

SbbHLH85, a bHLH Member, Modulates Resilience to Salt Stress By Regulating Root Hair Growth in Sweet Sorghum

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Abstract

Introduction: bHLH family proteins play an important role in plant stress response. However, the molecular mechanism regulating salt response of bHLH is largely unknown.

Materials and Methods: Here, we investigated the function and regulating mechanism of the sweet sorghum *SbbHLH85* during salt stress.

Results: Results show that *SbbHLH85*, different from its homologs in other species, is a new atypical bHLH transcription factor and is a key gene for root development in sweet sorghum. Knockout of *SbbHLH85* in sorghum by CRISPR-Cas9 results in the inhibition of root development. Overexpression of *SbbHLH85* resulted in significantly increased number and length of root hairs by participating in ABA and auxin signaling pathways, which can increase the absorption of Na^+ . While *SbbHLH85* plays a negative regulatory role in salt tolerance of sorghum. Through screening yeast two-hybrid library, we identified a potential interaction partner of *SbbHLH85* that is phosphate transporter chaperone PHF1, which modulates the distribution of phosphate. Both yeast two-hybrid and BiFC experiments confirmed interaction between *SbbHLH85* and PHF1.

Discussion: Based on these results, we suggest that the increase of Na^+ content and the decrease of Pi content result in the salt sensitivity of transgenic sorghum.

Introduction

The problem of soil salinization exists widely in the world. Salt stress caused by soil salinization affects the growth, development and harvest yield of plants, and in serious cases salt stress even leads to plant death (Asano et al., 2012; Sui et al., 2017). The most cost-effective way to utilize salinized soil is to develop salt-tolerant crop varieties based on well-established knowledge of molecular mechanisms for plant salt resistance, therefore promoting agricultural production. Sweet sorghum (*Sorghum bicolor* (L.) Moench) is a crop with high sugar content and high biomass yield, which has the laudatory name of second-generation sugarcane (Sui et al., 2015). In today's world, it is an important food, feed and energy crop. In addition to high biomass, sweet sorghum also has tolerance to various abiotic stresses, especially to salt (Schnippenkoetter et al., 2017). Many genes have been found to play an important role in regulating salt tolerance in sweet sorghum (Song et al., 2020; Wang et al., 2014b; Yang et al., 2018; Zheng et al., 2011). It is believed that the outcomes from studying characteristics of these genes can be applied to improve other crops for salt tolerance, which also is of great significance for understanding plant growth and development in saline alkali environment.

Salt stress increases the level of sodium ion (Na^+) and potassium ion (K^+), thus reducing the level of nutrient elements (e.g., N, P) and causing the imbalance of nutrient distribution in plants (Colla et al., 2008; Wang et al., 2020). It has been reported that the phosphorus content in pomelo and orange decreases significantly in response to salt stress (Ma et al., 2005). Phosphorus (P) is one of the indispensable elements required for plant growth and development (Dalen, 2012; Miao et al., 2009).

Phosphorus transfer between different tissues and subcellular organelles in plants are facilitated with a series of transport proteins having phosphorus transfer activity (Deng et al., 2017). The amount of phosphotransporters on the plasma membrane directly determines the absorption and distribution efficiency of phosphorus in plants (Mudge et al., 2002). PHT1 encodes a phosphoric acid (Pi) transporter, which is regulated by the plant-specific phosphate transport chaperone PHF1 during its transport to plasma membrane, and plays a fundamental role in the acquisition and reactivation of Pi (González et al., 2005; Shin et al., 2004).

The bHLH transcription factor proteins have a highly conserved basic/helix-loop-helix special domain, namely bHLH domain (Li et al., 2018; Maia et al., 2012; Yao et al., 2018). It has been found that bHLH transcription factors are involved in salt stress response (Chen et al., 2018; Chen et al., 2017; Zhou et al., 2009). Expression of both *AtbHLH92* and *OrbHLH001* was induced by salt, and their overexpression improved salt tolerance in plants (Jiang et al., 2009; Li et al., 2010). When the bHLH transcription factor *AtNIG1* was knocked out, the resulting mutant became more sensitive to salt stress in *Arabidopsis*. *AtNIG1* regulates salt tolerance by binding specifically to the E-box sequences in the promoter regions of many salt stress-related genes (Kim and Kim, 2006).

Studies have shown that the plant bHLH transcription factors are also involved in root hair formation (Gajewska et al., 2018; Yan et al., 2014). Root hair is a tubular protrusion formed by extension of root specific epidermal cells, which contains enzymes and nutrient transporters involved in nutrient absorption (Menand et al., 2007; Wei and Li, 2018). Root hairs increase the contact area between plants and soil and determine the efficiency of root absorption of water and nutrients (Krasilnikoff et al., 2003). The *Arabidopsis* bHLH transcription factors GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) have redundant functions. The number of root hairs in the single mutants of these two genes increased slightly, whereas the double mutants increased root hair number significantly (Bernhardt et al., 2005). The polar growth of root hair is initially triggered by *RHD6/RSL1* of the bHLH family, and then its elongation is activated by the expression of *RSL4/RSL2* (Vijayakumar et al., 2016). *RSL4* is essential for root hair elongation in *Arabidopsis*, which controls the final root hair cell size (Zhu et al., 2020). *RSL2* affects ROS production and root hair growth (Rymen et al., 2017).

In this study, we first cloned a bHLH gene, *SbbHLH85* in sweet sorghum, which is induced by salt stress and ABA. Some reports have shown involvement of the bHLH transcription factor in salt stress response and root hair formation. In order to investigate the molecular mechanism on how *SbbHLH85* regulates root hair development in response to salt stress in sweet sorghum, we first overexpressed *SbbHLH85* in sweet sorghum and *Arabidopsis*. Results showed that different from its homologs in other species, the sweet sorghum *SbbHLH85*, is a new atypical bHLH transcription factor and is a key gene for root development in sweet sorghum. Knockout of *SbbHLH85* in sorghum by CRISPR-Cas9 results in the inhibition of root development. Overexpression of *SbbHLH85* in sorghum and *Arabidopsis* significantly increase the number and length of root hairs. While salt resistance was significantly lower in those overexpression lines. Overexpression of *SbbHLH85* can influence the expression of the genes involved in ABA and auxin signal transduction (PYL and PIN3), peroxidase (PERs), and root hair development and

receptor-like proteins (RLKs). In addition, *SbbHLH85* interacts directly with *SbPHF1*, a phosphate transporter chaperone protein in sweet sorghum, affecting the transport of Pi. Based on these results, we suggest that *SbbHLH85* participates in regulation of ABA and auxin signal transduction pathways and distribution of nutrients to affect the development of root hairs, thus affecting the absorption of Na⁺ and the content of ROS, and mediating plant salt response.

Materials And Methods

Plant materials and growth conditions

In this study, wild type *Arabidopsis* was used as a control. The WT and mutant seeds were evenly seeded on 1/2 MS medium with corresponding antibiotics. The culture dish was placed at low temperature for 3 days and cultured in the tissue culture room (22°C-16 h light/18°C-8 h dark), and the screened positive seedlings were transferred to nutrient soil for further cultivation until the seeds mature. The harvested seeds were seeded with the same operation in 1/2 MS medium with salt, and the phenotype of the plants was observed.

Sweet sorghum (*Sorghum bicolor* (L.) Moench) cultivar M-81E was used in this experiment. The seeds of sweet sorghum were cultured in sand. Tap water was irrigated before emergence and 1/2 Hoagland nutrient solution was poured every day after emergence. When the seedlings grew to three leaves and one heart, Hoagland nutrient solution containing 0, 50, 100, 150 and 200 mM NaCl was applied to sweet sorghum under salt stress.

Cloning, bioinformatics and expression analysis of *SbbHLH85*

The *SbbHLH85* CDS sequence is obtained by comparing the *AtRSL2* sequence on the Ensembl website (<http://ensembl.gamene.org/>). Online website NCBI (<https://www.ncbi.nlm.nih.gov/>), SMART (<http://smart.embl-heidelberg.de/>) and software MEGA6 for nucleic acid and protein sequence analysis and the construction of evolutionary trees.

Root tissue of sweet sorghum was used for cloning and expression of *SbbHLH85*. Samples were immediately frozen in liquid nitrogen and stored -80°C before analysis. Three biological replicates were performed for RT-qPCR. Sweet sorghum internal reference gene *Sbactin-1* as a control, the primers are listed in supplementary table 1.

Subcellular localization of *SbbHLH85* protein

Using KpnI and BamHI sites to insert cloned *SbbHLH85* CDS into pCAMBIA1300-35S-sGFP vectors to produces 35 S-*SbbHLH85*-GFP constructs. It was transferred into *Agrobacterium tumefaciens* GV3101 and used to infect tobacco epidermal cells. GFP signal was observed by two-photon laser scanning confocal microscope (TCS S8MP, leica, germany). 35S: GFP transgenic tobacco was used as a localization control for expression in cytoplasm/nucleus. The primers are listed in supplementary table 1.

Generation of transgenic plants

To produce overexpressed *SbbHLH85* of *Arabidopsis*, *SbbHLH85* genes were linked into pROKII vectors with XbaI and KpnI and transferred to *Agrobacterium tumefaciens* GV3101 to obtain *SbbHLH85* overexpressed plants by infecting WT inflorescence. The transgenic *Arabidopsis* plants were screened with kanamycin (50 g/mL) and verified by RT-PCR. Insert the full-length cDNA of *SbbHLH85* into the pMWB110 vector through BamHI and KpnI sites to obtain pMWB110-*SbbHLH85* vector. pMWB110-*SbbHLH85* vector was introduced into sweet sorghum by *Agrobacterium*-mediated transformation. PCR, herbicide (glufosinate) spraying and bar rapid detection kit were used to detect transgenic plants. The primers used are listed in supplementary table 1.

Quantification of biomass, MDA content, Na⁺ and K⁺ content

For the measurement of biomass, we first take the whole plant out of the pot, wash it, weigh it and record it as fresh weight. Then they were dried in the oven for 7 days and then weighed again as dry weight. Fresh weight and dry weight of each treatment were measured 5 times (Song et al., 2019). The MDA contents were determined as described by Ma (Ma et al., 2013). As follows: the leaves of 0.2 g of each line were weighed, 5 ml 0.1% trichloroacetic acid (TCA) was added for grinding. Mix the homogenate with 5 ml 0.5% thiobarbituric acid (TBA), boil for 10 min and take it out, cool to room temperature, 3000 rpm/min centrifuge 15 min, absorb the supernatant and measure its volume. The absorbance of the solution at wavelength 532 nm and 600 nm was determined by UV spectrophotometer. Blank control was 0.5% TBA solution. For the determination of Na⁺ and K⁺, the specific steps are described by Song: each line treated with 0 and 100 mM NaCl. After 10 d, put 0.3 g roots in 5 ml ddH₂O, boil 2 h, filter plant residue and volume to 10 ml. The content of Na⁺ and K⁺ of each treated lines was determined by flame spectrophotometer (Song et al., 2020).

DAB and NBT staining

When *Arabidopsis* seedlings growing for about a month, treated with 0 or 100 mM NaCl in 1/2 concentration Hoagland solution for 48 h. Before staining leaves of rosette leaves of WT and overexpressed plants with the same growth, put in DAB or NBT dye solution, immerse the dye in the leaves, placed in the dark for more than 12 hours. Then put in the bleach (3:1:1 ethanol: acetic acid: glycerol), boil in boiling water for 10-15 minutes for decolorization, observe the color change of the blade and take the image.

Root hair experiment

The homologous gene of *SbbHLH85* is *AtRSL2* and *AtRSL4* in *Arabidopsis*. *AtRSL2* is closely related to root hair development and elongation, and it also has a functional redundancy gene *AtRSL4*. So we selected WT, M-81E, overexpression (*At-OX4*–*At-OX13*, *Sb-OX1*, *Sb-OX3*, *Sb-OX6*, *Sb-OX7*), *RSL2* mutant (*rsl2-1*–*rsl2-3*), *RSL4* mutant (*rsl4*), and *RSL2* and *RSL4* double mutant (*rsl2rsl4*) as experimental subjects, the main root hair development and root hair elongation were tested. Each *Arabidopsis* seed was on

demand in 1/2 MS medium. The sweet sorghum seeds are hydroponic. After 7 d in the lab, development and elongation of root hairs at the root tip 5 mm from the main root of each line were observed under electron microscope. The microscope was magnified by 40 times.

RNA-seq assay

The roots of WT, overexpression, mutant and double mutant were collected and preserved in liquid nitrogen. RNA-seq and differential gene expression analysis were carried out by BMK. Transcriptome analysis of 24 samples was completed, and 156.37 Gb of clean data was obtained. Use the HISAT2 system to sequenced the clean reads of each sample with the designated reference genome, and the reads on was assembled by StringTie comparison.

After the comparison and analysis, the reads on the sample were assembled and quantified by StringTie comparison. Based on the comparison results, the gene expression was analyzed. StringTie uses FPKM (fragments per kilobase of script per million fragments mapped) as an index to measure the expression level of transcripts or genes. The differentially expressed genes were identified according to their expression levels in different samples, and through GO database, KOG database and KEGG database for function annotation and enrichment analysis. KEGG pathway analysis was carried out for the common differential genes in each comparison group, and the heat map clustering analysis was performed for the expression level of selected genes, which were all completed on the platform of BMKCloud.

RT-PCR validation of genes related to plant salt resistance and root hair development

We extracted RNA from root tissues of WT, At-OX13, *rs/2-3* and *rs/2rs/4* lines which had been growing for about one month, and then reverse them into cDNA and then conduct RT-PCR. The primers used are in supplementary table 2. In sweet sorghum, M-81E and Sb-OX1 were treated with 0 mM and 100 mM NaCl for 48 h. Then RNA was extracted and transformed into cDNA, RT-PCR was performed. The primers used are in supplementary table 3.

Yeast two-hybrid experiment

The CDs of *SbbHLH85* were cloned into the bait vector pGBKT7 to obtain *SbbHLH85*-BD, and then transformed into yeast strain Y2HGold (Clontech). After detecting that the gene has no self-activating activity, we constructed a yeast two-hybrid library of sweet sorghum, and hybridized and screened according to the matching scheme described in Clontech's matchmaker tmgoldyeasttwo hybrid user's manual. After screening, the interaction between SbPHF1 and *SbbHLH85* was verified by yeast two-hybrid experiment. The full-length coding sequence (CDS) of *SbPHF1* was cloned into pGADT7 to obtain *SbPHF1*-AD, and *SbbHLH85*-BD and *SbPHF1*-AD were co-transformed into Y2HGold. Then the growth of the colony on the corresponding medium was observed. See supplementary table 1 for the primers used in yeast two-hybrid.

BiFC experiment

The CDs of *SbbHLH85* and the N-terminal of pSPYNE-35S were fused to obtain *SbbHLH85*-N-YFP, and the CDs of *SbPHF1* and the C-terminal of pSPYNE-35S were fused to obtain *SbPHF1*-C-YFP. The obtained plasmid was introduced into *Agrobacterium tumefaciens* (GV3101), and the method of transient transformation was used to infiltrate the tobacco. After 48 hours of normal culture, the fluorescence was observed under a confocal laser scanning microscope (Olympus). Supplementary table 1 lists the primers of BiFC.

Statistical methods

The statistical results are described as mean \pm standard deviation. Use the SPSS ver. 17.0 statistical software to analyze the data. One-way ANOVA was used as the designated package. Different letters indicate that there is a significant difference between the average (0.05) of the Duncan test.

Accession Numbers

The sequence data in this paper can be found in TAIR, NCBI or Ensembl database. The mutant numbers are as follows: *rs/2-1* (SALK_048849), *rs/2-2* (SALK_048857), *rs/2-3* (SALK_101872), *rs/2-4* (SALK_143203). The accession numbers are as follows: *SbbHLH85* (SORBI_3008G147800), *SbPHF1* (SORBI_3002G060900), *SbPYL4* (XM_002465362), *SbPIN3* (XM_002436716), *SbRSH2* (XM_021465244), *SbRSL4* (XM_002464182), *SbRHL1* (XM_021450304), *SbPER3* (XM_002437414), *SbGLO1* (XM_002466296), *SbPER4* (XM_002441658), *SbPER35* (XM_002466362), *SbRLK1* (XM_021446421), *SbRLK2* (XM_021466234), *SbRLK8* (XM_002455281), *AtPIN3* (AT1G70940), *AtSAUR50* (AT4G34760), *AtPYL6* (AT2G40330), *AtPRX33* (PRX33), *AtPRX37* (AT4G08770), *AtRCI3* (AT1G05260), *RSL4* (AT1G27740), *RHL1* (AT1G48380), *RHS18* (AT5G22410), *RSH3* (AT1G54130), *RHS15* (AT4G25220), *RHS19* (AT5G67400), *RHD3* (AT3G13870), *RHS17* (AT4G38390), *RHD6* (AT1G66470), *RHS10* (AT1G70460), *RHS12* (AT3G10710), *CLH1* (AT1G19670), *DGK5* (AT2G20900), *CYP97C1* (AT3G53130), *OSM34*(AT4G11650), *PR4*(AT3G04720), *PDC1*(AT4G33070).

Results

***SbbHLH85* is a salt stress responsive gene**

As a preliminary characterization of the function of *SbbHLH85*, we studied its conserved domain, expression pattern and subcellular localization. First, the phylogenetic tree analysis based on protein sequence showed that *SbbHLH85* was closely related to *A0A2S3HF40* in *Panicum hallii* and *A0A3L6RBM2* in *Panicum miliaceum*. *SbbHLH85* shares 33.15% and 32.18% homology with the *Arabidopsis* genes *AtRSL2* and *AtRSL4*, respectively (Figure 1A). After analyzing the domain structure, we found that they all have a bHLH domain at the C end (Figure 1B). The *SbbHLH85* gene contains a 951-bp coding sequence that encodes 316 amino acids. Different from *RSL2* and *RSL4*, the *SbbHLH85* protein contains a bHLH domain between amino acids 238 and 287, which contains a Q5-A9-R13 motif harboring a glutamine at the fifth position, alanine at the ninth position and arginine at the thirteenth position (Figure 1C; Supplementary Figure 1). By measuring the relative abundance of *SbbHLH85* in roots

of M-81E under different salt stress, the expression pattern of *SbbHLH85* was further studied. RT-PCR results showed that the expression of *SbbHLH85* in sweet sorghum roots decreased with the increase of salt concentration, dropping to the lowest level at 100 mM NaCl (Figure 1D). The subcellular distribution of *SbbHLH85* was studied by fusing *SbbHLH85* with the green fluorescent protein (GFP). In the lower epidermal cells of tobacco, signals were detected in the nucleus (Figure 1E). Therefore, *SbbHLH85* is a bHLH transcription factor that is negatively induced by salt and localized in the nucleus.

***SbbHLH85* actively regulates the development of root hairs**

To explore the effect of *SbbHLH85* on root hair development, we generated *SbbHLH85* overexpression lines At-OX4 and At-OX13 controlled by the CaMV 35S promoter in *Arabidopsis* (Supplementary Figure 2). For the knockout of *SbbHLH85* in sorghum by CRISPR-Cas9 results in the inhibition of root development, we use the *AtRSL2* mutants *rs12-1* and *rs12-3*, and the *AtRSL4* mutant *rs14* to test the function of *SbbHLH85* on root development. All the *Arabidopsis* mutants were purchased from the TAIR website (<https://www.arabidopsis.org/>). The double mutant *rs12rs14* was provided as a gift by Professor Hongwei Guo of Southern University of Science and Technology. Then, the complementing lines Crsl2-1, Crsl2-3, Crsl4, Crsl2rs14 were created by expressing *SbbHLH85* in *rs12-1*, *rs12-3*, *rs14* and *rs12rs14*, respectively. Figure 2A shows that the number and length of root hairs in the overexpression lines were the largest and the longest, whereas the control mutants *rs12-1*, *rs12-3* and *rs14* had fewer and shorter root hairs. Further, there was no root hairs observed in the double mutant *rs12rs14*. Thus, *SbbHLH85* is able to complement the root hair defect in the single and double mutants (Figure 2A-C).

To further explore the effect of *SbbHLH85* on root hair development, we constructed *SbbHLH85* overexpression lines controlled by CaMV 35S promoter in sorghum, and detected the relative expression of *SbbHLH85* in each line by RT-PCR. Compared with the wild type, *SbbHLH85* was highly expressed in Sb-OX1, Sb-OX3, Sb-OX6 and Sb-OX7 (Supplementary Figure 3). It was also found that *SbbHLH85* overexpression increased the number and length of root hairs in sweet sorghum (Figure 2D-F). We also attempted the gene-editing tool CRISPR-Cas9 to knockout *SbbHLH85* in sweet sorghum. However, we repeatedly observed inhibition of root growth in the transgenic plants carrying the *SbbHLH85*-Cas9 vector (Supplementary Figure 4) and as a result, we was unable to acquire the knockout mutants of sorghum. These findings suggest that *SbbHLH85*, different from *RSL2* and *RSL4* in *Arabidopsis*, is necessary for root development in sorghum.

***SbbHLH85* negatively regulates salt stress response**

To explore the role of *SbbHLH85* in regulating salt stress response in plants, we tested each of the *Arabidopsis* overexpression lines. We studied the effects of salt stress on plant growth, including germination rate and seedling survival. For the germinating plants, we sowed seeds of each line on 1/2 Murashige and Skoog (MS) medium containing 0, 100 or 150 mM NaCl. It was found that overexpression plants grew more slowly than WT and the mutant lines on the salt medium (Figure 3A). By measuring the germination rate and root length of each line, it was found that the germination rate and root length of overexpression plants were higher than that of WT and mutants no matter under the condition of non-

stressed or salt stress (Figure 3B, C). Under abiotic stress, plants are exposed to ion stress, oxidative stress and osmotic stress at the same time. Next, we compared the germination rate and root length of each lines under different stress conditions. The growth of seedlings in NaCl, LiCl and mannitol media showed different degrees of slow growth (Figure 3D). Among them, NaCl treatment resulted in the worst growth, the shortest taproot length and the lowest germination rate (Figure 3E, F). We also studied the salt tolerance of different lines at seedling stage. Salt stress reduced the biomass of each line, but the fresh weight and dry weight of mutant lines decreased less (Figure 3G, H). The contents of MDA increased more in WT and overexpression plants, but less in mutant lines (Figure 3I). Ion content analysis under salt stress showed that Na⁺ content in overexpression lines were higher than that in WT control (Figure 3J), while K⁺ content was opposite (Figure 3K). Diaminobenzidine (DAB) solution can react with H₂O₂, which can reflect the content of H₂O₂ in plants. Similarly, nitroblue tetrazolium (NBT) staining reflects the content of O₂⁻. Under salt stress, DAB and NBT staining of overexpression lines were the deepest, which indicated that overexpression of *SbbHLH85* caused more ROS production in plants (Figure 3L, M). We also transformed *SbbHLH85* to the mutant lines to investigate if *SbbHLH85* can rescue the salt sensitive phenotype. The results showed that the physiological indexes of the complementary lines Crsl2-1, Crsl2-3, Crsl4, and Crsl2rsl4 under salt stress recovered (Figure 4). Overall, these findings suggest that ectopic overexpression of *SbbHLH85* in *Arabidopsis* may affect the homeostasis of Na⁺ and K⁺, the content of ROS and the degree of membrane lipid peroxidation to improve salt tolerance.

To better understand the function of *SbbHLH85* in salt stress, we further tested the salt tolerance of the *SbbHLH85* overexpression lines in sweet sorghum. Under salt stress, the overexpression lines of sweet sorghum showed obvious weak growth, yellow leaves and even curly symptoms (Figure 5A). Next, we studied the biological processes of salt stress. The biomass accumulation of overexpression lines was lower than that of M-81E (Figure 5B-E). Under salt stress, the content of Na⁺ in overexpression lines increased significantly and the content of K⁺ decreased (Figure 5F-H). Compared with the WT of M-81E, MDA content of overexpression lines increased significantly after salt treatment (Figure 5I). This is consistent with the conclusion from *Arabidopsis* that *SbbHLH85* participates in salt stress response.

Transcriptome analysis of plants with altered *SbbHLH85* level

To reveal the molecular mechanism of *SbbHLH85* regulating plant response to salt stress and root hair development, we analyzed the RNA-seq data of WT, overexpression, mutant and double mutant plants under control and NaCl treatment. In order to verify the accuracy of transcriptome, we selected 22 representative genes for RT-PCR verification, and found that the transcriptome data were in high quality (Figure 6E). In the RNA-seq experiment, we used 48 h NaCl treatment and three biological replications. Under salt stress, there were 597 differentially expressed genes (DEGs) in WT, 244 DEGs in overexpression lines, 204 DGEs in single mutants and 278 DGEs in double mutants. We intersected these DGEs and got 156 DGEs (Figure 6A). Then, we used hierarchical clustering and correlation analysis to analyze the expression patterns of 156 DEGs genes. GO (Gene Ontology Consortium) analysis showed that these differential genes were involved in biological process, molecular function and cellular component, among

which they are mainly involved in biological process (Figure 6B). KEGG (Kyoto Encyclopedia of Genes and Genomes) and KOG (Cluster of Orthologous Groups of proteins) analysis showed that these differential genes were mainly related to phenylalanine metabolism, hormone signal transduction and secondary metabolite metabolism (Figure 6C, D). After further analysis of the genes in these pathways, we found that they are mainly involved in auxin signaling pathway (*AtPIN3*, *AtSAUR50*), ABA signaling pathway (*AtPYL6*), and also involved in the production of peroxidase (PER) (*AtPRX33*, *AtPRX37*, *AtRCI3*, *AT4G08780*) and receptor like kinases (RLK) (*AT4G00970*, *AT4G04570*) (Figure 6F) and development of root hairs (Figure 6G). This is consistent with our previous conclusion that *SbbHLH85* participates in salt stress and affects the development of root hair.

For the *SbbHLH85* has a unique Q5-A9-R13 motif, it can't directly bind to the promoter of the downstream genes. In order to further study the molecular mechanism of *SbbHLH85* regulating salt response and root hair development of sweet sorghum, all we can do is to identify all sweet sorghum genes homologous to the *Arabidopsis* ones. These genes are annotated as auxin signal, ABA signal, root hair development, PER and RLK. This study identified one ABA signal transduction gene (*SbPYL4*), one auxin signal transduction gene (*SbPIN3*), three root hair development related genes (*SbRSH2*, *SbRSL4*, *SbRHL1*), four PER genes (*SbPER3*, *SbGLO1*, *SbPER4*, *SbPER35*) and three RLK related genes (*SbRLK1*, *SbRLK2*, *SbRLK8*) in sweet sorghum (Figure 6H). This is consistent with the previous results, indicating that *SbbHLH85* is involved in root hair development and salt tolerance of sweet sorghum.

Interaction of *SbbHLH85* with *SbPHF1*

Increasing lines of evidence show that the bHLH proteins act by forming protein complexes with other interacting proteins (Abe et al., 2003; Oh et al., 2007). In order to find the potential chaperone of the bHLH85 protein, we used yeast two-hybrid system to screen for interactors. Firstly, the BD domain of pGBKT7 and bHLH85 was fused as bait. After we proved that bHLH85 had no self-activating activity (Figure 7A), the cDNA library containing the prey protein insert fused with GAL4-AD was used to co-transform the yeast cells with *SbbHLH85*-BD. Three colonies were positive for X-a-gal and Aurobasidin (AbA). Among these candidates, only the binding of *SbPHF1* and *SbbHLH85* was stable. *SbPHF1* encodes a SEC12-like protein and is homologous to *AtPHF1*. As a chaperone of phosphate transporter PHT1, *SbPHF1* helps PHT1 transport to plasma membrane. To confirm their interaction in yeast, *SbPHF1*-AD vector was co-transformed into Y2H competent state with *SbbHLH85*-BD vector. As shown in Figure 7B, the experimental group grew normally on the medium which was added with X-a-gal but lacked isoleucine and tryptophan (SD/-L/-T/X), and the colony turned blue. Similarly, the experimental group grew normally on the medium added with the X-a-gal and AbA but lacking isoleucine, tryptophan, histidine and adenine (SD/-L/-T/-H/-Ade/X/A) and the colony turned blue. This shows that *SbbHLH85* has a strong interaction with *SbPHF1* (Figure7B).

To determine whether the interactions also exist in plant cells, we then used a BiFC system to verify the above observation. The agrobacterium *tumefaciens* of *SbPHF1*-C-YFP and *SbbHLH85*-N-YFP were mixed and transformed into tobacco leaves. At the same time, the empty carrier was combined with each fusion

structure and injected into tobacco leaves. After incubation for 2 days, YFP signal was observed with a fluorescence microscope. The co-transformed samples showed YFP fluorescence in the nucleus, while none of the control samples showed any YFP signal (Figure 7C). This demonstrates that *SbbHLH85* and *SbPHF1* were co-localized and interacted in plant nucleus.

Discussion

How plants find a balance between environmental stress and plant growth is a new and important research topic (Monlau et al., 2015). A large number of indirect evidences have confirmed that the bHLH proteins are involved in the process of salt tolerance in plants (Babitha et al., 2015; Long et al., 2010; Waseem et al., 2019). Here, the overexpression of *SbbHLH85* is proved to increase the salt sensitivity of sorghum. The mechanism is possibly that *SbbHLH85* and *SbPHF1* interaction affects the development of root hair through ABA and auxin signal transduction pathways, and then affect the distribution of nutrients in plants, thus regulating salt tolerance of sweet sorghum. This observation enriches the regulation network of salt stress response, and it is of great significance for improving crop productivity under adverse environmental conditions.

***SbbHLH85*, different from its homologs in other species, is a new atypical bHLH transcription factor**

The bHLH transcription factors belong to the second largest family of transcription factors in plants, named so for its bHLH domain (Herbst and Kolligs, 2008). It plays a key role in plant growth and abiotic stress (Jiang et al., 2009). In animals, bHLH transcription factors can be divided into six categories: A-F (Wang et al., 2010). Most bHLH transcription factors in plants belong to class B. only 11% of plant bHLH proteins have a conserved motif: Q5-A9-R13, which is not found in animals (Sailsbery and Dean, 2012). With the development of molecular biology, more and more bHLH transcription factors have been found, especially the identification of new atypical bHLH transcription factors, which makes this family more diverse. By analyzing the protein sequence of *SbbHLH85* and bHLH transcription factors in other species, we found that *RSL2* and *RSL4* is the most closely related gene in *Arabidopsis*. But different from *RSL2* and *RSL4*, *SbbHLH85* has a Q5-A9-R13 motif as shown in Supplemental Figure1, indicating that *SbbHLH85* is a new atypical bHLH transcription factor. On the one hand, it enriches the diversity of bHLH family in sweet sorghum. On the other hand, it indicates that *SbbHLH85* may play a unique role in promoting salt tolerance and growth of sweet sorghum, which may be of great significance for crop improvement.

***SbbHLH85* affects plant salt tolerance and root hair growth through ABA and auxin signal transduction pathways**

ABA and auxin signal transduction pathways play an important role in regulating plant salt tolerance (Huang et al., 2012; Min et al., 2015; Sun et al., 2016). *PYR/PYL* were found in 2009 to act as ABA receptors and bind to PP2C family members represented by *ABI2* and *ABI1* to regulate phosphorylation of downstream protein kinases and thus initiate the ABA signal transduction pathway (Fujii et al., 2009; Nishimura et al., 2009; Raghavendra et al., 2010; Umezawa et al., 2009). *PYL4* and *PYL6* belong to the

second subgroup of ABA receptors and participate in plant salt stress response in *Arabidopsis* (Aleman et al., 2016). *PbPYL4* in *Pyrus pyrifolia* is homologous to *PYL4* from *Arabidopsis*, and the expression of *PbPYL4* in roots, stems and leaves was activated by salt stress, which suggests its involvement in plant response to salt stress (Wang et al., 2014a).

Existing studies have shown that auxin regulation processes affect plant growth and development by regulating other gene expression, including the small auxin-up RNA (SAURs) transcription factor family (Van et al., 2017). The overexpression of *TaSAUR75* increased the root length and survival rate, thus improving the salt tolerance of *Arabidopsis* (Guo et al., 2018). The overexpression of *TaSAUR78* enhanced the salt tolerance of transgenic *Arabidopsis* and reduced the accumulation of ROS under salt stress (Guo et al., 2019). Similarly, the PINFORMED (PIN) protein family is a recognized auxin output carrier responsible for transporting auxin from the intracellular to the extracellular (Harrison and Masson, 2008). The *PIN1*, *PIN2*, *PIN3*, *PIN4* and *PIN7* genes were isolated from *Arabidopsis* and were shown to be involved in root orientation, root elongation and growth, and plant salt tolerance (Ganguly et al., 2012; Lewis et al., 2011; Liu et al., 2015; Lv et al., 2018; Schlicht et al., 2008; Sun et al., 2008).

In recent years, increasing number of evidences have shown that ABA and auxin pathways are related to root hair growth. For example, low phosphorus treatment increased root length and root hair density of wild rice seeds (Giri et al., 2018; Josefine and Matthias, 2016). However, overexpression of *OsPIN2* resulted in the loss of sensitivity of roots to phosphorus deficiency, and the root length of rice was shortened by increasing the distribution of auxin in root epidermis (Sun et al., 2019). Similarly, overexpression of *SAPK10* (Stress/ABA-activated protein kinase 10) resulted in longer root hairs in rice, while overexpression of *OsABI2* (OsABI-Like 2) resulted in weakened ABA signal transduction and shorter root hairs in rice plants (Wang et al., 2017).

bHLH transcription factors also affect the growth of root hairs. The *Arabidopsis* bHLH gene family members *AtRHD6* and *AtRSL1* regulate the development of root hair cells downstream of the transcriptional regulation complex that determines the fate of epidermal cells. A single mutant of the *AtRHD6* gene has a small amount of root hairs; a single mutant of the *AtRSL1* gene has normal root hairs, while the double mutants of these two genes have no root hairs (Menand et al., 2007). *RSL4/RSL2* is related to the development of root hairs, and their expression is also regulated by both exogenous hormones and environmental signals. Auxin can activate *RSL4* expression and control ROS related genes, including four peroxidases (*PER1*, *PER44*, *PER73*) (Mangano et al., 2017). Peroxidase genes (PERs) encodes class III peroxidase, and its activity is related to root hair elongation and ROS clearance (Kwon et al., 2015; Wanapu and Shinmyo, 1996). Similarly, *RSL2* can inhibit the growth promoting effect of auxin by inhibiting ROS produced by peroxidases (PERs) (Mangano et al., 2017). In this study, the expression of ABA and auxin pathway genes, peroxidase, receptor like protein kinase and root hair development related genes were changed by *SbbHLH85* overexpression under salt stress (Figure 6F-H). In conclusion, *SbbHLH85* participates in ABA and auxin signaling pathways, controls the development of root hair and affects the content of ROS.

***SbbHLH85* negatively regulates plant salt resistance by affecting plant nutrient distribution**

Under salt stress, the uptake of K^+ by root cells decreases and the absorption of Na^+ increases, which leads to the imbalance of ion homeostasis (Song et al., 2020). Recent studies have shown that plants can protect themselves by reducing the length and density of root hair and reducing the absorption area of excessive Na^+ when they sense stress signals (Lv et al., 2018; Wang et al., 2008).

Salt stress also can cause imbalance of nutrient distribution in plants, including phosphorus (P) (Yang et al., 2003). P is a key element of many biomolecules (nucleic acids, ATP, phospholipids) in many metabolic pathways; it is one of the important nutrients needed for plant growth and development (Marschner et al., 2005). Plant phosphorus uptake and transport through phosphate transporters, and phosphate transporters encoded by the phosphate transporter family (PHT1) genes are important proteins for plant acquisition and transport of phosphates (Shin et al., 2004). PHT1;5 is strongly induced in the root, leading to Pi mobilization changes between plant roots/grounds. Compared to the WT, PHT1;5 overexpression enhances root hair formation (Nagarajan, 2010).

The transport of PHT1 protein to plasma membrane is regulated by plant specific companion protein PHF1. *PHF1* gene encodes a plant specific protein related to SEC12 protein structure, which locates in endoplasmic reticulum, and is strongly expressed in plant root cuticle, root hair and its cortical cells (Bayle et al., 2011). In 2005, a mutant of PHF1 was isolated in *Arabidopsis*. Studies on the mutant showed that the mutation of PHF1 reduced the accumulation of *PHT1;1* transporter in plasma membrane, destroyed the transport of Pi and reduced the accumulation of Pi. In this study, *SbbHLH85* is proved to interact strongly with *SbPHF1*. Therefore, we believe that the interaction between *SbbHLH85* and *SbPHF1* can destroy the transportation and accumulation of Pi in plants under salt stress, and aggravate the uneven distribution of nutrient elements, especially phosphorus, caused by high salinity, which greatly reduces the salt resistance of sweet sorghum.

In conclusion, *SbbHLH85*, different from *RSL2* and *RSL4* in *Arabidopsis*, is a necessary gene for root development in sweet sorghum. Knockout of *SbbHLH85* in sorghum by CRISPR-Cas9 results in the inhibition of root development. We then show the molecular mechanism of *SbbHLH85* regulating the salt response of sorghum by directly interacting with the specific companion protein PHF1, which can affect the distribution of Pi. *SbbHLH85* participates in the ABA and auxin pathways-mediated root hair development by affecting the expression of ABA and auxin pathway genes. The increase in the root hair number and length can promote the absorption of Na^+ . The increased Na^+ absorption and the decreased Pi content can ultimately result in the salt sensitive phenotype of sweet sorghum (Figure 7D). In the future, it will be an effective measure to improve the salt tolerance of crops through properly reducing the number and length of root hairs upon presence of the stress.

Declarations

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Author contributions

Na Sui and Yushuang Song planned and designed the research; Yushuang Song, Simin Li, Yi Sui, Hongxiang Zheng, Wenjing Yang and Xi Sun performed experiments; Na Sui, Yushuang Song, Guoliang Han, Hailian Wang, Kunyang Zhuang and Fanying Kong collected data and carried out all analyses; Na Sui and Qingwei Meng wrote the paper. All authors read and approved the final manuscript.

Declaration of Competing interests

The authors declare no competing interests.

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Figures

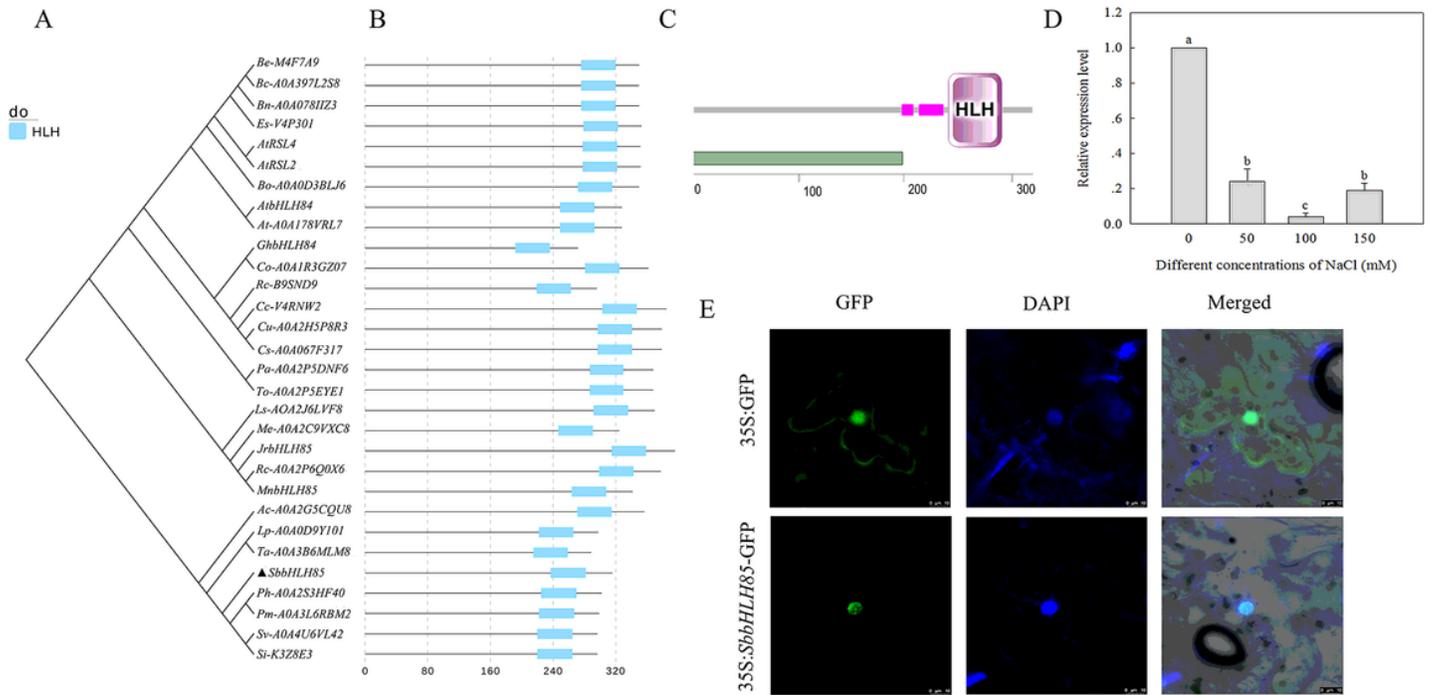


Figure 1

SbbHLH85 is a bHLH TF and is negatively induced by salt. (A) Phylogenetic tree based on SbbHLH85 and homologous proteins. (At: *Arabidopsis thaliana*; Sb: *Sorghum bicolor*; Be: *Brassica rapa* subsp. *Pekinensis*; Bc: *Brassica campestris*; Bn: *Brassica napus*; Es: *Eutrema salsugineum*; Bo: *Brassica oleracea* var. *oleracea*; Gh: *Gossypium hirsutum*; Co: *Corchorus olitorius*; Rc: *Rosa chinensis*; Cc: *Citrus clementina*; Cu: *Citrus unshiu*; Cs: *Citrus sinensis*; Pa: *Parasponia andersonii*; To: *Trema orientale*; Ls: *Lactuca sativa*; Me: *Manihot esculenta*; Jr: *Juglans regia*; Rc: *Ricinus communis*; Mn: *Morus notabilis*; Ac: *Aquilegia coerulea*; Lp: *Leersia perrieri*; Ta: *Triticum aestivum*; Ph: *Panicum hallii*; Pm: *Panicum miliaceum*; Sv: *Setaria viridis*; Si: *Setaria italica*). (B) Analysis of the protein domain of SbbHLH85 and the homologous genes. (C) Structural domain analysis of SbbHLH85. (D) Expression of SbbHLH85 in sweet sorghum roots under salt stress. (E) Subcellular localization of SbbHLH85.

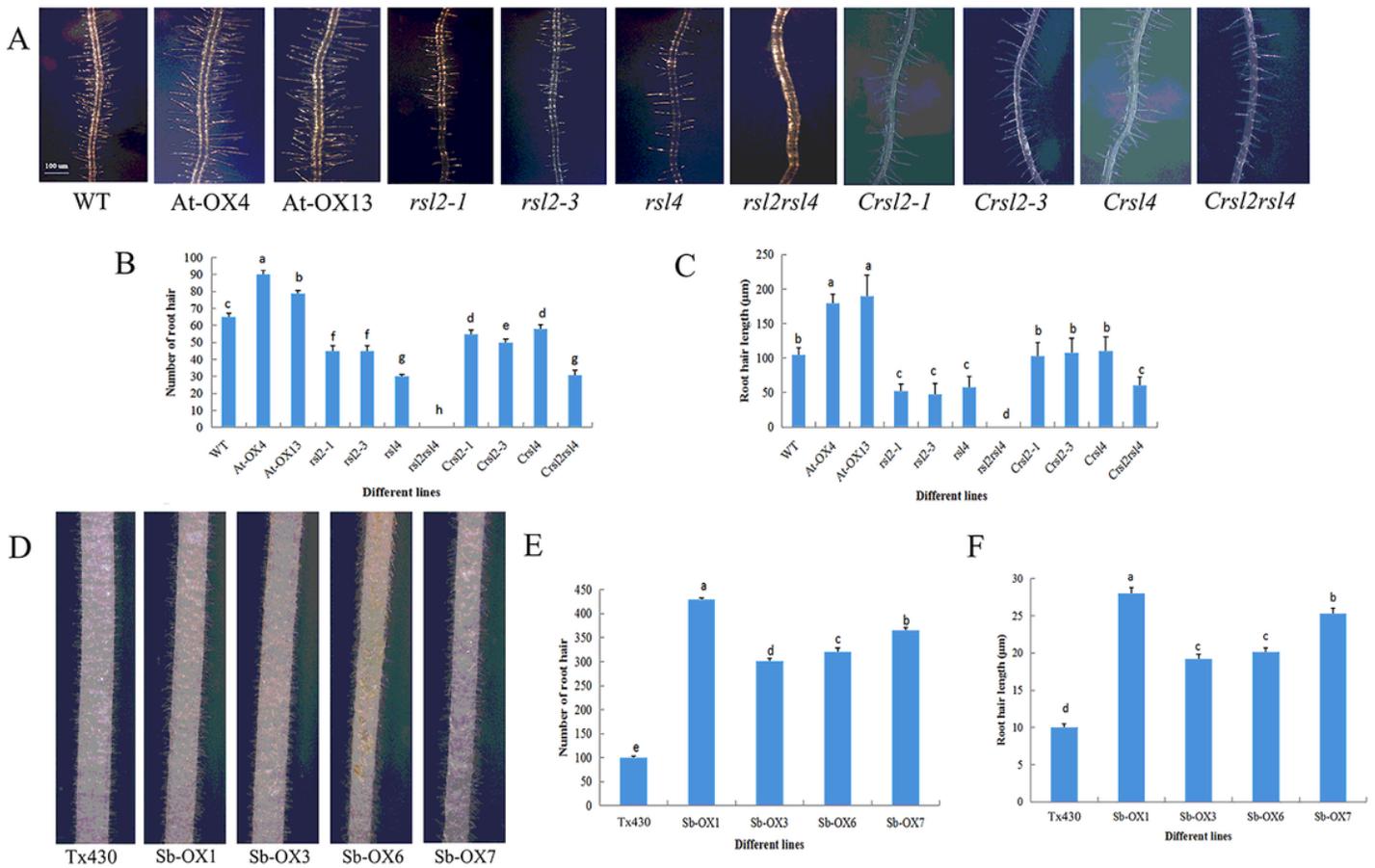


Figure 2

SbbHLH85 regulate root hair development. (A) Root hair phenotypes of different Arabidopsis lines. (B) Root hair numbers statistics of different Arabidopsis lines. (C) Root hair length statistics of different Arabidopsis lines. (D) Root hair phenotypes of different sweet sorghum lines. (E) Root hair numbers statistics of different sweet sorghum lines. (F) Root hair length statistics of different sweet sorghum lines.

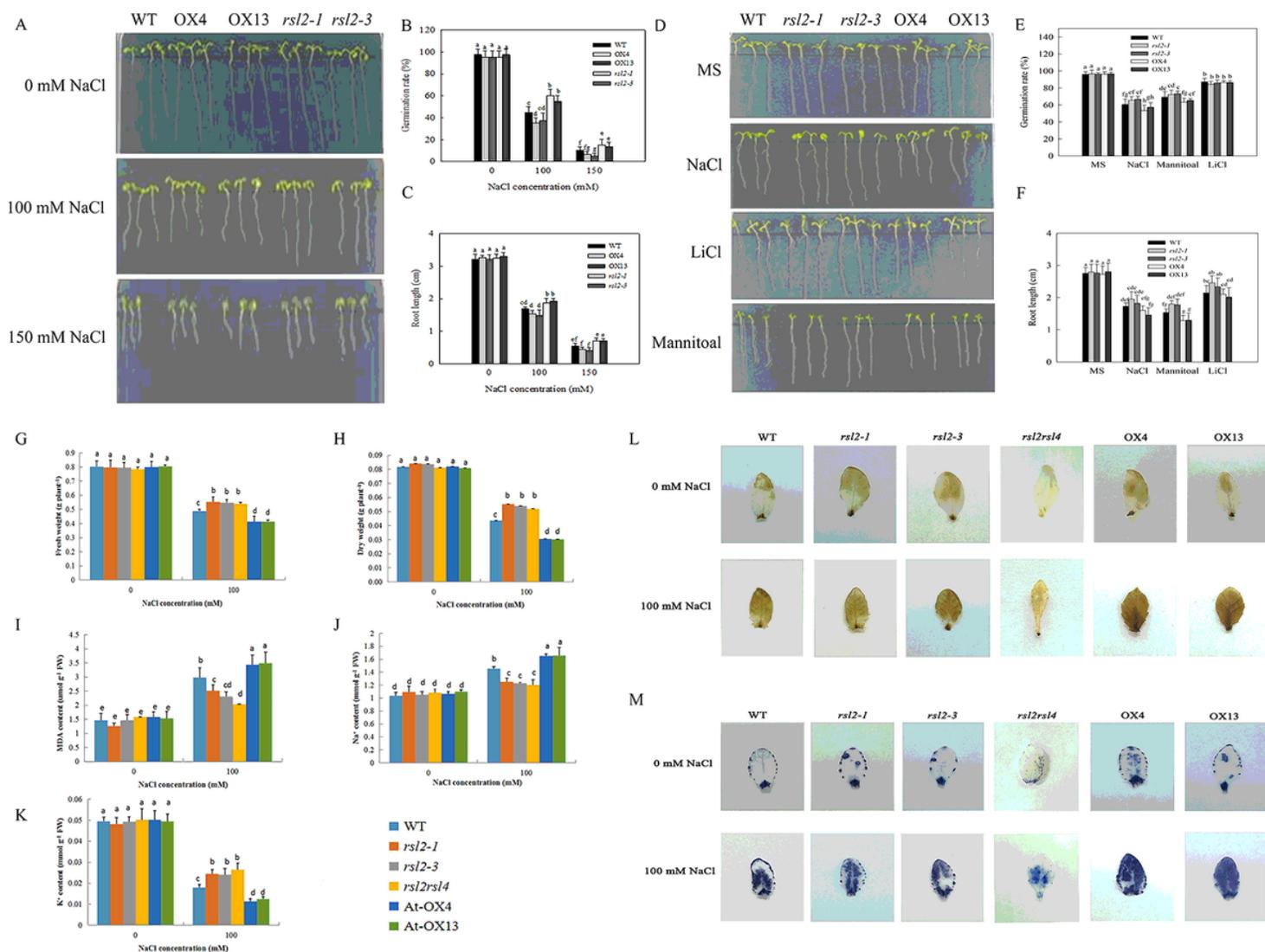


Figure 3

SbbHLH85 negatively regulates salt tolerance in Arabidopsis. (A) The phenotypic characteristics of Arabidopsis lines under different salt concentrations. (B) Germination rate statistics of Arabidopsis lines under different salt concentrations. (C) Root length statistics of Arabidopsis under different salt concentrations. (D) The phenotype of Arabidopsis lines under NaCl, LiCl, Mannitol treatment conditions. (E) Germination rate statistics of Arabidopsis lines under NaCl, LiCl, Mannitol treatment conditions. (F) Root length statistics of Arabidopsis under NaCl, LiCl, Mannitol treatment conditions. (G and H) Biomass of WT and transgenic plants under salt stress. (I) The MDA content of WT and transgenic plants under salt stress. (J and K) Na⁺ and K⁺ contents of WT and transgenic plants under salt stress. (L and M) DAB and NBT in WT and transgenic plants under salt stress. Data are presented as the mean \pm SD of five measurements. Means with different letters are significantly different at $P < 0.05$.

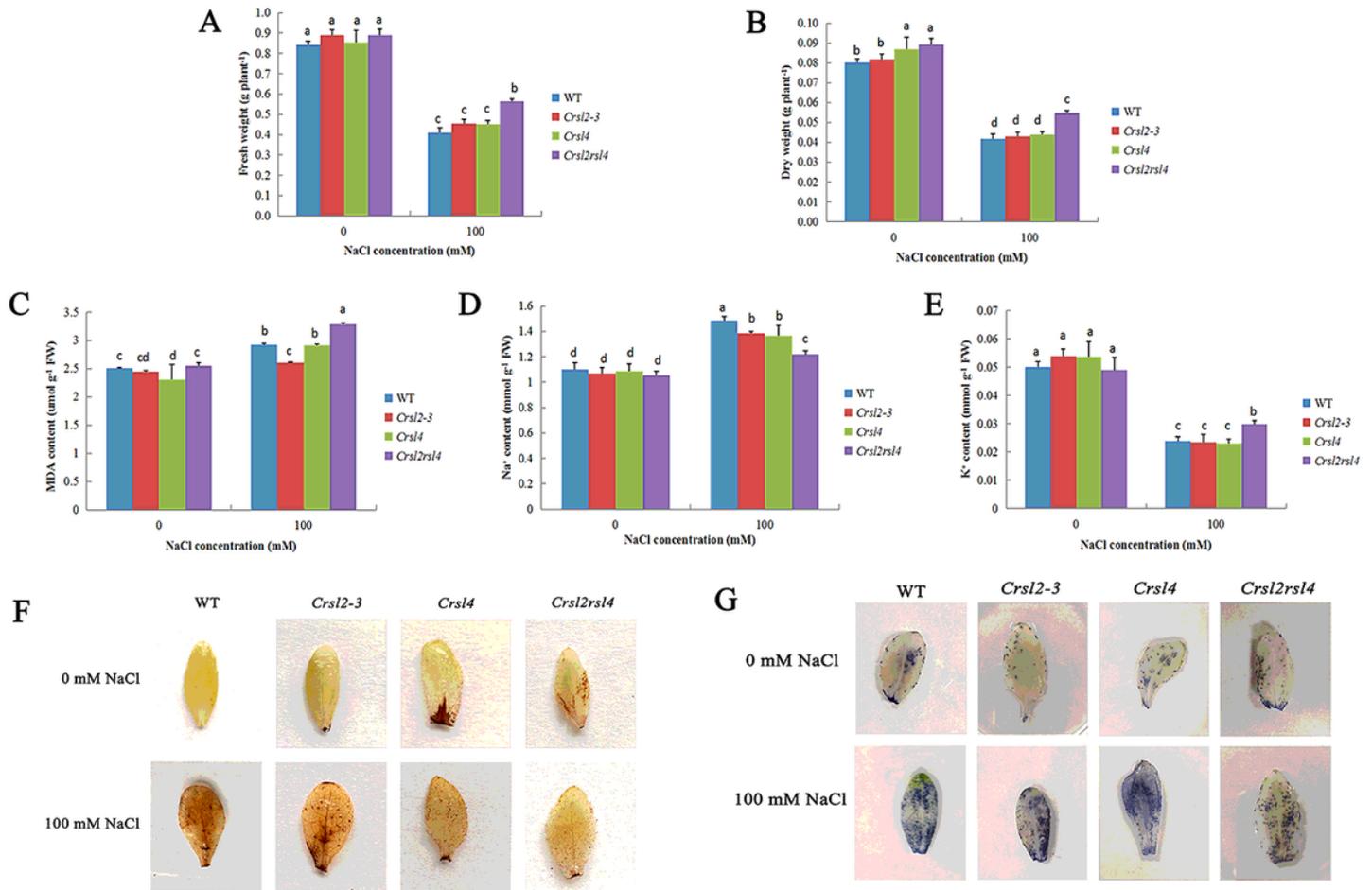


Figure 4

SbbHLH85 can recover salt-sensitive phenotypes in Arabidopsis. (A and B) Biomass of WT and restocking lines under salt stress. (C) The MDA content of WT and restocking lines under salt stress. (D and E) Na⁺ and K⁺ contents of WT and restocking lines under salt stress. (F and G) DAB and NBT in WT and restocking lines under salt stress. Data are presented as the mean \pm SD of five measurements. Means with different letters are significantly different at $P < 0.05$.

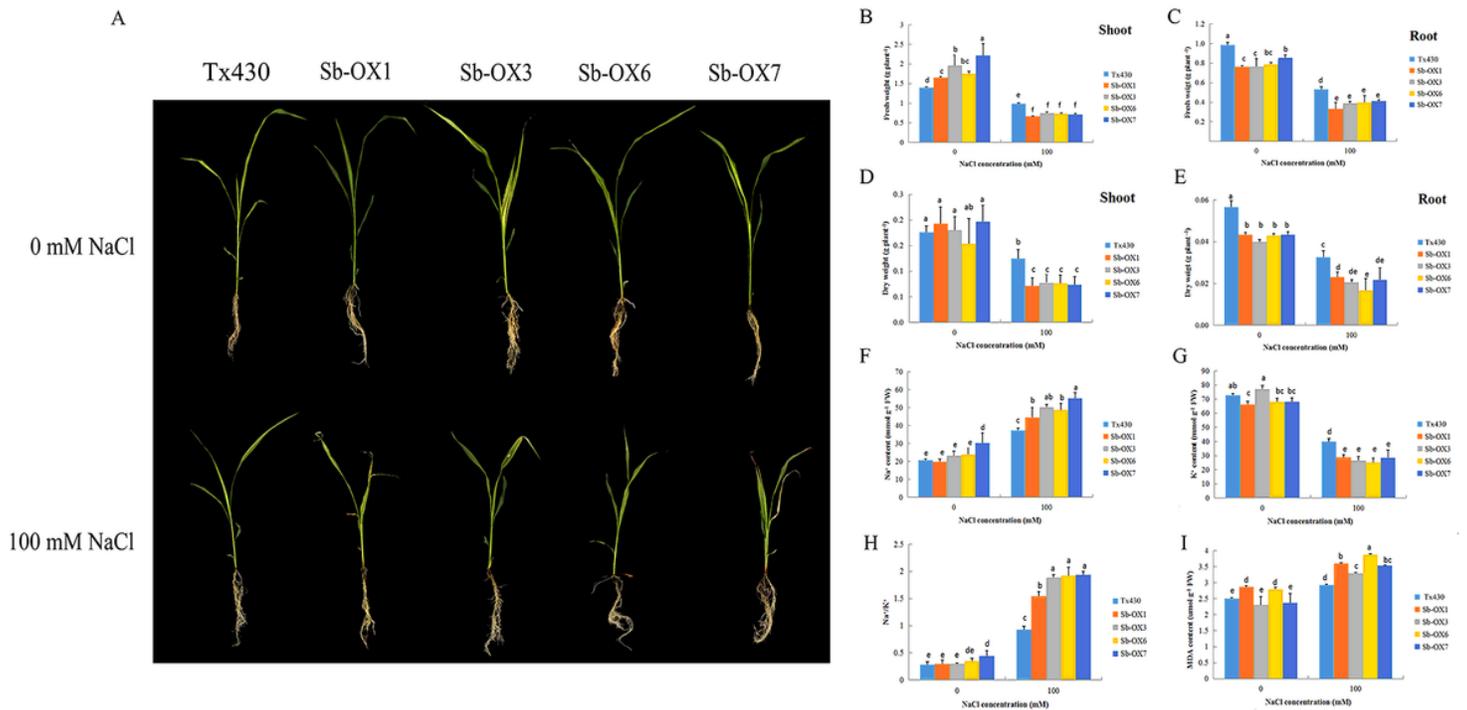


Figure 5

SbbHLH85 negatively regulates salt tolerance in sorghum. (A) Growth status of transgenic sweet sorghum lines under salt stress. (B and C) Fresh weight of WT and overexpression lines of sweet sorghum under salt stress. (D and E) Dry weight of WT and overexpression lines of sweet sorghum under salt stress. (F-H) Na⁺ and K⁺ contents and Na⁺/K⁺ ratio of WT and overexpression lines of sweet sorghum under salt stress. (I) MDA content of WT and overexpression lines of sweet sorghum under salt stress. Data are presented as the mean \pm SD of five measurements. Means with different letters are significantly different at $P < 0.05$.

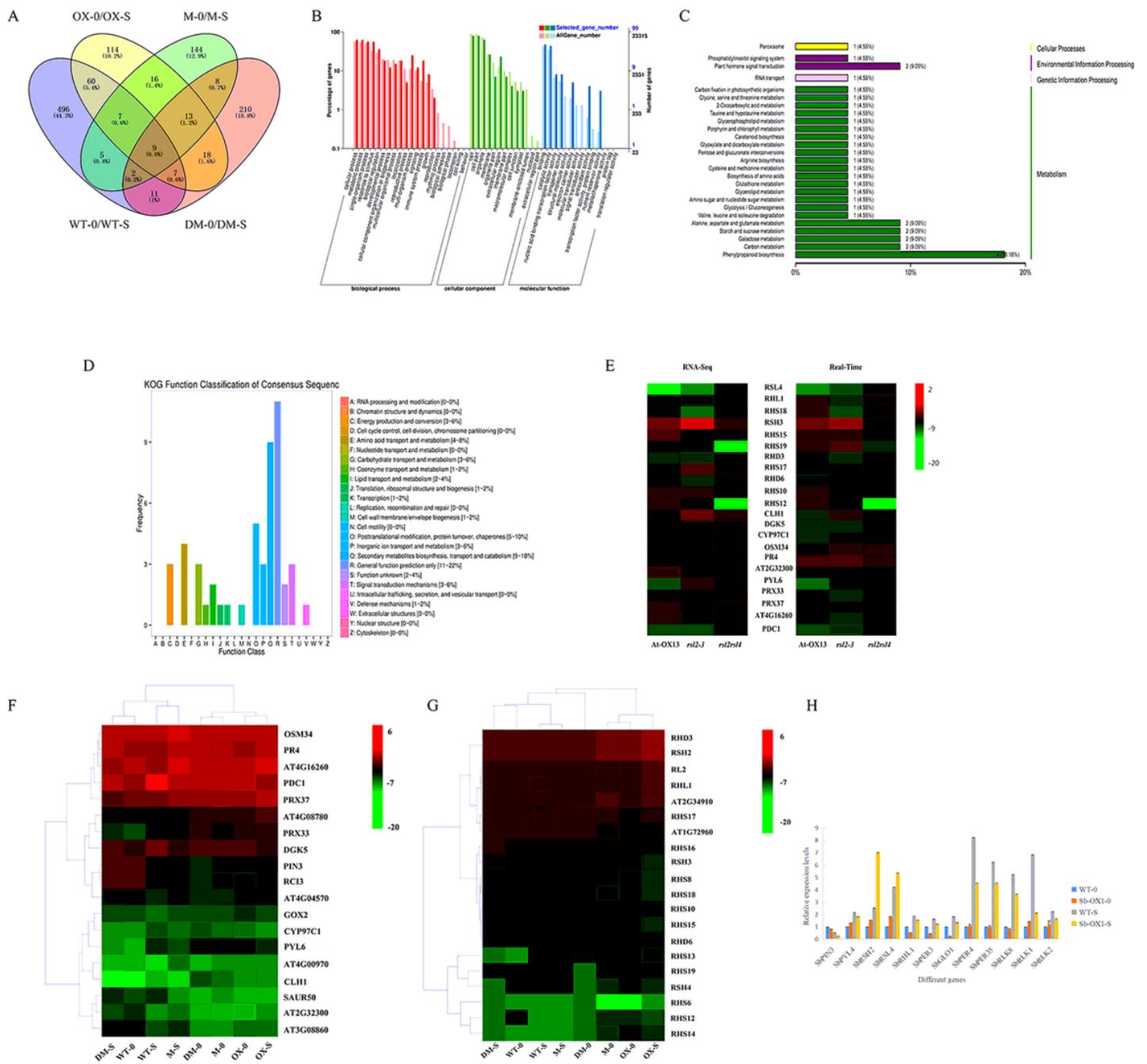


Figure 6

RNA-seq analysis of the WT, At-OX13, rsl2-3, rsl2rsl4 lines before and after salt stress. (A) Venn diagram of the numbers of expressed genes in the WT, At-OX13, rsl2-3, rsl2rsl4 lines before and after salt stress. (B) Gene Ontology Consortium analyses of the differentially expressed genes (DEGs) between WT, At-OX13, rsl2-3, rsl2rsl4 lines before and after salt stress. (C) Kyoto Encyclopedia of Genes and Genomes analyses of the differentially expressed genes (DEGs) between WT, At-OX13, rsl2-3, rsl2rsl4 lines before and after salt stress. (D) KOG analyses of the differentially expressed genes (DEGs) between WT, At-OX13, rsl2-3, rsl2rsl4 lines before and after salt stress. (E) The RNA sequences of 22 representative genes were verified by qRT-PCR. (F and G). Expression analysis of phenylalanine metabolism, high salt response and

root hair growth and development related genes. (H) Quantitative analysis of homologous genes in sweet sorghum.

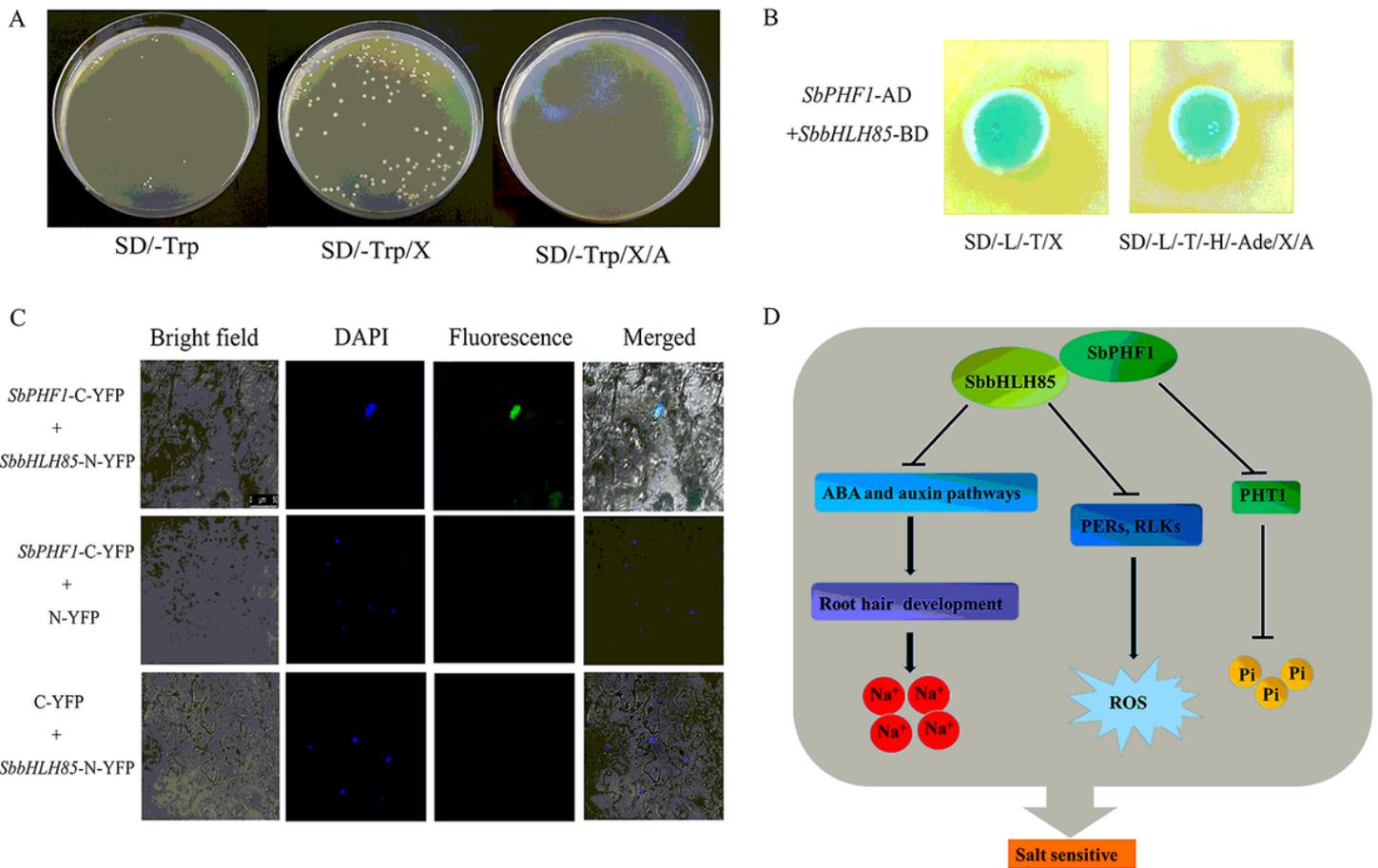


Figure 7

SbbHLH85 interacts with *SbPHF1* to participate in salt tolerance. (A) The self activation activity of *SbbHLH85* was verified. *SbbHLH85* was used as bait protein, the self activation activity of *SbbHLH85* was verified by yeast system on the lacked tryptophan medium (SD/-Trp), added with X-a-gal but lacked tryptophan medium (SD/-Trp/X), and added with the X-a-gal and AbA but lacking tryptophan (SD/-Trp/X/A) medium. (B) Yeast-two-hybrid assays. Interaction was indicated by the ability of cells to grow on SD/-L/-T/X and SD/-L/-T/-H/-Ade/X/A medium. The Gal4 DNA binding domain was fused with *SbbHLH85* (shown as *SbbHLH85-BD*) and the Gal4 activation domain was fused with *SbPHF1* (shown as *SbPHF1-AD*). (C) BiFC analysis. Fluorescence was observed in the nuclear chamber of lower epidermal cells of tobacco leaves. The C-terminal part of YFP is fused with *SbPHF1* (*SbPHF1-C-YFP*), and the N-terminal part of YFP is fused with *SbbHLH85* (*SbbHLH85-N-YFP*). No signals were observed from the negative controls. DAPI, 4',6-diamidino-2-phenylindole. (D) A proposed model of *SbbHLH85* regulating root hair growth and salt tolerance in sweet sorghum. Under salt stress, *SbbHLH85* and *SbPHF1* combine with each other, they regulate the genes related to ABA and auxin signal transduction pathway and root

hair growth, resulting in damage to root hair growth, thus destroying the distribution of phosphorus transport in plants and improving the salt tolerance of plants.

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