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Ectopic Expression of Kenaf (*Hibiscus cannabinus* L.) *HcWRKY50* Improves Plants' Tolerance to Drought Stress and Regulates ABA Signaling in *Arabidopsis*

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Abstract: Kenaf (*Hibiscus cannabinus* L.) is an environmentally friendly, multipurpose fiber crop suitable for osmotic stress tolerance studies. However, the mechanisms of tolerance remain largely unknown. Here, we identified a stress-responsive *HcWRKY50* gene from kenaf (*Hibiscus cannabinus* L.) and studied its function and tolerance under drought stress. HcWRKY50 is a nuclear-localized protein. The overexpression of *HcWRKY50* in *Arabidopsis* showed higher drought tolerance, exhibiting increased root length and lateral root number, and reduced stomatal density compared with the control lines. The seed germination and seedling growth of *HcWRKY50* transgenic plants showed less sensitivity to ABA but they became more sensitive to ABA in their stomatal aperture. Furthermore, qRT-PCR analysis revealed that *HcWRKY50* regulated ABA signaling by promoting the expression of several key ABA-responsive and stress-responsive genes such as *RD29B* and *COR47* in transgenic lines. Taken together, this study demonstrated that the kenaf transcription factor *HcWRKY50* regulates seed germination and seedling growth and improves drought stress tolerance via an ABA signaling pathway.

Keywords: WRKY; kenaf (*Hibiscus cannabinus* L.); drought; ABA; stomata

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1. Introduction

As sessile organisms, plants have evolved various intricate mechanisms to help overcome the disadvantages of abiotic and/or biotic stress [1,2]. To deal with these environmental stresses, plants have developed multiple defense strategies, which include physiological and biochemical modification, cellular reprogramming, and immune activation [3]. Simultaneously, numerous stress-responsive genes are activated to mitigate these environmental challenges. These genes can be divided mainly into effectors and regulators [1,2]. Among them, transcription-factor-mediated regulation is a critical step during the activation of various stress responses [3]. Therefore, stress-related transcription factors have been adopted to genetically improve the stress tolerance of several crops in order to sustain the development of the agricultural environment.

WRKY transcription factors, characterized by a highly conserved WRKYGQK heptapeptide domain, are reported to be involved in various aspects of plant growth, plant development, and abiotic and/or biotic stress responses [4–6]. Increasing evidence shows that *WRKY* genes play master regulatory roles in responding to diverse sources of abiotic stress [6,7]. For example, *AtWRKY63* and *AtWRKY57* enhance drought tolerance by regulating the ABA signaling pathway [7,8]. Similarly, *OsWRKY24*, *OsWRKY45*, *OsWRKY72*,

and *OsWRKY77*, which are involved in ABA signaling, also improve tolerance to stress in rice [9]. The ectopic expression of *TaWRKY2* and *TaWRKY19* in *Arabidopsis* improves plants' tolerance to salt, drought, and freezing stresses by regulating the expression levels of stress-responsive *DREB2A*, *RD29A*, RD29B, and *Cor6.6* genes [10]. *LtWRKY21* is also highly induced under drought stress and activates the ABA signaling pathway [11]. Coinciding with these results, the overexpression of *GsWRKY20* also improves drought tolerance by decreasing water loss rate and stomatal density via the ABA signaling pathway in *Arabidopsis* [12]. These observations demonstrate that the established stress tolerance mechanisms have a close relationship with ABA signaling.

The ABA signal pathway has been reported to involve various molecular and cellular responses [12,13]. ABA receptors perceive the ABA signal and subsequently trigger downstream signaling cascades to induce different physiological responses. When ABA is present, ABA can bind to the cytosolic ABA receptor proteins PYR/PYL/RCAR and recruit PP2Cs, leading to the release of SnRK2 protein kinases, and subsequently activating downstream target genes [12,13]. In *Arabidopsis*, WRKY genes can directly function on ABA downstream regulators by binding to the promoters of target genes that are involved in the ABA signaling pathway, such as *ABFs*, *ABI4*, *ABI5*, and *DREBs* [4]. Increasing evidence suggests that *WRKY* genes positively bind and/or act upstream of the well-known stress-responsive genes of the ABA signaling pathway, such as *RD29A*, *RD29B*, and *COR47* [3,8,10,12,14]. However, the exact roles that the *WRKY* genes play in response to stress are lesser known in crop plants.

Kenaf (*Hibiscus cannabinus* L.) belongs to the *Malvaceae* family and is an annual herbaceous fiber crop. Kenaf is mainly grown in regions of the Asia-Pacific [15,16]. Kenaf grows very fast and can grow to a height of 4–6 m within a 4-month growth period, yielding huge fiber biomasses of up to 100–150 t per hectare [15,16]. Due to its colossal fiber yield and biodegradability, kenaf has been explored for widespread utilization in papermaking, building materials, bio-composites, animal feed, and recycled plastics [15,17]. More importantly, kenaf possesses excellent performance with a high tolerance to drought stress, salinity, and barrenness [15,18,19]. Therefore, kenaf could be used in the phytoremediation of saline–alkali soil and/or as an osmotic-stress-tolerant crop. However, its tolerance mechanism is still unclear, and how the WRKY genes in kenaf plants regulate tolerance remains obscure.

In this present study, a WRKY transcription factor from kenaf (*HcWRKY50*) was identified and was functionally characterized as a positive regulator of drought stress. The seed germination and seedling growth of *HcWRKY50* overexpression in *Arabidopsis* were insensitive to ABA but stomatal closure became more sensitive. The *HcWRKY50* transgenic *Arabidopsis* exhibited greater tolerance to drought stress with reduced stomatal density, a decreased water loss rate, and an increased root length and lateral root number. The quantitative reverse transcription PCR (qRT-PCR) analysis revealed that *HcWRKY50* is involved in the ABA signaling pathway and positively regulates the expression levels of key ABA-responsive genes and well-known stress response genes (*RD29B* and *COR47*). Our results reveal that *HcWRKY50* plays a positive role in improving drought tolerance and regulating ABA signaling.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Kenaf (*H. cannabinus* L. cv. Fuhong992) seeds were sown in nutrient-rich soil and cultured in a growth chamber (28 °C for 16 h light cycle and 26 °C for 8 h dark cycle, ~270 μmol m^{-2} s^{-1} light intensity, and 65–75% relative humidity). Seedlings of 2-week-old kenaf plants were collected for different treatments, as described by Niu et al. [17]. For the tissue-specific expression analysis, roots, stems, and leaves were harvested separately from 2-week-old kenaf seedlings. For the osmotic and salinity stress treatments, seedlings were cultured in solution buffers containing 15% (*w*/*v*) PEG6000 and 200 mM NaCl, respectively [20]. Each treatment contained three independent biological replicates.

Arabidopsis ecotype (Col-0) was grown in a greenhouse at 21–24 °C under a 16 h light/8 h dark cycle with a relative humidity of 65%. For ABA and/or stress-responsive marker gene expression analyses, 2-week-old control and *HcWRKY50* overexpression lines were subjected to 15% (*w*/*v*) PEG6000 (osmotic stress) and 100 mM ABA [1]. Each treatment contained three independent biological replicates. The samples were harvested and stored at −80 °C for RNA isolation.

2.2. RNA Isolation and qRT-PCR

Total RNA was isolated using the Ultrapure RNA kit (CW0597, China). First-strand cDNA was synthesized using the Reverse Transcription Kit (Pimerscript^{TM} RT DRR037S TaKaRa, Otsu, Japan). Reverse transcription PCR (RT-PCR) was performed to amplify the full-length cDNA of *HcWRKY50*. The primers are listed in Supplementary Table S1. The PCR products were cloned into a pMD18-T vector and transformed into Escherichia coli DH5α cells for sequencing. For the qRT-PCR analysis, the qPCR SuperMix TransStart Top Green (TransGen, AQ132-11) and the Bio-Rad CFX-96 detection system were used to detect patterns of gene expression, with the following steps: 94 °C for 30 s, 40 cycles of 94 °C for 5 s, 60 °C for 15 s, and a melting curve cycle from 65 °C to 95 °C. Kenaf *HcTUBα* and 18S *rRNA* [17] and *Arabidopsis AtACT2/7* were selected as the reference genes for normalization, respectively. All assays were performed in three independent technical and biological replicates.

2.3. HcWRKY50 Sequence Cloning and Subcellular Localization

The coding regions (CDS) of *HcWRKY50* (GWHPACDB063666) were identified from the kenaf genome database (https://ngdc.cncb.ac.cn/gwh/Assembly/1033/show, accessed date 25 March 2020) [21]. *HcWRKY50* was cloned into the pENTR$^{TM/D}$-topo vector. Then, the positive clones were recombined with the pGWB605 vector and were finally transformed into *Agrobacterium tumefaciens* GV3101. The 35S::*HcWRKY50*-GFP and the control 35S::GFP *Agrobacterium* were infiltrated into the leaves of *Nicotiana benthamiana*, as described by Chen et al. [1]. The GFP fluorescence signal was examined at a wavelength of 488 nm with a confocal microscope (Olympus FV500, Olympus, Japan).

2.4. Plant Transformation and Stress Treatments

The resulting 35S::*HcWRKY50*-GFP and 35S::GFP *Agrobacterium* tumefaciens GV3101 were also used for *Arabidopsis* transformation via the vacuum infiltration method [22]. The T1 transgenic plants were screened by herbicide, and the seeds of each positive T1 plant were individually harvested. T2 plants were selected for propagation, and the T3 homozygous lines were used for further analysis. Meanwhile, the 35S::GFP empty vector transformation *Arabidopsis* were used as the control lines.

For seed germination, leaf opening, cotyledon greening, and survival rate assays, the seeds from the control and homozygous overexpression *Arabidopsis* plants were surface-sterilized and then germinated on 1/2 MS agar medium (with or without 0.5 μM and 0.8 μM ABA, and 200 mM and 300 mM mannitol). For the root length assay, the seedlings from 5-day-old transgenic homozygous *Arabidopsis* were transferred to a fresh medium (with or without 10 μM and 20 μM ABA, and 300 mM mannitol, respectively) for vertical growth. After 7 d, the root length was measured and photographed. For the survival rate assay in soil, the 3-week-old seedlings were transferred into pots treated with or without drought treatments, water was withheld for 2 weeks in a growth chamber [10,12]. The survival rates for the control and *HcWRKY50* transgenic lines were calculated. All experimental data were obtained from the three independent biological replicates.

2.5. MDA Content, Electrolyte Leakage, and Water Loss Rate

The MDA content and the relative electrolyte leakage were evaluated according to the methods described by Chen et al. [1]. In order to measure the rate of water loss, the rosette leaves of 4-week-old seedlings were sampled and weighed, placed in 24 °C with

60% relative humidity, and weighed at assigned time intervals. The relative water loss rate was calculated based on the initial fresh weight of leaves. This was replicated three times.

2.6. Stomatal Conductance and Density

Stomatal closure assays were performed as described previously [12]. Briefly, rosette leaves of the same relative position and age were sampled from the *HcWRKY50* overexpression and control lines. For stomatal opening, the leaves were positioned to float on their abaxial side in the opening buffer for 3 h under lights. The leaves were then transferred to the different ABA concentration solutions [12]. The stomatal aperture, size, and density were examined by scanning electron microscopy (SEM). Approximately 180–350 stomata were statistically analyzed in the *HcWRKY50* transgenic and control lines, and the stomatal length/width ratios were simultaneously determined.

2.7. Statistical Analysis

All experiments were performed after three replications (either three biological repeats or three technical repeats). The results are represented by the mean value ± standard error from each assay. ANOVA was used to analyze the significant differences between each assay and the corresponding controls. *** indicated $p < 0.01$, and * indicated $p < 0.05$.

3. Results

3.1. Identification and Sequence Characteristic of HcWRKY50 Gene

The bioinformatic analysis showed that *HcWRKY50* has an open reading frame (ORF) of 858 bp. A homology search analysis showed that *HcWRKY50* is homologous to bonafide WRKY genes, especially to *AtWRKY50*, *GhWRKY50*, and *OsWRKY50* (Figure 1). Similarly, the phylogenetic analysis demonstrated that *HcWRKY50* is a member of the WRKY IIc subgroup, so it was designated as *HcWRKY50* (Supplemental Figure S1). Multiple sequence alignments showed that *HcWRKY50* did not share a highly conserved WRKYGQK motif, but instead shared an alternative WRKYGKK motif accompanied by a C-terminal C2H2 zinc finger motif (Figure 1).

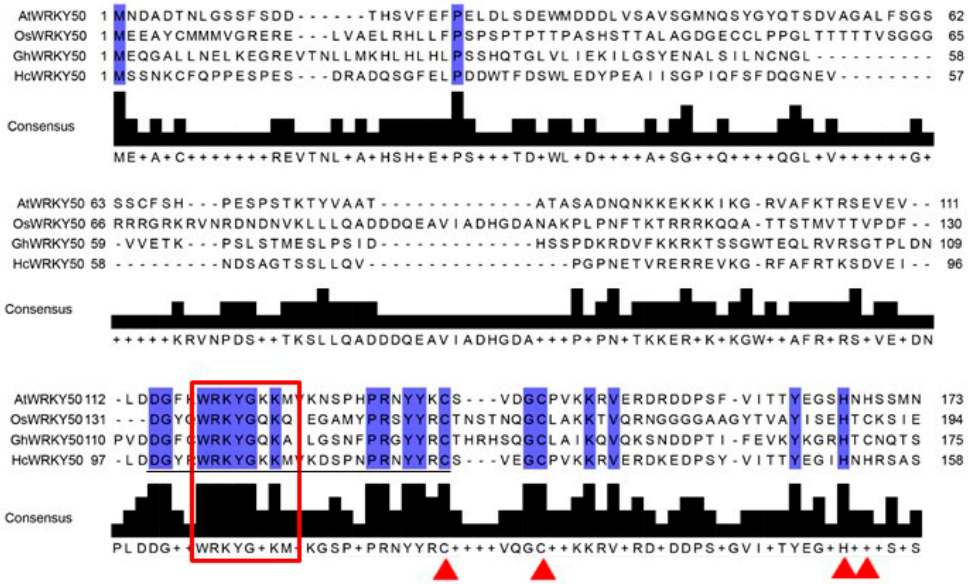

Figure 1. Sequence analysis of *HcWRKY50*. Multiple sequence alignment of deduced amino acid sequences of HcWRKY50 with other WRKYs from *Arabidopsis* (AtWRKY50, NP_197989), rice (OsWRKY50, DAA05116), and cotton (GhWRKY50, AIE43840). Sequences were aligned using ClustalX. The typical WRKY domain is underlined, and the WRKYGQ(K)K amino acids that were completely conserved were boxed. Arrowheads indicate the cysteine and histidine residues of the putative zinc finger motif.

3.2. HcWRKY50 Was Differentially Expressed in Tissues and Induced by Different Stresses

To characterize the *HcWRKY50* function, the level of expression of *HcWRKY50* was examined using qRT-PCR analysis across different tissues and different stress conditions. The results showed that *HcWRKY50* was expressed differentially in different tissues. *HcWRKY50* was mainly expressed in the roots of kenaf, with an expression level 2.5-fold greater that of the epidermis and leaves (Figure 2A). Then, we also checked the level of expression of *HcWRKY50* in response to salinity, drought, and cold stress. We found that the expression level of *HcWRKY50* significantly increased in response to the salinity and drought stimuli at 12 h and 24 h, respectively (Figure 2B,D). However, cold stress reduced the level of expression of *HcWRKY50* (Figure 2C). These results indicate that *HcWRKY50* is induced in response to salinity, drought, and cold treatments, with different induction patterns (Figure 2B–D).

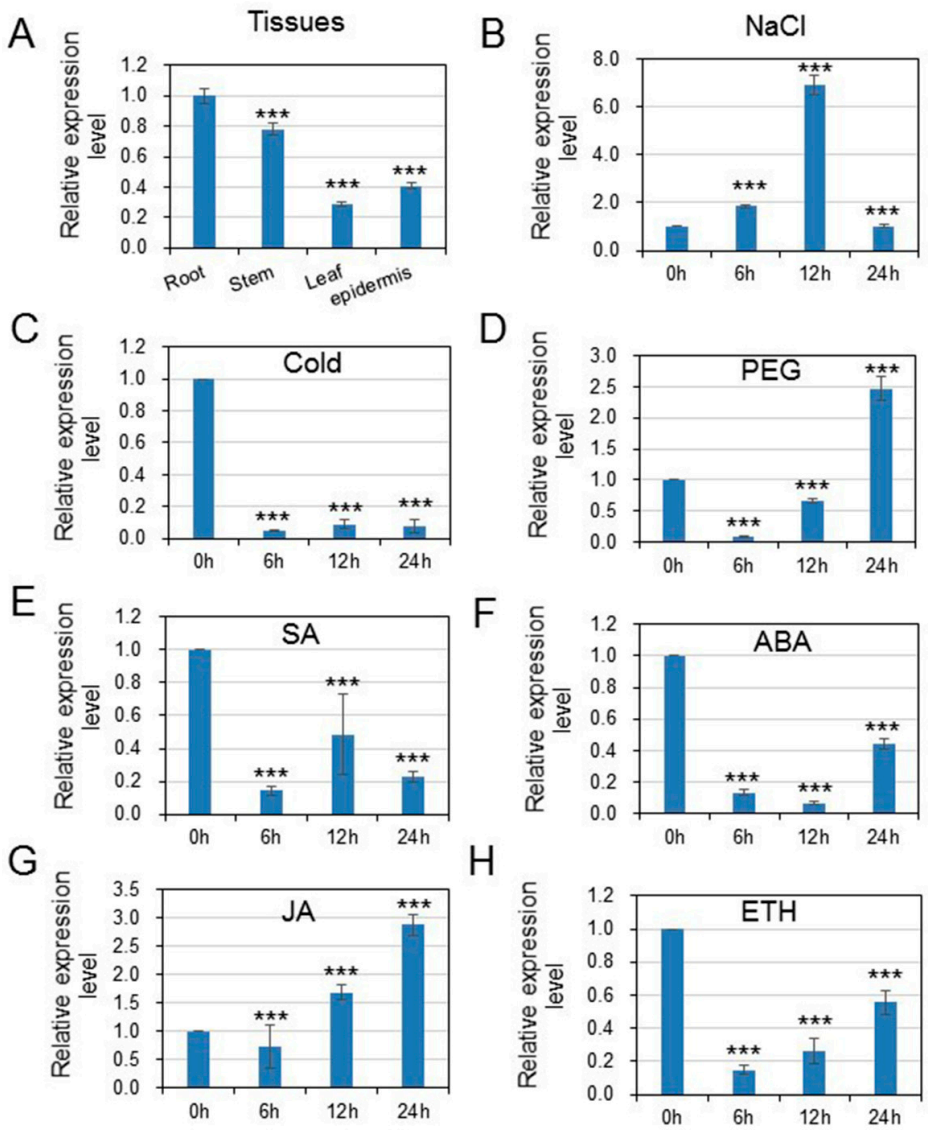

Figure 2. Expression profiles of HcWRKY50 under various abiotic stresses. HcWRKY50 is differentially expressed in different tissues (**A**), and seedlings of 2-week-old kenaf are induced by different stresses, including salinity stress (200 mM NaCl) (**B**), cold stress (**C**), osmotic stress (15% PEG6000) (**D**), and different hormone treatments such as SA (**E**), ABA (**F**), JA (**G**), and ETH (**H**). Leaves were harvested for further analysis. The TUBα and 18S rRNA genes were used as standard controls to show the normal number of templates in PCR reactions. ANOVA was used to analyze the significant differences between each assay and the corresponding controls. *** indicated $p < 0.01$.

The expression levels of genes across different treatments showed the potential functions of these genes in response to different hormone pathways. We also examined the level of expression of *HcWRKY50* in response to hormones (SA, ABA, JA, and ETH) to check whether there was any correlation between abiotic stress and hormone responses. The results showed that *HcWRKY50* transcripts dramatically decreased 6 h after contact with SA and ETH stimuli, followed by an increase at 12 h and a return to lower expression levels at 24 h (Figure 2E,H). For the ABA treatment, *HcWRKY50* mRNA expression was reduced at 6 h and 12 h and then accumulated at 24 h (Figure 2F). JA stimulation also induced *HcWRKY50* transcripts and reached the highest level of expression at 24 h (Figure 2G). Together, these results indicate that *HcWRKY50* might be involved in abiotic stress and hormone stimulation.

3.3. HcWRKY50 Protein Localizes in Cell Nuclei

For the subcellular localization of HcWRKY50, the 35S::*HcWRKY50*-GFP fusion protein was generated (Figure 3A). The resulting constructs and the positive control 35S-GFP *Agrobacterium* were transformed into the leaves of *N. benthamiana*. The results showed that the 35S::*HcWRKY50*-GFP fusion protein was exclusively localized in the nuclei of the epidermal cells of *N. benthamiana* (Figure 3B). In contrast, the control GFP protein was found in both the nucleus and the cell membrane (Figure 3B). This result indicates that HcWRKY50 is a nuclear-localized protein.

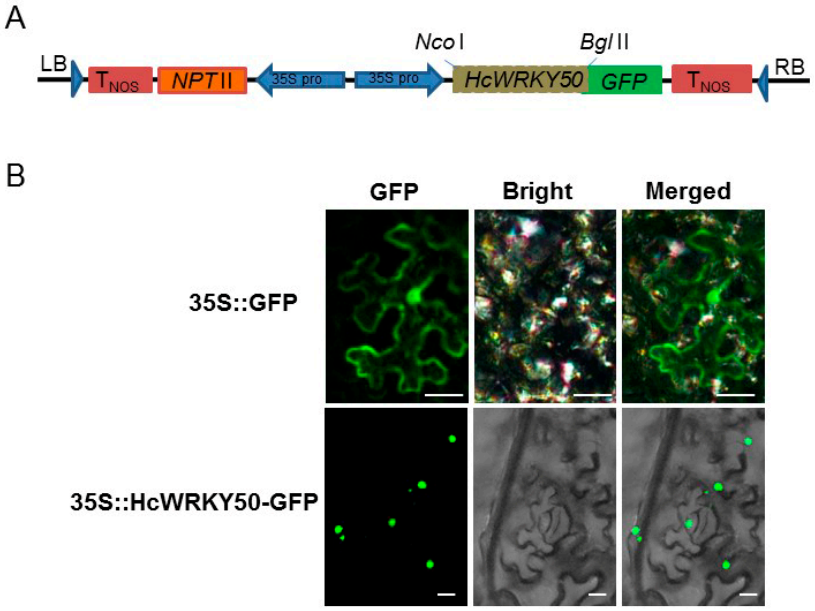

Figure 3. Subcellular localization of HcWRKY50-GFP fusion protein. (**A**) Schematic representation of the 35S::*HcWRKY50*-GFP fusion construct and the 35S::GFP construct. The *HcWRKY50* CDS was fused into the GFP expression vector. (**B**) Subcellular localization of the 35S::*HcWRKY50*-GFP and 35S::GFP constructs was checked by a confocal laser scanning microscope. Scale bar = 20 μm.

3.4. HcWRKY50 Overexpression Plants Had Decreased Sensitivity to ABA

To study the function of *HcWRKY50*, the constructs of *35S::HcWRKY50*-GFP and 35S-GFP empty vectors were also transformed into *Arabidopsis* plants. Three independent homozygous lines for *HcWRKY50*, with relatively high expressions of the transgenes, were selected for further phenotypic analysis (Figure 4A). These transformation lines showed a similar phenotype under normal conditions. To examine the ABA response of *HcWRKY50* transgenic lines, the seeds from OE50-1#, OE50-2#, and OE50-3# were germinated on 1/2 MS medium with 0.5 μM and 0.8 μM ABA treatments. For the mock group, both control and *HcWRKY50* overexpression lines showed a comparable germination rate, but OE50-1#, OE50-2#, and OE50-3# showed higher germination rates than the control lines after 3 days

of germination (Figure 4B,C). However, for the ABA treatment, the seeds from OE50-1#, OE50-2#, and OE50-3# lines showed a much faster germination rate than the control lines (Figure 4B,C). Furthermore, the OE50-1#, OE50-2#, and OE50-3# lines showed significantly greater performance, with developed healthy cotyledons, and more open and green leaves than the control lines following treatment with different concentrations of ABA for 7 days (Figure 4B,E).

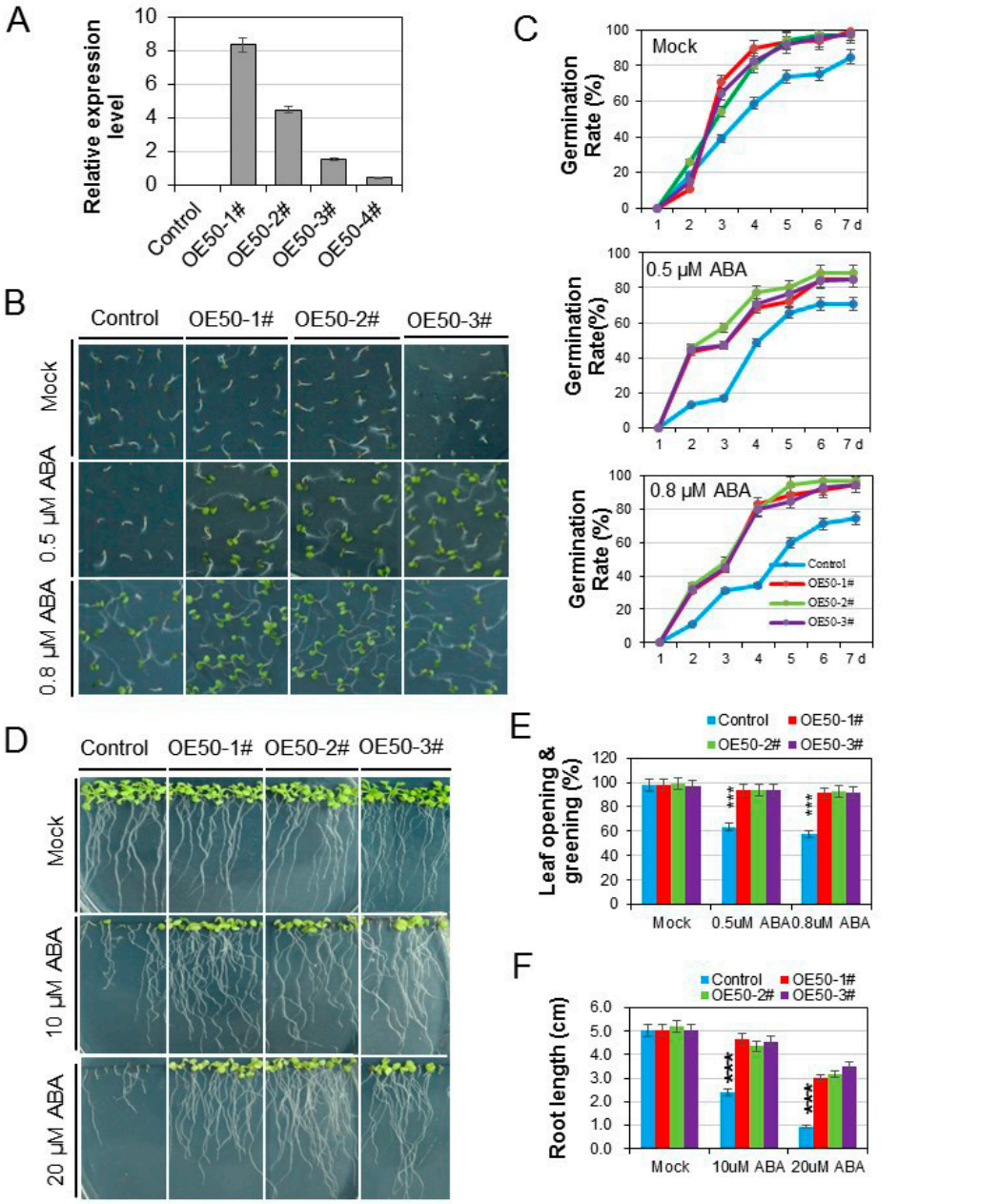

Figure 4. Overexpression of *HcWRKY50* in *Arabidopsis* reduced plant sensitivity to ABA. (**A**) qRT-PCR analysis of *HcWRKY50* transcript levels in the homozygous *35S::HcWRKY50* line. *Actin 2/7* were used as normalization factors for qRT-PCR. (**B**,**C**,**E**) The seed germination rate, the quantitative evaluation of leaf opening, and the greening rate on 1/2 MS with or without different concentrations of ABA in different *HcWRKY50* transgenic lines (OE50-1#, OE50-2#, and OE50-3#) and control lines. Photographs were taken 7 d after sowing. (**D**,**F**) Five-day-old seedlings grown on 1/2 MS were transferred to new plates supplemented with 0, 10, and 20 μM ABA. The roots of transgenic and control seedlings grown on ABA supplemented plates were photographed and measured. All values are means (±SD) from three independent experiments. *** $p < 0.01$ by ANOVA analysis.

To confirm the inhibition effects of different ABA concentrations, the ABA sensitivity of root growth was also examined. The 5-day-old seedlings were transferred onto vertical agar plates supplemented with 10 μM and 20 μM ABA. As expected, the root growth rates of the control and *HcWRKY50* overexpression lines were comparable when grown on mock MS mediums (Figure 4D,F). On the contrary, root growth of the control lines was significantly inhibited when treated with different ABA concentrations, however the *HcWRKY50* overexpression lines were resistant and showed a longer root length than the control lines (Figure 4D,F).

3.5. HcWRKY50 Overexpression Plants Increased the Tolerance to Drought Stress

The qPCR results showed that the *HcWRKY50* transcript is enriched in the root (Figure 2A), and is strongly induced under drought, salinity, and ABA treatments (Figure 2B,D,F). This prompted us to investigate whether *HcWRKY50* contributes to osmotic stress and ABA signaling pathways. To test this hypothesis, three independent positive transgenic lines (OE50-1#, OE50-2#, and OE50-3#) were selected and their stress tolerance performance was evaluated. Drought stress was mimicked using mannitol irrigation. The germination rates of the *HcWRKY50* overexpression and control lines were not significantly different on the control 1/2 MS media. However, after mannitol treatment, the OE50-1#, OE50-2#, and OE50-3# lines showed a faster germination rate than the control lines, with good seed coat breakage and radicle emergence (Figure 5A,B). Moreover, these transgenic lines had a faster root growth rate than the control lines, with longer root length and a more lateral root number (Figure 5C–E). The drought tolerance performance of *HcWRKY50* overexpression lines was further investigated in soil. Soil-grown 2-week-old transgenic lines (OE50-1#, OE50-2#, and OE50-3#) and control plants were completely dehydrated after receiving no water for 2 weeks. They were then re-watered and their survival rates were determined. Most of the leaves of the transgenic lines (OE50-1#, OE50-2#, and OE50-3#) remained green and thrived, while the leaves of the control plants became yellow, dry and/or wilted (Figure 5F). Finally, the *HcWRKY50* overexpression lines displayed a higher survival rate than the control lines (Figure 5G). Moreover, the root length of OE50-1# and OE50-2# transgenic lines were significantly longer than that of the control plants (Supplemental Figure S2A,B). The root–shoot ratio also showed similar results (Supplemental Figure S2A,C). Two indicators (electrolyte leakage and MDA) reflecting membrane injury after stress were also adopted, elevating the stress tolerance of the transgenic and control lines. As shown in Figure 5H,I, the electrolyte leakage percentage of the OE50-1#, OE50-2#, and OE50-3# lines was lower compared to the control lines, and the MDA contents were also significantly reduced in the overexpression lines (OE50-1#, OE50-2#, and OE50-3#) under drought stress. Collectively, these results suggest that *HcWRKY50* positively contributes to drought stress tolerance.

3.6. HcWRKY50 Regulated ABA-Induced Stomatal Closure and Decreased the Stomatal Density

Water loss rate is an essential factor for evaluating drought tolerance. In line with the increased drought tolerance of the *HcWRKY50* overexpression lines, the leaves of these transgenic lines showed a lower water loss rate than the leaves of the control lines (Figure 6A). Since water loss is associated with stomatal aperture, the stomatal aperture was further examined. The length/width ratio of stomata is a significant indicator of stomatal closure. Without ABA treatment, the transgenic lines (OE50-1#, OE50-2#, and OE50-3#) displayed the same length/width ratio of stomata as the control lines. However, after 10 μM ABA treatment, the length/width ratios of stomata in the transgenic lines (OE50-1#, OE50-2#, and OE50-3#) were significantly higher than the control lines (Figure 6B). Larger length/width ratios of stomata indicate a smaller stomatal aperture. This means that the stomatal aperture of the *HcWRKY50* overexpression lines was more sensitive to ABA stimuli. In addition, stomatal size and stomatal density were also compared between the *HcWRKY50* transgenic and control lines (Figure 6C). The results showed no significant difference in stomatal size between the transgenic and control lines (Figure 6D). On the

contrary, the stomatal density of the *HcWRKY50* transgenic lines was significantly reduced compared to that of the control lines (Figure 6E). This reduced stomatal density correlates with reduced water loss in the *HcWRKY50* overexpression lines. Together, these results show that the *HcWRKY50* overexpression lines exhibit insensitivity to ABA during seed germination and root growth, increased sensitivity to ABA in stomatal closure, and fewer stomatal numbers. With their good drought tolerance performance, this suggests that *HcWRKY50* could play an essential role in ABA-mediated drought response pathways.

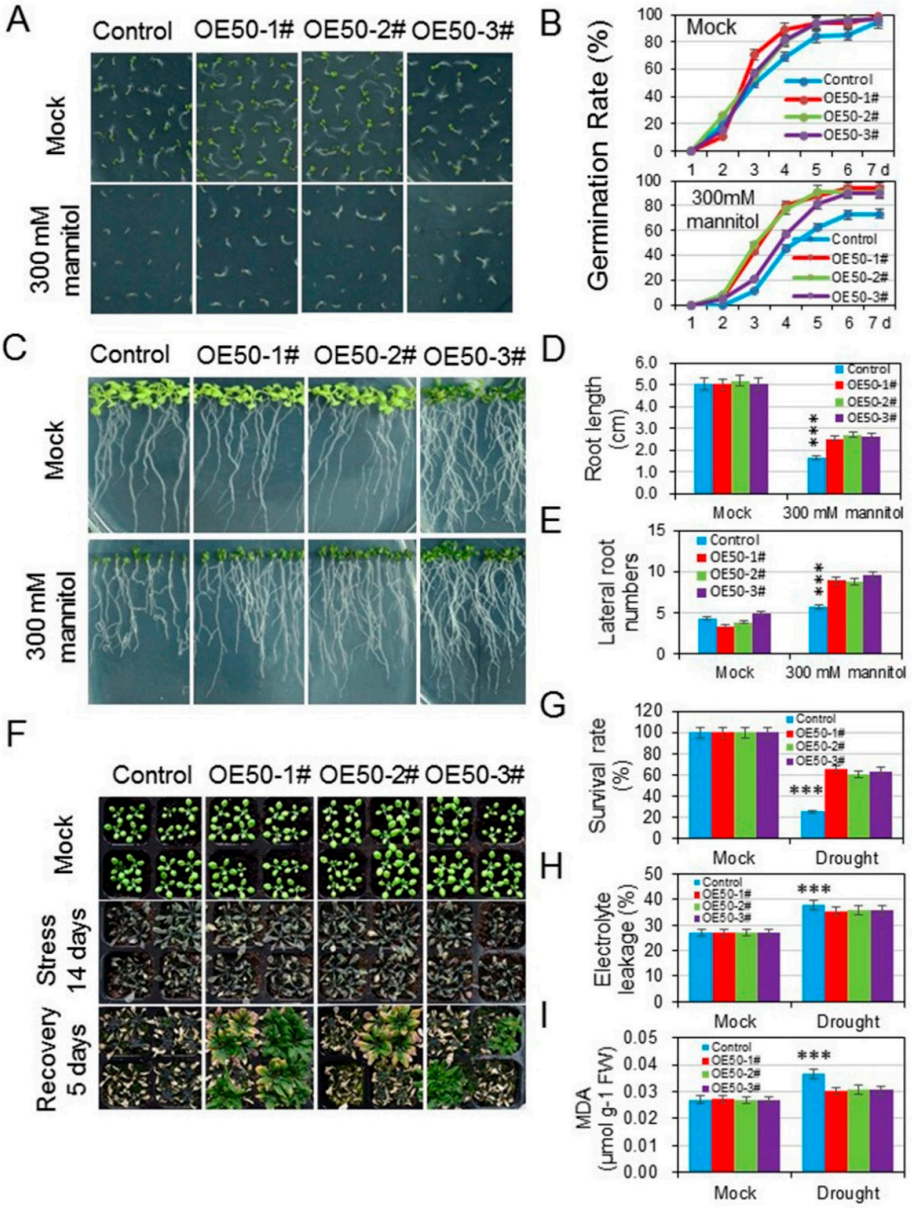

Figure 5. Overexpression of *HcWRKY50* in *Arabidopsis*-enhanced drought tolerance. (**A**,**B**) The seed germination rates of *HcWRKY50* transgenic and control lines was compared when grown on 300 mM mannitol. Photographs were taken 7 d after germination. (**C**–**E**) The root lengths and lateral root numbers of control and transgenic seedlings were photographed and measured after treatment with 300 mM mannitol. (**F**–**I**) Three-week-old control and transgenic *Arabidopsis* seedlings were grown without water for 2 weeks and were then re-watered for 5 days (**F**). Plant survival rate measurements were taken under drought treatment (**G**), and electrolyte leakage (**H**) and MDA contents (**I**) were measured after 14 d of drought stress. All values represent the means of three independent biological replicates; error bars indicate the SD. Significant differences are represented by *** $p < 0.01$, respectively, by ANOVA analysis.

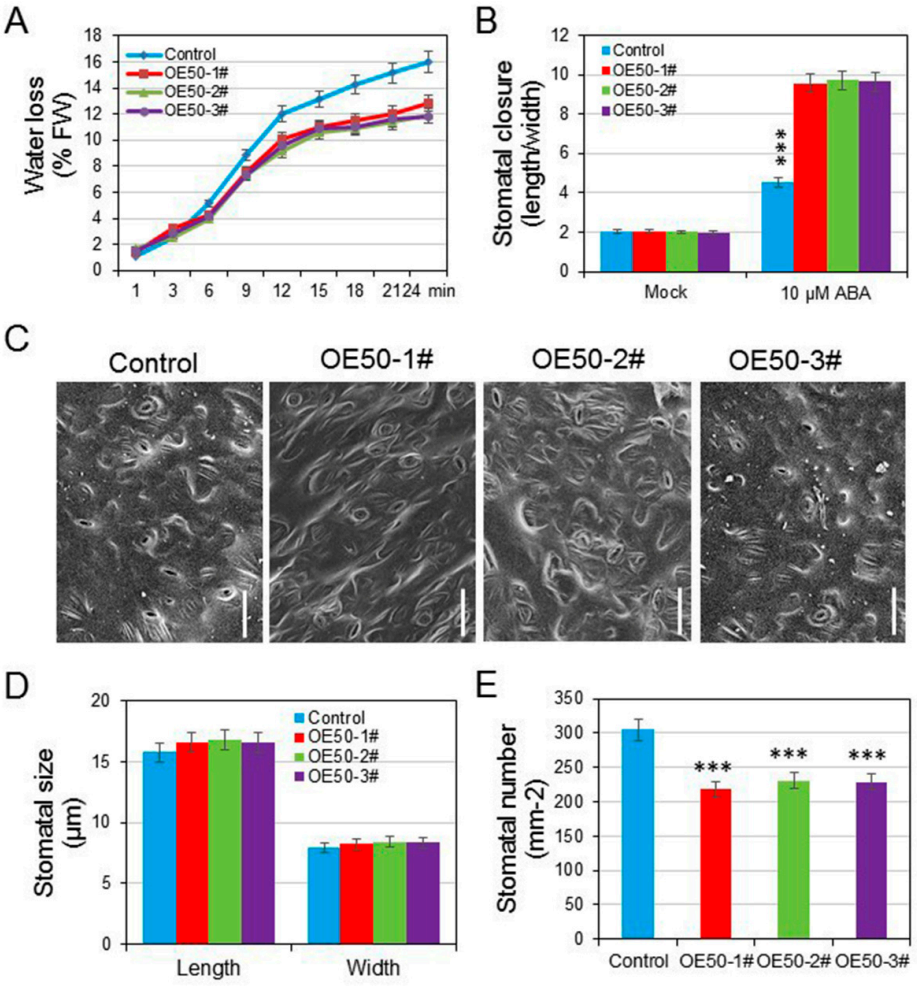

Figure 6. *HcWRKY50* promoted ABA-induced stomatal closure and decreased the stomatal density. (**A**) Water loss from 0.5 g detached leaves of the *HcWRKY50* transgenic (OE50-1#, OE50-2#, and OE50-3#) and control lines. Three replicates were repeated at each time point. (**B**) The stomatal length/width ratio was used to evaluate stomatal closure. (**C**,**D**,**E**) Stomatal size and stomatal density (**D**,**E**) were photographed (**C**) and compared between the control and *HcWRKY50* transgenic lines. Values represent the means (±SD) from three independent replicates. Significant differences are represented by asterisks corresponding to *** $p < 0.01$ by ANOVA analysis.

3.7. HcWRKY50 Positively Regulated the Expression of ABA- and Stress-Responsive Genes

To investigate the functional role of *HcWRKY50* in ABA-mediated pathways, the expression profiles of genes involved in the ABA signaling pathway were examined in the *HcWRKY50* transgenic and control plants. After 7 days of drought treatment, the ABA-responsive genes, such as ABA insensitive 1 and 2 (*ABI1* and *ABI2*), *COR47*, and *NCED3*, were significantly up-regulated in the OE50-1#, OE50-2#, and OE50-3# lines compared to the control lines (Figure 7A,B,E,H). In contrast, ABA-responsive element binding factor 4 (*ABF4*), *COR15A*, and *RD29A* expression were down-regulated in the transgenic lines (Figure 7C,D,I). The stress-related marker genes (*KIN1*, *NCED3*, *RD29B*, and *P5CS*) were also positively regulated in the OE50-1#, OE50-2#, and OE50-3# lines (Figure 7G,H,J,L), while the *STZ* gene was down-regulated (Figure 7K). However, regulation of the *DREB2A* gene did not change significantly (Figure 7F). The higher transcript levels of ABA and stress-responsive genes in the transgenic lines, compared to the control lines, indicated that *HcWRKY50* is essential for ABA-related stress response pathways.

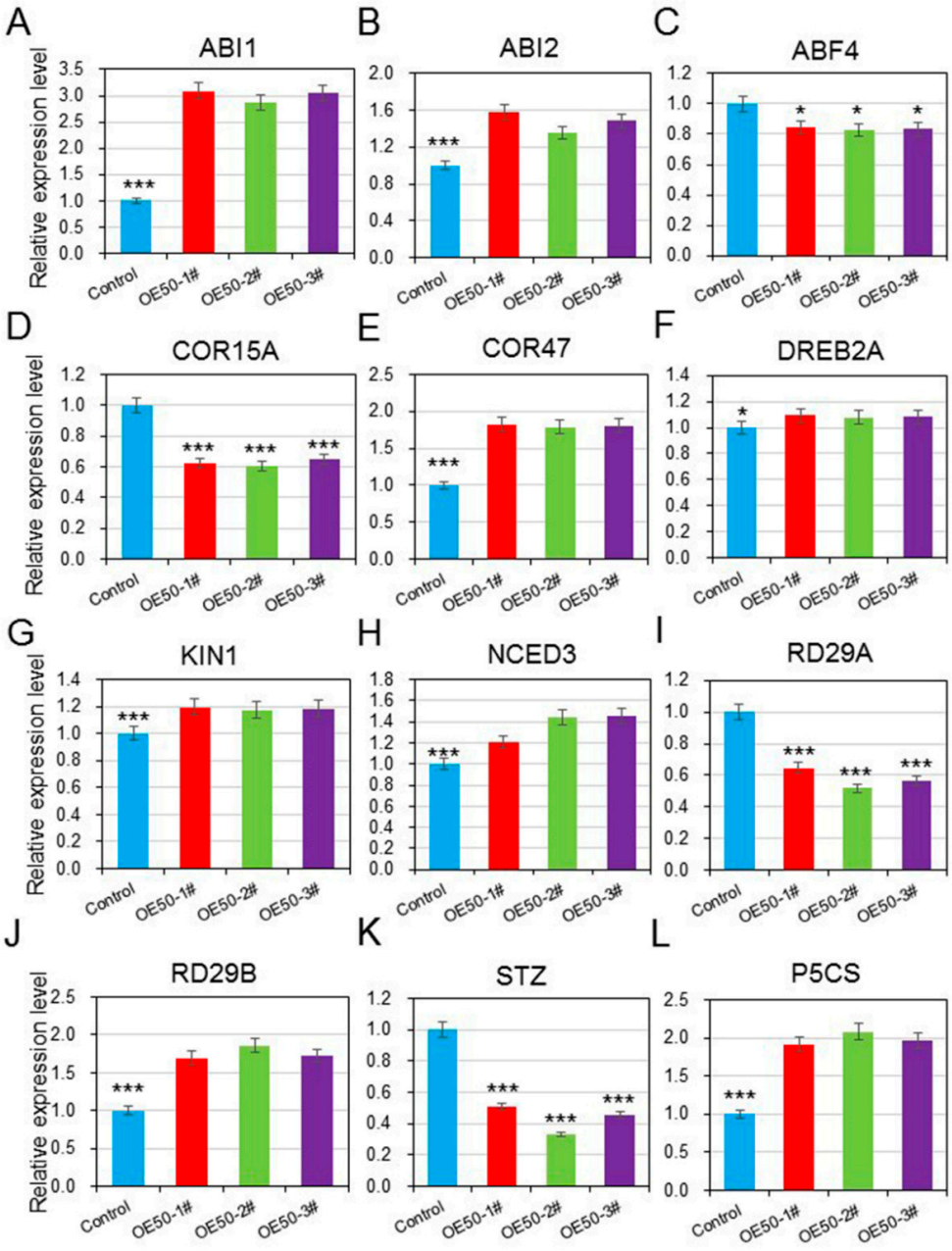

Figure 7. *HcWRKY50* regulated the expression of ABA- and stress-related regulators. (**A**–**E**) ABA-mediated genes (*ABI1*, *ABI2*, *ABF4*, *NCED3*, and *COR47*) were used to analyze ABA-responsive gene expression, (**F**–**L**) stress-responsive genes (*DREB2A*, *KIN1*, *NCED3*, *RD29A*, *RD29B*, *STZ*, and *P5CS*) were selected and their expression levels were evaluated in the OE50-1#, OE50-2#, and OE50-3# lines and in the control lines. Each value represents the mean ± SD of three independent replicates. Significant differences are represented by asterisks corresponding to * $p < 0.05$ and *** $p < 0.01$ by ANOVA analysis.

4. Discussion

Drought stress can affect plant growth and development, and further limits crop productivity and geographical distribution. Meanwhile, plants have evolved various mechanisms to adapt and/or tolerate drought stress. Among these modifications, the most distinct changes include the transcriptional regulation of stress-responsive genes, the accumulation of metabolites and osmoprotectants, the biosynthesis of antioxidants, and the maintenance of water potential [12,23]. Among these physiological traits, stomatal aperture, size, and density are essential indicators of water loss [23]. Furthermore, many

studies have documented that the phytohormone ABA induces stomatal aperture in order to regulate water status in plant cells [13]. Several transcription factors and their target genes are reported to be involved in the ABA-mediated signal transduction and regulation of many molecular and cellular responses to drought stress [2,11,13].

Here, we found an atypical WRKY gene (*HcWRKY50*) in kenaf that contains a WRKYGKK motif and is highly homologous to *AtWRKY50* and *GhWRKY50*. Due to the difficulty of kenaf transformation, to study the functional characteristics of this gene in plants, we transformed the *HcWRKY50* into *Arabidopsis*. *Arabidopsis* has been widely used in transgenic studies for the analysis of gene function in plants for whom gene transformation is difficult [10,12]. In our study, the overexpression of *HcWRKY50* improved drought stress tolerance in transgenic lines, as is evident by their higher survival rate, longer root length, more lateral roots, decreased water loss and reduced stomatal density. The performance of drought tolerance in these *HcWRKY50* transgenic lines was probably due to the reduction of stomatal density, leading to reduced water loss during drought stress. Interestingly, the reduced stomatal density in the *HcWRKY50* overexpression lines did not affect transgenic plant growth and development, although transpiration rate may be influenced. This result was in line with the many previous studies that show that the alteration of stomatal density has no directly concomitant effect on transpiration rate [12,24]. Additionally, *HcWRKY50* promotes drought tolerance by regulating several physiological parameters, such as lowering the MDA contents and electrolyte leakage percentage, thereby protecting the cell membrane from osmotic damage. These results indicate that *HcWRKY50* enhances plant tolerance to osmotic stress by protecting the cell membrane against injury resulting from lipid peroxidation.

Previous studies demonstrate that drought stress is closely associated with ABA-mediated signaling pathways [2,4,11,13]. Critical ABA regulatory factors such as *ABI1*, *ABI2*, and *WRKY40* may inhibit the expression of ABA-responsive genes, and further, may regulate ABA-induced physiological change and development [25–27]. In rice, *OsWRKY50* mediates ABA-dependent seed germination and seedling growth and enhances salt tolerance via ABA-independent pathways [28]. In this present study, *ABI1* and *ABI2* were up-regulated by *HcWRKY50*, whereas *ABF4* was down-regulated in the *HcWRKY50* overexpression lines. Notably, the upregulation of *ABI1* and *ABI2* and/or downregulation of *ABF4* was consistent with the ABA phenotypes that seed germination and seedling growth were insensitive to in the *HcWRKY50* transgenic lines. However, stomatal closure in the *HcWRKY50* transgenic lines indicated that these lines were more sensitive to ABA than the control lines. Previous studies have suggested that ABA biosynthesis and accumulation are associated with drought stress induction [12,13]. Therefore, the better drought tolerance performance of the *HcWRKY50* transgenic lines could be attributed to ABA- and/or drought-induced stomatal closure.

In addition, the stress-responsive marker genes were also regulated in these *HcWRKY50* transgenic lines. For example, the expression of *COR47*, *DREB2A*, *KIN1*, *RD29B*, and *P5CS* were positively regulated compared with expression of these genes in the control lines. Among these stress-induced genes, many studies have revealed that the constitutive overexpression of *DREB2A* may induce *RD29A* and *RD29B* expression to improve stress tolerance in transgenic *Arabidopsis* lines [29]. On the other hand, *RD29A* and *RD29B* genes are the targets of *DREB* genes, encoding hydrophilic proteins and endowing plants with higher resistance to osmotic stress [30,31]. This result is also verified in our study. In this study, the *COR15A*, *RD29A*, and *STZ* were downregulated in the *HcWRKY50* transgenic lines under stress. Previous studies have indicated that downregulation or knockdown of the *STZ* gene could improve plant tolerance to abiotic stress [32,33]. Therefore, the *HcWRKY50* genes most likely improve drought stress in transgenic plants through the direct or indirect regulation of ABA- and stress-related gene expression. However, the relationship between *HcWRKY50* and ABA- and stress-associated genes should be further elucidated to confirm their direct or indirect regulation.

5. Conclusions

Altogether, our study identified a stress-inducible kenaf WRKY transcription factor gene, *HcWRKY50*, and characterized it as a nuclear-localized protein that can respond to salinity, drought, cold, and phytohormones (ABA, SA, JA, and ETH). The overexpression of *HcWRKY50* improved plant tolerance to drought stress through the negative or positive transcriptional regulation of ABA-mediated and/or stress-responsive gene pathways as shown in our hypothetical model (Figure 8). The detailed mechanisms of *HcWRKY50*-mediated drought tolerance require further elucidation. Our study provided new information regarding the WRKY gene response to drought stress in kenaf plants, and offered ways to improve the drought tolerance of kenaf and other crops by genetic manipulation.

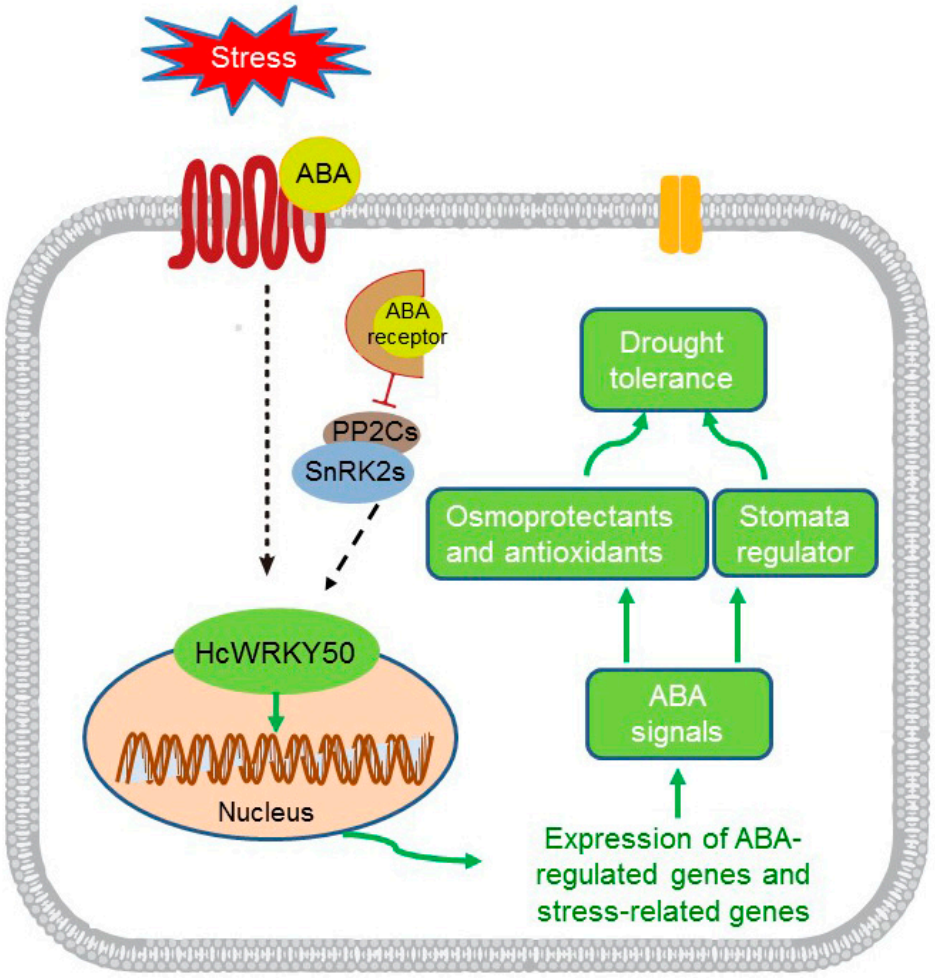

Figure 8. Proposed model for the *HcWRKY50*-mediated regulation of drought stress. Upon drought stress, the ABA–receptor complex senses the stress signal and transduces the stress signal downstream. The activated *HcWRKY50* then binds to promotors of the downstream target genes and triggers a signaling cascade, resulting in regulation of the expression of ABA-regulated genes and stress-related genes, altering ABA signaling and regulating osmoprotectants, antioxidants and stomata regulator to enhance drought tolerance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12051176/s1, Figure S1: Phylogenic analysis of different groups of WRKY proteins from different plants; Figure S2: Overexpression of *HcWRKY50* in *Arabidopsis* facilitated the root development and improved root-shoot ratio; Table S1: The primers used in this study.

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