1] Reactive oxygen species (ROS) accumulation dramatically influences the plant growth and, which are known to produce under normal as well as in an oxidative environment.[2] Usually ROS such as hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}), singlet oxygen species ($^1O_2$), and hydroxyl radical (OH^-) are produced as a byproduct of metabolic processes, including photosynthesis, dark respiration, and photorespiration, however, its accumulation greatly enhanced under abiotic stress environment conditions.[3,4,5] ROS also acts as signaling molecules under optimal growth conditions and also identified as a critical endogenous messenger in the complex ABA-signaling network.[6] The ABA-signaling network comprises diverse regulators, such as NADPH oxidases (Respiratory Burst Oxidase Homologues, RBOHs) and several other transcription factors.[7,8] In contrast to this the ROS detoxification system includes enzymes such as superoxide dismutase (SOD), catalases (CAT), and ascorbate peroxidases (APX) involved in detoxification of H_2O_2 .[9,10,11] The expression of such stress-responsive genes, which are regulated by a complex network of transcription factors (TFs) in response to such signals is well documented.[12] Heat Shock Transcription Factors (HSFs) are crucial gears of the signal transduction pathways umpiring the activation heat stress regulatory pathways or genes under heat stress.[13,14,15,16] Recent studies have shown that the HSFs are not only known to regulate the heat stress responsive pathways but also modulate the expression of a considerable number of other stress-responsive pathways. [17,18,19, 20]

An exogenous ABA, salt, and drought have been reported to induce the *HSFA6a* expression; surprisingly, the *HSFA6a* transcript is not significantly altered under heat and cold stresses.[21] ABA-responsive element binding factor (ABRF), a key regulator of the ABA-signaling pathway has been shown to be involved in the transcriptional regulation of HSFA6a and under stress conditions is localized in the nucleus.[21] Additionally, *HSFA6a* overexpressing plants exhibited hypersensitivity toward ABA and tolerance against salt and drought stresses at both the seed germination and seedling stage. Furthermore, an abscisic acid (ABA) signaling has been identified to be involved in thermos tolerance, and *HSFA6b* offers increased salinity, osmotic, and cold tolerance, but not heat stress.[12] *HSFA6b* has also been known to be involved in ABA-mediated stress responses.[12,21] Again NAC transcription factors bind to the promoters of *HSFA6a* and *HSFA6b* and provide thermo-tolerance.[22] HSFA6a is reported as a transcriptional activator of stress-responsive genes via the

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ABA-dependent signaling pathway, and HSFA6b acts as a downstream regulator of the ABA-mediated stress response as well as being required for heat stress resistance.[12,21] Unfortunately, the functional redundancy between *HSFA6a* and *HSFA6b* and their response to ABA and osmotic stress have not been adequately addressed, and neither has their role under these stresses[23] or their role in an ABA-mediated stress response.

In recent decades, the post-genomic era has confronted researchers with the urgent need to develop an efficient tool for functional gene analysis.[24] Reverse genetics approaches such as gene silencing have been widely used to elucidate gene function as well as to decipher regulatory mechanisms and metabolic pathways.[25,26] Genome editing technology (GET) can alter plant genome sequences by targeted-modification in genes, which leads to an understanding of the specific gene function as well as the functional diversity of a gene.[27] CRISPR-Cas9 technology has rapidly spread as a third-generation genome editing technology, which is different from ZFN and TALEN in the protein recognition mechanism.[28] The CRISPR/Cas9 system is now running with the mainstream of genome editing technology.[26] Unfortunately, the approach has never been applied to decode the functional redundancy if there is any between the *HSFA6a* and *HSFA6b* transcription factors as well as decode their role in ABA-signaling system.

Taking resource to the information described above and the preliminary work of our laboratory in the screening of ABA-regulated transcription factors we used the CRISPR/Cas9 technique for the first time for the generation of double mutants of *HSFA6a* and *HSFA6b*. The two main aim of the study is to examine the functional redundancy between the genes (*HSFA6a* and *HSFA6b*) for identification of a more obvious phenotype; and to facilitate an in-depth understanding of the ABA-signaling pathway. Based on our verdicts, it can be concluded that the *HSFA6a* and *HSFA6b* double mutant show a more obvious phenotype compared to the single mutant as well as WT under ABA and osmotic stresses. The study provides a theoretical platform for exploring the diversity as well as the functional complexity of heat-shock transcription factors in response to plant stresses.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used in this study. 1% bleach solution (0.1% mercury) was used for sterilization of seeds for 10 minutes and washed five times with purified water. The cleaned seeds were sown on one half-strength Murashige and Skoog (MS) agar medium with 1.5% w/v sucrose and 0.8% w/v agar, pH 5.7 for germination. The plates were then kept in the dark at 4°C for 3 days to stratify before being placed in a growth chamber for germination. Seedlings were transferred to soil (nutrient/vermiculite 1:1.5) and grown to maturity. The plants were grown under extended day conditions (16 h light/8 h dark cycle) at 20 ± 2° C, light intensity 100–120 μmol $m^{-2}s^{-1}$ and at 70% relative humidity.

Generation and screening of CRISPR/Cas9 edited lines

The CRISPR-Cas9 cloning plasmids pHSN401 and pCBC-DT1T2 were used in the study.[29] 23 base pair (5′-N20 NGG-3′) target sites were selected from the exonic regions of *HSFA6a* and *HSFA6b* from the *Arabidopsis* genomic DNA database (http://www.arabidopsis.org/), and the Cas-Finder database was searched for the evaluation of specific screens for highly specific target site sequences (http://www.rgenome.net/cas-offinder/). The two sgRNAs were constructed to enable simultaneous site-directed editing of *HSFA6a* and *HSFA6b* of *Arabidopsis*. Therefore, while designing the primers for sgRNA, the 19 nucleotides sequence up-stream to the PAM region was used for forward (DT1-BsF) and the reverse (DT2-BsR) primers, respectively. The CRISPR/Cas9 expression vector was constructed by using pCBC-DT1T2 as a template with the use of two sets of primers (DT1-BsF/DT2-F0 and DT2-R0/DT2-BsR) using nested PCR to obtain two sgRNAs, U6-29 promoter, and other essential elements. The length of the amplified target sequence was approximately 626 bp. Following the PCR amplification, the fragment was purified. The purified fragment and the pHSN401 vector were digested with *Bsa*I restriction enzyme (NEB, UK). The digested-purified PCR product and the vector were ligated by using T4 DNA fast ligases (NEB, UK). The *E.coli* cells were transformed with the ligation product, and the recombinant was selected by picking a single clone, PCR amplification, and sequencing. The obtained positive recombinant construct was transformed into *Agrobacterium* GV3101 by the freeze-thawing method and verified by colony PCR. GV3101 was cultured on solid YEP medium containing Kan 50 mg L^{-1}, Rif 30 mg L^{-1}, and Tet 20 mg L^{-1} for 2 days at 28°C in an orbital shaking incubator (New Brunswick, USA). Colony PCR was performed, and positive colonies were cultured in liquid to LB-medium. The construct was introduced into WT *Arabidopsis* by the *Agrobacterium tumefaciens* strain GV3103 mediated floral infiltration method. The genomic DNA of the mutant and wild-type *Arabidopsis* leaves was extracted using the CTAB method. The primers with flanking regions were designed to amplify the target sites, and sequencing screen the positive mutants. The sequencing chromatograms were decoded by using DSDecode web-based software.[30] The sequences of the primers used are provided in Table S1.

Genetic stability of Cas9-generated T2 mutants

The Cas9-generated T1 heterozygous lines were planted on one-half MS medium for germination, after 10 days the seedlings were transplanted into the soil for the screening of positive T2 mutants. DNA was extracted by the CTAB method from the rosette leaves of 24 randomly chosen plants. Two pairs of primers, including Hyg-IDF/R and zCas9-IDF/R (Shown in Table S1), were used to analyze lines without the Cas9 T-DNA insertion, target sites of non-transgenic T_2 plants were also amplified by using gene-specific primers as described above. The genotypes of these plants were analyzed by sequencing commercially.

Gene expression analysis

RNA was extracted from 7-days-old seedlings, which were treated with or without 100 μM ABA for 4 h. Total RNA was isolated using Trizol reagent (Invitrogen, USA) and Moloney Murine Leukemia Virus reverse transcriptase (M-MLV, Promega, USA) was used for the synthesis of the first-strand cDNA. Real-time PCR was performed with GoTaq® qPCR and RT-qPCR Systems (Promega, USA) by using the synthesized cDNA as a template and *ACTIN2/8* as an internal. All the PCR reactions were performed in triplicate, and the relative expression of the gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

Stress application and phenotypic analysis

For stress study, the harvested T_2 homozygous seeds were surface-sterilized to break dormancy and were sown on one-half MS medium. The plates were placed vertically at 4°C for 2 days in the material room for vernalization. The seedlings elongated to 1 cm were transferred into one-half MS medium and one-half MS medium with 150 mM or 200 mM mannitol, 150 mM or 200 mM NaCl, and 30 μM or 50 μM ABA after 7 days. The difference in root growth and the length was recorded from five independent biological replicates, and statistical analysis was conducted.

Water loss measurement and stomatal aperture under ABA treatment

The water loss measurement analysis was conducted using a method as described previously[31] from the detached rosette leaves, which were grown for 4 weeks under short-day conditions (8 h light/16 h dark cycle). The leaves were placed on a clean plate, immediately weighed after excision, and periodically weighed every 5 min by the Water loss measurement system [Sartorius, Germany]. The results are shown as a percentage of initial fresh weight. Each sample was three replicates at the same time. More than three independent experiments were performed.

The stomata aperture analysis was conducted as per the method described by [31]. Four-week plants were used for the stomatal aperture assay. Epidermal strips were peeled and were incubated in the stomata buffer (50 mM KCl, 10 μM $CaCl_2$, 10 mM Mes-Tris, pH 6.15) in light for 2 h at 22°C to fully open the stomata. After adding 10 μM ABA to the buffer and incubating for an additional 2 h in the light, images were obtained under a microscope (Olympus BX53). The stomata in the photographs were measured using Image J software (Broken Symmetry Software). More than 100 stomatal apertures for each treatment were measured. Three independent experiments were conducted.

ROS accumulation analysis

7-day-old seedlings of Col-0 and mutants were treated in liquid MS with or without 10 μM ABA for 5 h before the seedlings were used for the accumulation analysis of ROS. NBT (Nitro Blue Tetrazolium; Sigma-Aldrich, USA) staining was used to detect O^{2-} accumulation in tissues as per user manual instruction. The seedlings were incubated in a reaction buffer containing 1 mM NBT (Sigma-Aldrich), 20 mM K-phosphate, and 0.1 M NaCl for 2 h in the dark. The chlorophyll was removed from the seedlings by infiltrating them with lacto-glycerol-ethanol (1: 1: 4 volume) and 5 min boiling. 2′,7′-dichlorodihydrofluorescin diacetate ($DCFH_2$-DA) staining was used to detect H_2O_2. The seedlings were incubated in a buffer containing 20 μM $DCFH_2$-DA (Sigma-Aldrich) and 20 μM K-phosphate at pH 6.0 in the dark for 30 min. The roots were then photographed by using a Carl Zeiss LSM510 META confocal microscope with an excitation at 488 nm.

Epidermal strips of 4-week-old plants were used for the stomatal fluoresce assay or ROS accumulation analysis in guard cells. The strips were treated with 10 μM ABA to the buffer and incubating for an additional 1 h in the light, and then was stained with $DCFH_2$-DA buffer for 30 min. The photographs are taken by confocal mentioned as mentioned earlier.

Results

Construction and verification of *Arabidopsis* CRISPR/Cas9 binary expression vectors

The target site was selected in each exon region by sequence analysis of *HSFA6a* and *HSFA6b*. The cohesive end of *Bsa*I was added to the 5′ end as the target site sequence of sgRNA of *HSFA6a, HSFA6b* (Figure 1a-b; Table S1). Using the pCBC-DT1T2 vector as a template, the two sgRNAs were simultaneously amplified and targeted by a nested PCR reaction (Table S2). The target product obtained after PCR was approximately 630 bp, which was consistent with the expected results (Figure S1B). After the two sgRNA tandem PCR fragments and the pHSN401 vector were digested with *Bsa*I, the CRISPR/Cas9 system vector containing two-site editing was obtained and transformed into *E. coli*. A single colony of *E. coli* was verified by colony PCR and the positive ones were used for plasmid extraction and confirmed by sequencing. The correct plasmid was transformed into *Agrobacterium tumefaciens* GV3101.

Transformation and screening of CRISPR/Cas9 lines

The *Agrobacterium*-mediated inflorescences method was used for the genetic transformation of wild-type *Arabidopsis thaliana*. Following the obtaining of the T0 seeds, 19 potential T1 mutant plants were selected on hygromycin (25 μg/mL) resistant medium (Data not shown) and transplanted into soil. The positive mutants were subjected to DNA isolation, PCR amplification, and sequencing analysis by using the specific genomic amplification primers *HSFA6a* LP and *HSFA6a* RP, *HSFA6b* LP and *HSFA6b* RP (Table S1) in the flank of the target sequence of about 400 bp to detect the mutation of the target sequence. Sequencing results showed that the transgenic-positive plants contained three types of mutants: lines of only heterozygotes of *HSFA6a*, lines of only heterozygote of *HSFA6b*, and lines of heterozygous of *HSFA6a* and *HSFA6b* at the same time (partial sequencing results see Figure S1A). These results confirm that the nucleotide sequence of the target site was edited efficiency.

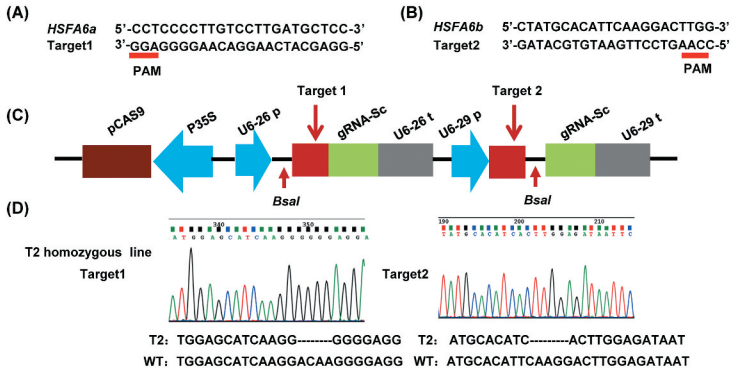

Figure 1. Construction of the CRISPR/Cas9 plasmid for targeting two sites in *hsfa6a* and *hsf6ab*. (a) and (b) The schematic map of the constructed target sites of *hsfa6a* and *hsf6ab*. The PAM belongs to NGG sequence. (c) The pictorial diagram of sgRNA cassette. (d) and (e) The sequencing chromatograms show the positions of the deletion in *hsfa6a* and *hsf6ab* mutants.

Stable integration of CRISPR/Cas9 cassettes in *Arabidopsis*

To determine whether these mutations are heritable or not, we analyzed T2 generation *Arabidopsis*. To exclude the effect of the inserted T-DNA on the subsequent phenotypic analysis, non-transgenic plants were identified by PCR reverse selection using two pairs of primers (Table S1), including the hygromycin resistance gene (Figure S1C) and Cas9 gene (Figure S1D). As shown in Figure S1C the arrows in the gel image were the plants to be selected. Sequencing results in Figure 1d suggest the successful inheritance of edited genes in the T2 generation. In the T2 generation non-transgenic plants, the mutations occurred near the PAM sites (Figure S2E), and the insertion, deletion, and substitution mutations were observed with frequency as shown in Table 1. The homozygous lines *hsfa6a, hsfa6b, hsfa6a/hsfa6b-1*, and *hsfa6a/hsfa6b-2* were selected for the phenotype experiments. The genotypes of the target genes are shown in Table S2. To detect the expression levels of *HSFA6a* and *HSFA6b* genes from mutants, the wild-type and generated mutants of *Arabidopsis* seedlings were subjected to real-time quantitative PCR analysis. The expression results suggest that the levels of *HSFA6a* and *HSFA6b* genes remained unaffected in wild-type. However, the levels were reduced significantly from the *hsfa6a, hsfa6b, hsfa6a/hsfa6b-1*, and *hsfa6a/hsfa6b-2* mutants (Figure S2). The result hence indicates the successful editing of the genes.

Table 1. Determination of mutation types obtained by transformation with CRISPR/Cas9 vector in *Arabidopsis thaliana*.

HSFA6a	Mutation type	Description	No. of clones with mutations	Mutation rate %	Total mutation rate
	Deletion	1 bp	1	7.14%	50.00%
		2 bp	3	21.43%	
		4 bp	3	21.43%	
	Insertion	1 bp	1	7.14%	7.14%
	substitution	1 bp	6	42.86%	42.86%
HSFA6b	Deletion	4 bp	3	23.08%	53.85%
		5 bp	2	15.38%	
		7 bp	2	15.38%	
	Insertion	1 bp	3	23.08%	23.08%
	substitution	1 bp	1	7.69%	23.08%
		2 bp	1	7.69%	
		3 bp	1	7.69%	

The abiotic stress tolerance phenotypes of the edited lines

For the characterization of WT and the mutant's phenotype over different abiotic stresses, primary root growth in the WT and the mutant were analyzed in ABA, mannitol, and NaCl. In standard MS medium no significant difference in the root growth of WT and mutants were observed (Figure 2). While the seedlings were grown in 30 and 50 μM ABA containing MS medium, the root growth of WT, and mutants (*hsfa6a* and *hsfa6b*) were inhibited, and the extent of inhibition was similar, with no significant difference (Figure 2). However, the root elongation of *hsfa6a/hsfa6b* was significantly different (Figure 2). Compared with 50 μM ABA, lower concentrations ABA of 30 μM caused a severe difference in the primary root elongation in both wild-type and mutants. Therefore, it can be concluded that *hsfa6a/hsfa6b* are resistant to ABA stress. Furthermore, similar results were also obtained when subjecting 150 and 200 mM mannitol, except that the difference between root length of *hsfa6a/hsfa6b* and WT was reduced (Figure S3). With increasing concentrations of mannitol, the WT seedlings showed reduced primary root elongation, but the double mutant changed slightly. It warrants mentioning that there was no significant difference in root growth of WT, *hsfa6b, hsfa6a/hsfa6b*, and *hsfa6a* observed under 120 and 200 mM NaCl (Figure S4). These results imply *hsfa6a/hsfa6b* mutant tolerant to the ABA and osmotic stress.

HSFA6a and *HSFA6b* are involved in ABA-regulated seed germination and stomatal movement

To investigate whether *hsfa6a* and *hsfa6b* are involved in other ABA-regulated responses, we next examined seed germination, stomatal movement, and water loss (Figure 2–3). Seeds were spotted on MS and MS medium supplemented with different concentrations of ABA. The germination rate was measured after 7 days (Figure 2). Seeds of the WT and the mutants germinated normally on MS medium (Figure 2c). However, in medium supplemented with 0.5 or 1 μM ABA, the seed germination of the WT was slower than that of the mutants (Figure 2c), with a significant difference in 1 μM ABA. Moreover, lower germination rates of the two single mutants were also found when different

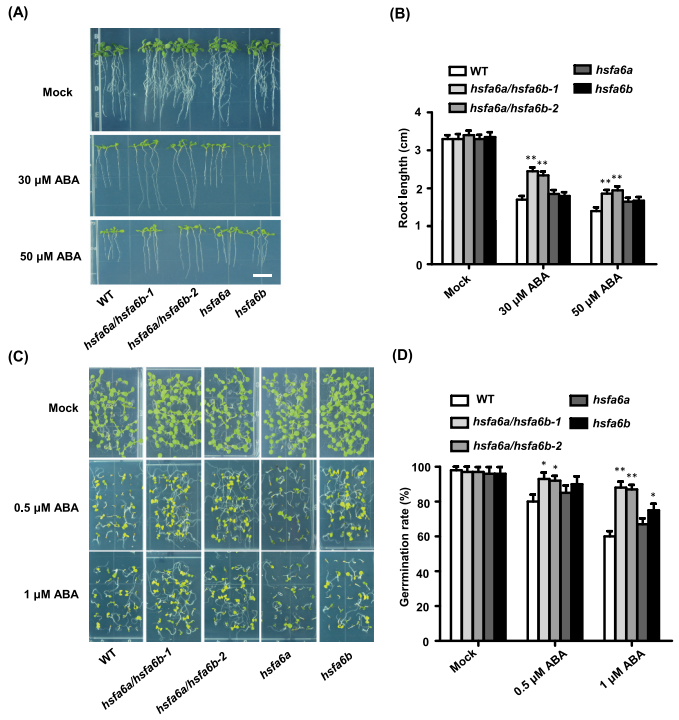

Figure 2. *HSFA6a/HSFA6b* edited lines are tolerant to ABA stress. (a) Phenotypic analysis of single (*hsfa6a* & *hsfa6b*) as well as double mutant (*hsfa6a/hsfa6b*) under ABA stress. WT and mutants seeds were germinated on MS medium and grown for 5 d before being transferred to MS medium containing different ABA concentrations. Bar = 1 cm. (b) Statistical analysis of root length of a single (*hsfa6a* & *hsfa6b*) as well as double mutant (*hsfa6a/hsfa6b*) and the wild-type. Three independent experiments were performed with three biological replicates. Five roots from one plate were measured for each repeat. Values are in means ±SE, n = 3. **: $P < .01$. (c) Seed germination of wild-type, *hsfa6a, hsfa6b* single mutants, and double mutant *hsfa6a/hsfa6b* in response to ABA. All seeds compared on MS medium with and without 0.5 μM ABA, 1 μM ABA for 7 d. (d) The percentage of germination in wild-type and mutants in response to different ABA concentrations. Values represent means ± SD (n = 3) from three biological replicates. A t-test determined statistical significance between the wild-type and mutants: *P < .05 and **P < .01.

concentrations of ABA were applied, with rates of 60%, 96%, 95%, 68% and 74% for WT, *hsfa6a/hsfa6b-1, hsfa6a/hsfa6b-2, hsfa6b, hsfa6a* respectively, under 1.0 μM ABA treatment (Figure 2d). These results suggest that the seed germination of *hsfa6a/hsfa6b* is more insensitive to ABA than the single mutant, and they are redundant in function.

ABA identified as involved in stomatal closure. Thus, we examined the ABA-dependent stomatal movement phenotypes of the mutant (Figure 3). The stomatal apertures of WT and the mutant were the same under control conditions (Figure 3a). However, on application with 10.0 μM ABA for 2 h, the stomata in WT and the single mutants (*hsfa6b* and *hsfa6a*) were closed, while the stomata in the double mutants (*hsfa6a/hsfa6b-1* and *hsfa6a/hsfa6b-2*) remained open (Figure 3a). Furthermore, the measurement of the stomatal pore size confirms our findings (Figure 3b). Thus, the results of the stomatal movement and root growth physiology of the double mutant (*hsfa6a/hsfa6b*) suggest that the double mutants of the *hsfa6a* and *hsfa6b* plants are more insensitive to exogenous ABA than the WT and their single mutants.

To obtain a direct measure of transpiration, we tested the drought-resistance by measuring the water loss from the detached leaves. The water loss of the efficiency of the double mutants, i.e., *hsfa6a/hsfa6b-1*, and *hsfa6a/hsfa6b-2* was higher than the WT and their single mutants, i.e., *hsfa6a* and *hsfa6b* (Figure 3c). Following drying after 300 min, the double mutants exhibited a significant difference compared to the WT plants, while the single mutant *hsfa6a* possessed no difference compared to WT. The water loss efficiency fresh weight of WT, *hsfa6a/hsfa6b-1*, and *hsfa6a/hsfa6b-2* were recorded to be approximately 32%, 40%, and 41%, respectively (Figure 3c). Collectively, these results indicate that *HSFA6a* and *HSFA6b* act as positive regulators of the ABA and drought-stress response.

A reduced ROS accumulation in double mutant *hsfa6a/hsfa6b*

To investigate the probable reason behind the insensitivity of double mutants over the single under selected stresses, have measured the ROS levels as ABA and chosen stresses are known to involved in the regulating the ROS levels. On comparing the concentration of H_2O_2 as well as superoxide by using $DCFH_2$-DA and NBT staining of root tips of the wild-type and mutants, reduced accumulation of H_2O_2, as well as superoxide, recorded form double mutants (*hsfa6a/hsfa6b-1*, and *hsfa6a/hsfa6b-2*) to than the wild-type and single mutants (Figure 4a-b). Furthermore, to confirm the ABA insensitivity and stomatal physiology in the double mutant, we also measured the level of ROS level in guard cells of single, double mutant as well as WT by suing epidermal peels (Figure 4c). The fluorescence results identified that the on exposure with ABA, a significant fluorescence was detected from the guard cells of WT and single mutant than the double mutants (Figure 4c).

HSFA6a and *HSFA6b* regulates the transcription of ABA, osmotic stress-related and ROS-scavenging/signaling genes

To investigate the probable mechanism of the ABA and osmotic stress insensitivity of double mutants, the expression profiles of ABA signaling, osmotic, and ROS-antioxidative defense components genes were analyzed from 7-day-old seedlings treated with ABA (Figure 5). We investigated the expression levels of *RD29A, KIN1*, and *COR47*, which have been identified as positive regulators of the ABA-signaling pathway, *DREB1A*, which is a positive regulator of osmotic stress, *RBOH, CAT*, and *CSD3* which are well known involved in ROS signaling pathway. ROS-scavenging enzymes coding genes such as superoxidase mutase (*CSD3* and *CSD2*) and catalases (*CAT3* and *CAT1*) were up-regulated in double mutant to than those of WT and single mutant (Figure 5). However, the expression of respiratory burst oxidase homologs (RBOHD and RBOHF) was upregulated in single mutant and WT.

Moreover, the expression levels of *RD29A, KIN1, COR47*, and *DREB1A* were up-regulated in all lines including WT under ABA treatment (Figure 5). However, a registered-reduced expression level of these genes in double mutants compared with WT and their single mutants on ABA treatment was observed (Figure 5). The expression of *COR47, DREB1A*, and *RD29A* in *hsfa6a/hsfa6b-1* and *hsfa6a/hsfa6b-2* decreased significantly. These results indicate that the loss of

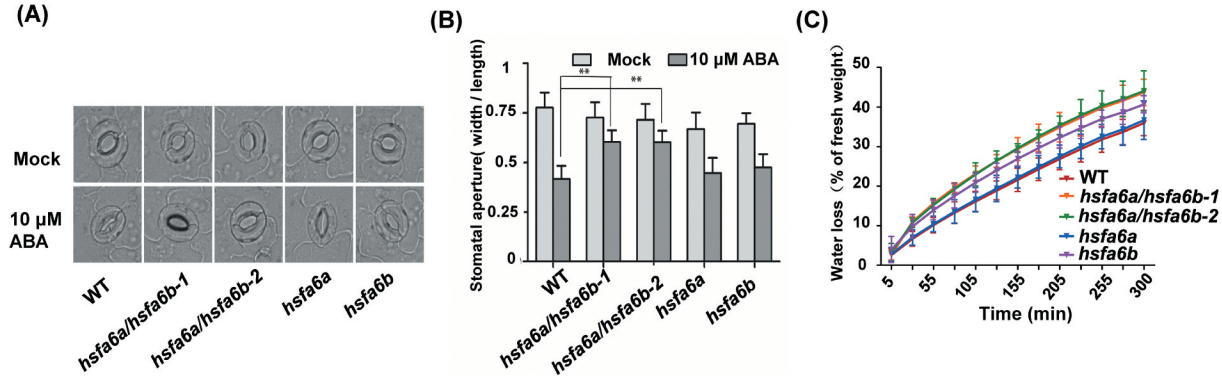

Figure 3. *HSFA6a* and *HSFA6b* double mutant have a high transpiration rate. (a) Stomatal images of WT, single and double mutants before and 2 h after treatment with 10 μM ABA. Epidermal strips with fully open stomata were treated with or without 10 μM ABA for 2 h. The leaves from 4-week-old plants were incubated with stomatal buffer in the dark for 30 min and then exposed to the light for 2 h. (b) The stomatal closure rate was observed at 2 h of ABA treatment. The data were obtained from >100 stomata. Values represent means ± SD (n > 100) from five biological replicates. Statistical significance between the wild-type and mutants was tested by t-test: *P < .05 and **P < .01. (c) Time courses water loss from detached leaves of WT, single (*hsfa6a*& *hsfa6b*) as well as double mutant (*hsfa6a*/*hsfa6b*). Four-week-old plants were used for relative water loss measurements. The experiments were repeated three times to get similar results. Each data point represents the means ± SD (n = 3).

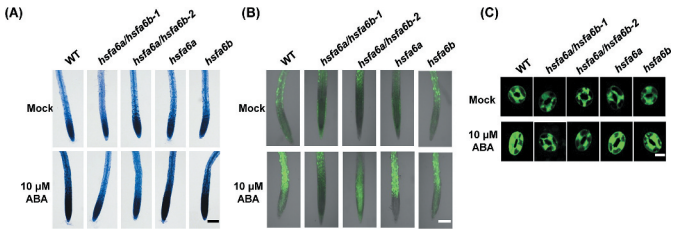

Figure 4. A reduced ROS accumulation in root and guard cells of double mutant (*HSFA6a* and *HSFA6b*) on exposure with ABA. (a) and (b) 7-day-old seedlings of WT and mutants were treated with or without 50 μM ABA for 5 h before the seedlings were stained with NBT and H2DCF-DA to visualize the ROS. Three independent experiments were performed, and about 10 seedlings were examined in each analysis. Bar = 100 μm. (c) Epidermal peels and roots treated with 10 μM ABA and 50 μM ABA for 2 h respectively were incubated in H2DCF-DA for 30 min. Stomatal images of WT, single and double mutants before and 2 h after treatment with 10 μM ABA. Bar= 5 μm.

function of *HSFA6a* and *HSFA6b* results in the weakening of ABA response in the mutants and gene expression was markable inhibited in double mutations.

Discussion

The CRISPR/Cas9 system is an emerging targeted genome editing technique that has not only caught the attention of scientists because of its high efficiency but has also been successfully applied in various organisms including plants.[32,33,34,35,36] Most of the gene functional analysis studies in *Arabidopsis* lies mainly on T-DNA insertion lines. However, the differences in insertion sites have a significant influence on phenotype, but also limitations due to different ecological backgrounds. This study uses the CRISPR/Cas9 binary vector system, which is very helpful for choosing target sites, the construction of expression vectors, mutation site analysis, evaluation of heritability, and the elaborated editing of the *Arabidopsis thaliana* genes.[29]

In this study, we have successfully generated the *hsfa6a* and *hsfa6b* double mutants by using CRISPR-Cas9 (Figure S1-2; Figure 1). The statistical analysis of the obtained progenies suggests that base insertions or deletions, substitution mutations were mainly generated at two or three bases upstream of the PAM region, while the deletions of one or several bases were identified as more frequent mutations (Figure S1-2; Table 1). The insertion of different numbers of bases was observed. However, the number of bases was not more significant, and no long fragments of additions and deletions were found (Figure S1). Also, the homozygous mutants were also screened during the detection of T2 plants by the Cas9 gene and hy gene (Figure S1). Our results demonstrate that we have successfully applied the CRISPR/Cas9 system for the simultaneous successful generation of mutants of two target genes in *Arabidopsis*, and this provides technical support for the study of a family of genes or the research of components of signal transduction pathways. The results of the differential gene expression analysis showed that the expression levels of *hsfa6a* and *hsfa6b* genes between mutants *(hsfa6a, hsfa6b, hsfa6a/hsfa6b-1 and hsf6a/hsf6b-2)* and wild-type *Arabidopsis* lines were remarkably different (Figure S2). This difference is probably due to the insertion of bases in the target gene, causing frameshift mutations, which leads to a protein with no functionality.[37] Compared with previously reported T-DNA insertion mutants, our edited mutants' lines showed a complete loss of functionality of genes and had distinct advantages for further functional analysis in the future.

The phenotypic analysis of mutants and WT in response to abiotic stresses such as ABA, NaCl, and mannitol in *Arabidopsis thaliana* regarding root length suggest that *hsfa6a*/*hsfa6b* is insensitive to ABA, NaCl, and mannitol compared to the WT (Figure 2; Figure S3-4). It has been reported that the overexpression of *HSFA6a* leads to sensitivity toward ABA in germination.[21] While its knockout mutant is unable to show any significant phenotypic changes.[21] Hence, the results are consistent with Hwang et al. Furthermore, we have demonstrated that *HSFA6a* also plays a vital role in the inhibition of root elongation by ABA and osmotic stress and the other ABA relative phenotype through analysis of double mutants. [12], identified that *HSFA6b* is directly involved in ABA-induced seed germination, cotyledon afforestation, and the root growth process. Moreover, in response to salt and drought, tolerance

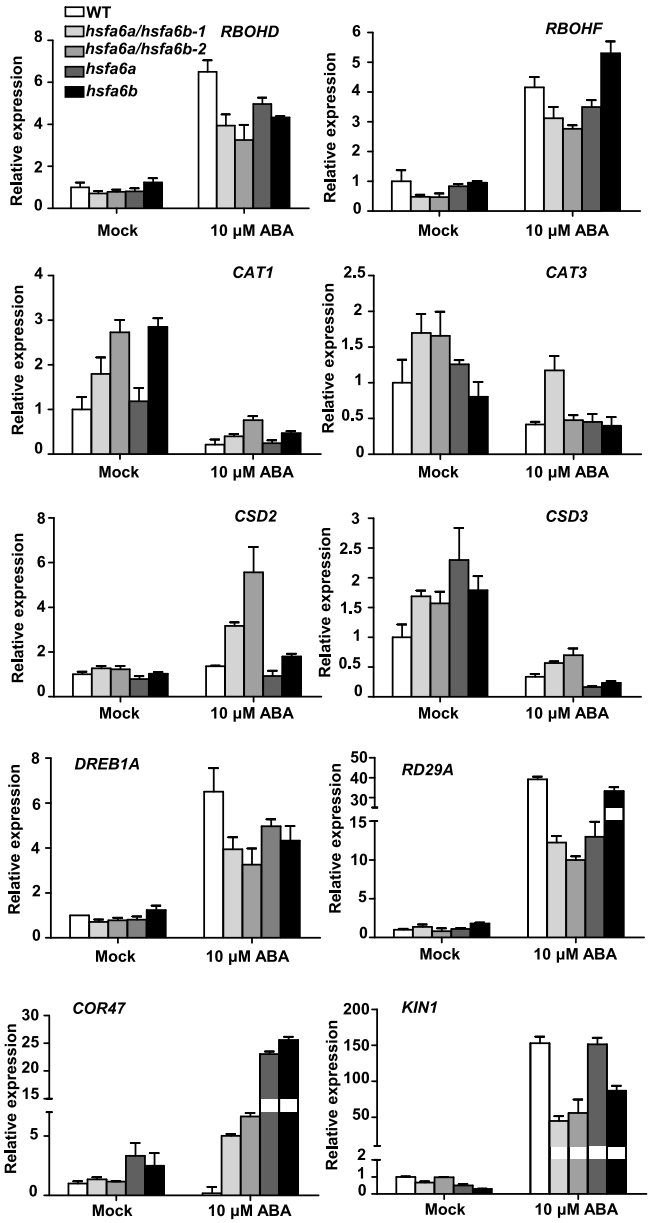

Figure 5. The Relative expression analysis of ABA, osmotic stress-related and ROS-scavenging/signaling genes from the seedlings of wild-type (WT) and mutants (Single & double) under ABA. Statistical significance between the wild-type and mutants was tested by t-test: *P < .05 and **P < .01. *ACTIN2/8* was used as a control. Values are in mean ± SD of three independent biological replicates.

was observed through the overexpression of *HSFA6b*, while its mutations identified *HSFA6b* as a positive regulator involved in ABA-mediated salt and drought resistance.[12] However, the results of our study suggest that a significantly reduced seed germination following ABA treatment and inhibition of root elongation by mannitol in *hsfa6b* and wild-type (Figure 3).

Moreover, *hsfa6b* showed an insignificant change in stomatal conductance and root elongation on ABA treatment (Figure 3). While the phenotype of the double mutant showed a registered difference both in germination, root elongation, and stomatal movement (Figure 2–3), the results are indicative of *hsfa6a* and *hsfa6b* functional redundancy in response to ABA and osmotic stress (NaCl and mannitol). The mutation of the only *HSFA6b* suggests that the *hsfa6b* is statistically insignificant and differs from wild-type regarding seed germination and root elongation (Figure S3-4; Figure 2–3). Therefore, the results of our study also provide phenotypic evidence of the evolutionary relationship between *hsfa6a* and *hsfa6b* and indicate that during seed germination and root elongation, as well as stomatal movement, these genes might play a crucial role in ABA and osmotic stress response in a functionally redundant manner.

Moreover, the investigation of ABA insensitivity of double mutants over the single under selected stresses, identified double mutant accumulate significantly less ROS (Figure 4). Therefore, it can be inferred that the ABA treatment significantly enhanced the production of H_2O_2 and superoxide in single mutants as well as in wild-type. Notwithstanding above the ABA insensitivity and stomatal physiology imaging-based analysis confirms that the ROS level in guard cells of double mutant found to be a lower than the WT and single mutants (Figure 4). These results vividly confirmed that the simultaneous editing of *hsfa6a* and *hsfa6b* leads to dysfunction of ABA-mediated ROS signaling pathways genes in double mutant than the WT and single mutant, which might be responsible for the ABA insensitivity of double mutant.

Real-time relative expression profiling of ABA (*RD29, KIN1, COR47*), osmotic stress (*DREBA1*) and ROS related (*RBOHD* and *RBOHF*) as well as antioxidative defense (*CAT1, CAT3, CSD2, CSD3*) system responsive genes were carried out, to identify the probable downstream signaling and regulatory component of these stress-responsive pathways (Figure 5). The ABA-independent path (*RD29A*), ABA-signaling pathway (*KIN1*, and *COR47*) and osmotic stress regulatory pathway (*DREB1A*) have been identified to involve in these signaling events[38,39,40,41,] The results of the gene expression analysis suggest that there is a complete transcriptional regulation pathway in double as well as single mutant modulated (Figure 5). The expression levels of these genes inhibited at different degrees in mutants. These results suggest that the mechanism of *HSFA6a* and *HSFA6b* action in plant stress tolerance may be attributed through the ROS signaling pathway, thereby affecting the expression of stress-related genes, and altering the stress adaptation process.

In conclusion, inducing multigenic mutations by CRISPR/Cas9 is an effective method for functional gene analysis, and this study has laid down a solid foundation for further research on *hsfa6b* gene function as well as the targeting of it signaling components.

Author contributions

D.P. and W.W. conceived and designed research; W.W., Y.H.and Q.C. performed the experiments. W.W. and P.K.S. analyzed as well as interpreted the data and wrote the manuscript. All the authors read and approved the final submission of the manuscript.

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Disclosure statement

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