E2 conjugases UBC1 and UBC2 regulate MYB42-mediated SOS pathway in response to salt stress in Arabidopsis

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Summary

- Histone H2B monoubiquitination (H2Bub1) is recognized as a crucial eukaryotic regulatory mechanism that controls a range of cellular processes during both development and adaptation to environmental changes. In Arabidopsis, the E2 conjugated enzymes UBQUITIN CARRIER PROTEINS (UBCs) -1 and -2 mediate ubiquitination of H2B. Here, we elucidated the functions of UBC1 and -2 in salt-stress responses and revealed their downstream target genes.
- Real-time quantitative PCR assays showed that UBC1 and -2 positively regulated the salt-induced expression of MYB42 and Mitogen-Activated Protein Kinase 4 (MPK4). Chromatin immunoprecipitation assays revealed that H2Bub1 was enriched weakly on the chromatin of MYB42 and MPK4 in the ubc1,2 mutant. We further determined that UBC1/2-mediated H2Bub1 enhanced the level of histone H3 tri-methylated on K4 (H3K4me3) in the chromatin of MYB42 and MPK4 under salt-stress conditions.
- MPK4 interacted with and phosphorylated MYB42. The MPK4-mediated MYB42 phosphorylation enhanced the MYB42 protein stability and transcriptional activity under salt-stress conditions. We further showed that MYB42 directly bound to the SALT OVERLY SENSITIVE 2 (SOS2) promoter and mediated the rapid induction of its expression after a salt treatment.
- Our results indicate that UBC1 and -2 positively regulate salt-stress responses by modulating MYB42-mediated SOS2 expression.

Introduction

Salt stress is a major environmental factor that hinders plant growth and development. Plants have evolved a set of complicated and precise mechanisms for altering physiological and biochemical processes to adapt to saline conditions. The maintenance of Na⁺ : K⁺ homeostasis is essential for plant growth and development under saline conditions (Zhu, 2001). The famous salt overly sensitive (SOS) pathway in Arabidopsis thaliana plays a crucial role in regulating Na⁺ : K⁺ homeostasis under salt conditions. The SOS pathway includes three important genes, SOS1, SOS2 and SOS3, which function together to prevent excessive Na⁺ accumulation in the cytosol (Zhu, 2016; Yang & Guo, 2018). SOS3 (Liu & Zhu, 1998), encoding a Ca⁺⁺ binding protein, senses the Ca⁺⁺ concentration in cells elicited by salt stress and interacts with SOS2, a serine/threonine protein kinase (Halfter et al., 2000; Quintero et al., 2002). The SOS3–SOS2 complex further activates SOS1 (Shi et al., 2000), which encodes a Na⁺ : H⁺ exchanger, to facilitate Na⁺ efflux (Qiu et al., 2002; Shi et al., 2002; Quan et al., 2007). The SOS pathway has been investigated thoroughly, yet the upstream transcriptional regulators of the SOS genes remain poorly known.

Histone H2B monoubiquitination (H2Bub1) is an important histone modification that is associated with transcriptional regulation, including transcriptional activation and elongation (Pavri et al., 2006; Weake & Workman, 2008). In Arabidopsis, histone H2B is monoubiquitinated by two RING E3 ligases (HISTONE MONOUBIQUITINATION1 (HUB1) and HUB2) and two E2 conjugases (UBQUITIN CARRIER PROTEIN (UBC1) and UBC2) (Cao et al., 2008). The hub1, hub2 and ubc1,2 mutants show the loss of H2Bub1 (Liu et al., 2007; Cao et al., 2008). HUB1 and 2 regulate multiple developmental processes in plants, including seed dormancy (Liu et al., 2007), leaf and root growth (Fleury et al., 2007), flowering (Cao et al., 2008; Gu et al., 2009; Xu et al., 2009), photomorphogenesis and circadian rhythms (Bourbousse et al., 2012; Himanen et al., 2012), defense and immune responses (Dhawan et al., 2009; Hu et al., 2014; Zou et al., 2014). A genome-wide analysis revealed that H2Bub1 associates with active genes distributed throughout the genome and marks chromatin regions, notably in combination with H3K4me3 and/or with H3K36me3, in Arabidopsis (Roudier et al., 2011). HUB1 and -2 regulate the expression levels of FLOWERING LOCUS C (FLC) and some circadian clock genes (e.g. CCA1 and TOC1) by enhancing the H3K4me3 deposition in their chromatin (Cao et al., 2008; Himanen et al., 2012; Malapeira et al., 2012). In addition, H2Bub1 directly modulates the expression of the R gene SNC1 and impacts plant immune responses (Zou et al., 2014). H2Bub1...
is associated with H3K4me3 at the GbDREB locus, and a greater level of H3K4me3 modification in chromatin regions of GbDREB triggers more rapid responses to drought stress in AtHUB2 transgenic cotton (Chen et al., 2019). Moreover, HUB1 and HUB2 play important regulatory roles in responses to salt stress (Zhou et al., 2017). However, it is unclear how H2Bub1 regulates the expression of target genes involved in salt tolerance. UBC1 and -2 are functionally redundant in the control of flowering time in Arabidopsis (Cao et al., 2008; Xu et al., 2009), whereas their regulatory mechanisms in biotic and abiotic stress responses in plants are not fully understood.

The mitogen-activated protein kinase (MAPK) cascade is a universal module of signal transduction from the cell surface to the nucleus, and it plays a crucial role in altering physiological and biochemical processes in response to extracellular stimuli (Ichimura et al., 2000; Li et al., 2017; Zhao et al., 2017). Moreover, MKK4/5–MPK3/6 and MEKK1–MKK1/2–MPK4 constitute two important MAPK signaling pathways that are involved in plant responses to diverse external stimuli, including biotic and abiotic stresses (Petersen et al., 2000; Teige et al., 2004; Meng & Zhang, 2013; Pitzschke et al., 2014). MPK4 and MPK6 are activated by salt stress in plants, and MPK3/MPK6 participates in the salt-stress response (Ichimura et al., 2000; Li et al., 2014).

MYB proteins are members of a superfamily of transcription factors that are encoded by c. 339 genes in Arabidopsis, and they contain a highly conserved DNA-binding (MYB) domain (Feller et al., 2011). Plant MYB proteins are involved in many processes, including secondary metabolism (Zhong et al., 2008) and development (Baumann et al., 2007; Wang et al., 2009), as well as in mediating hormone actions (Li et al., 2009; Zheng et al., 2012) and abiotic stress responses (Nguyen & Cheong, 2018; Du et al., 2019). MYB42 belongs to the R2R3–MYB group. However, the function of MYB42 remains obscure.

In the present study, we revealed that UBC1 and -2 play positive roles in regulating plant salt tolerance. UBC1/2-mediated H2Bub1 modulated the expression of MYB42 and MPK4 during salt stress by enhancing the levels of H3K4me3 in their chromatin. MYB42 acted downstream of UBC1/UBC2 to mediate salt tolerance in Arabidopsis. In addition, MPK4 interacted with and phosphorylated MYB42, which enhanced the MYB42 protein’s stability and its transcriptional activity under salt-stress conditions. MYB42 also mediated the salt-induced expression of the SOS2 gene by binding to its promoter in vitro and in vivo. These results indicate that UBC1 and -2 positively regulate MYB42-mediated SOS2 expression in response to salt stress in Arabidopsis.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana plants were grown under a 16 h : 8 h, light : dark photoperiod at 21 ± 2°C on 0.8% agar plates containing ½ Murashige & Skoog medium. ubc1-1 (SALK_109196), ubc2-1 (SALK_060994) and ubc1,2 (ubc1-1ubc2-1) were described previously (Cao et al., 2008) (UBC, UBIQUITIN CARRIER PROTEINs). The myb42-1 (SALK_003422) mutant was obtained from the Arabidopsis seed platform (State Key Laboratory of Plant Physiology and Biochemistry, China Agricultural University). sos2-2 (SOS, SALT OVERLY SENSITIVE) (Guo et al., 2004) and pAIR-HA-MPK4ΔD1/Col-0 (an active mutant of Mitogen-Activated Protein Kinase 4 (MPK4) driven by a steroid-inducible promoter, Berri et al., 2012) were used in this study. The 35S:UBC, 35S:UBC1/ubc1-1, 35S:UBC2/ubc2-1, 35S:MYB42/Col-0, ProMYB42: MYB42/myb42-1 and 35S:MYB42/ubc1,2 transgenic plants were generated through Agrobacterium-mediated transformation. The myb42-1ubc1-1ubc2-1 triple mutant was generated by crossing. The primers used for genotyping the mutants are listed in Supporting Information Table S1.

Salt sensitivity assay

Seeds were grown for 5 d under normal conditions and transferred into medium supplemented with 175 mM NaCl. After 4 d, plants were photographed, and the survival rates of the seedlings were scored. Seedling death was scored as complete bleaching of cotyledons and leaves (Zhu et al., 1998). To compare the root lengths after the salt treatment, 5-d-old seedlings were transferred into medium containing 100 mM NaCl for 5 d and then photographed. The root lengths were measured using IMAGEJ software. At least 30 seedlings were observed per line, and each experiment was repeated three times.

Measuring Na⁺ and K⁺ concentrations

The measurement protocols for Na⁺ and K⁺ contents were as described previously (Yang et al., 2009). The samples were dried at 80°C to constant weights for 24 h and the dry biomasses were weighed. Then, the dry plant tissues were incinerated in a muffle furnace at 300°C for 3 h and 575°C for 6 h. The ashes were dissolved in 10 ml 1% hydrochloric acid and then further diluted with 1% hydrochloric acid into different concentrations. The Na⁺ and K⁺ contents were determined using a 4100-MP AES device (Agilent, Santa Clara, CA, USA).

 Transcriptome sequencing

In order to compare the transcriptomes of wild-type (WT) and ubc1,2 mutant, 7-d-old seedlings were treated with 200 mM NaCl or water for 3 h. Total RNA was extracted using the TriZOL reagent (Invitrogen). Two micrograms of total RNA from each sample were used for transcriptome sequencing at Novagene (http://www.novogene.com/), employing the high throughput sequencing platform highseq4000. The clean data for each sample amounted to 4.0 G. Differential gene expression was determined using RNA-seq DGE tools (Trapnell et al., 2010).
RNA extraction and real-time reverse transcription quantitative PCR (qRT–PCR)

Total RNAs from 7-d-old seedlings were extracted using a Plant Total RNA Purification Kit (GeneMark, Taiwan, China), followed by reverse transcription using a Prime Script™ Reverse Transcription Reagents kit (TaKaRa, Dalian, China). The qRT-PCR was performed using a SYBR Green PCR Master Mix kit (TaKaRa). The quantification of the transcript levels of the cDNA fragments was normalized to the expression of the ACTIN2 gene in Arabidopsis at several points after the NaCl treatment. The specific primers used are listed in Table S2.

Chromatin immunoprecipitation (ChIP)-based qPCR assays

ChIP was performed as described previously with some modifications (Cao et al., 2008; Li et al., 2017). In brief, 2-wk-old seedlings were fixed with 1% formaldehyde solution for 10 min in a vacuum, and the process was terminated by the addition of 2 M glycine (final concentration of 0.125 M) under vacuum conditions for 5 min. The chromatin was then isolated and sonicated. The DNA fragments combined with target proteins were co-immunoprecipitated using anti-Myc antibody, anti-H2Bub1 (histone H2B monoubiquitination), anti-H3K4me3 (histone H3 tri-methylated on K4) and anti-H3 (all Cell Signaling Technology, Danvers, MA, USA), and the enriched DNA fragments were assessed by qRT-PCR using the primers listed in Table S3.

Green fluorescent protein (GFP) fluorescence assay

In order to determine the localization of MYB42 in protoplasts, 35S:MYB42-GFP was transformed into Arabidopsis mesophyll protoplasts as described previously (Yoo et al., 2007). The 35S:GFP construct was used as a control. The GFP signal was visualized under a confocal microscope (Zeiss LSM 710) after a 16-h incubation at 22°C. Additionally, 7-d-old stable transgenic plants expressing 35S:MYB42-GFP were treated with NaCl for 30, 60 or 90 min, and the GFP signals in root epidermal cells were visualized using a confocal microscope. The 35S:MYB42-GFP and pBI-HA-MPK4DE (a constitutively active form of MPK4) constructs were transiently co-expressed in tobacco leaves after a NaCl treatment, and the GFP signal was visualized under a confocal microscope.

Transcriptional activation assays in yeast

The MYB42 coding sequence was cloned into the pGBK7T7/BD vector (Clontech) containing the GAL4 DNA-binding domain. pBD-MYB42 or the negative control pBD vector was transformed into the yeast strain AH109 (Clontech). The transformed strains were cultured on SD/-Trp plates, and then were spotted on the medium without both Trp and His (SD/-Trp-His) to determine whether these clones could activate the expression of the reporter gene HIS3 (Rheece-Hoyes & Wallhout, 2012).

Yeast-two-hybrid (Y2H) assay

The MPK4 coding sequence was cloned into the pGBK7T7 vector (Clontech) as the bait construct, and the MYB42 coding sequence was cloned into the pGAD7T7 vector as the prey construct. Bait and prey constructs were co-transformed into yeast strain AH109. Yeast strains containing the bait and prey plasmids were cultured in SD/-Trp-Leu medium. Positive clones were identified by the ability to grow on SD/-Trp-Leu-His-Ade and turn blue on SD/-Trp-Leu-His-Ade containing 6 mM 3-amino-1,2,4-triazole and 20 μg ml⁻¹ X-α-gal.

Co-immunoprecipitation assays

Transgenic 35S:MYB42-Myc plants were used to detect the interactions of MYB42 and MPK4. The total proteins extracted from stable transgenic plants were immunoprecipitated with anti-Myc magnetic beads (Thermo Fisher Scientific, Waltham, MA, USA). The immunoprecipitates were separated on a 10% SDS-PAGE gel and detected with anti-MPK4 (Sigma-Aldrich).

Luciferase complementation imaging (LCI) assays

The MPK4 and MYB42 coding sequences were amplified and cloned separately into the nLuc and cLuc vectors. The primers for PCR fragment amplification are listed in Table S4. The empty vectors were used as negative controls. nLuc-SGT1 + RAR1-cLuc was used as a positive control (Chen et al., 2008). Agrobacterium tumefaciens strain GV3101 containing each construct was independently transiently transfected into Nicotiana benthamiana. Leaves were collected 2–3 d after infiltration. The LCI assay was performed as described previously (Chen et al., 2008).

Preparation of recombinant proteins and in vitro phosphorylation assay

MYB42 and MPK4 cDNAs were PCR-amplified, independently cloned into the pET-30a vector and then separately transformed into Escherichia coli (BL21). The primer sequences are listed in Table S4. The proteins were expressed at 16°C for 12 h using 0.3 mM isopropyl β-D-1-thiogalactopyranoside and purified using anti-His magnetic beads. MPK4 and MPK4DE were used to phosphorylate the recombinant MYB42 protein (1:10) in the reaction buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 25 mM ATP and 1 mM DTT) at 28°C for 30 min. The reactions were stopped by the addition of 5 X SDS buffer. ProQ diamond staining of phosphoproteins was performed as described previously (Sheikh et al., 2016). After separation by SDS-PAGE, the gel was fixed with 50% methanol and 10% acetic acid for 1 h. The gel was washed with water for 30 min and stained with Pro-Q diamond stain (Invitrogen) in the dark for 2 h. The gel was destained four times for 30 min each with 20% acetonitrile and 50 mM sodium-acetate (pH 4.2). The gel was washed again with water for 10 min and scanned using a Typhoon Scanner (GE Healthcare, Pittsburgh, PA, USA).
Immunoblot assay

Immunoblot assays were performed as described previously (Zhou et al., 2017). Briefly, 8-d-old stable transgenic plants expressing 35S:MYB42-MYC treated as described in Fig. 6 were used to extract total protein. Anti-Myc antibody (Cell Signaling Technology) was used to immunoblot the MYB42-Myc protein.

Cell-free protein degradation assay

The cell-free protein degradation assay of MYB42 was performed as described previously (Li et al., 2017). Briefly, total proteins were extracted from 10-d-old seedlings in degradation buffer (50 mM Tris-MES (pH = 8.0), 500 mM sucrose, 1 mM MgCl2, 10 mM EDTA (pH = 8.0), 5 mM DTT). Equal amounts of proteins from pAI-HA-MPK4DE/Col-0 and WT plants were incubated with recombinant MYB42-His protein for different time periods. The MYB42 protein was detected by immunoblotting with an anti-His antibody.

Yeast-one-hybrid (Y1H) assay

The Y1H assay was performed as described previously (Li et al., 2010). The full-length cDNA of MYB42 and a DNA fragment of the SOS2 promoter were amplified and separately cloned into pB42AD and pLacZi. All of the primer sequences are listed in Table S4. Empty vectors pB42AD and pLacZi were used as controls. The fusion constructs were co-transformed into yeast strain EGY48. The transformants were grown on SD/-Ura-Trp dropout plates containing 20 μg ml⁻¹ X-β-gal for blue color development.

Transcriptional activity assay

Transcriptional activity assays were performed as described previously (Li et al., 2017). Nicotiana benthamiana leaves were co-transfected with pSOS2:GUS combined with 35S:MYB42-MYC and pBb:HA-MPK4DE (GUS, β-glucuronidase). The 35S:LUC was included for all the samples as an internal transfection efficiency control. The firefly luciferase (LUC) activity was detected using luciferase as a substrate (Promega). The GUS activity was analyzed using methyl umbelliferyl glucuronide (Sigma–Aldrich). The GUS : LUC ratio was used to determine the SOS2 promoter activity.

Transient dual-LUC reporter assay

A 1.5-kb promoter sequence of SOS2 was amplified and cloned into the pGreen II 0800-LUC vector, and used as a reporter plasmid (Hellens et al., 2005). The coding sequence of MYB42 was amplified and cloned into pGreen II 62-SK, and used as an effector plasmid. Arabidopsis protoplasts were prepared and co-transfected with the constructs following the instructions for the Dual-Luciferase Reporter Assay System (Promega). The ratio of LUC to renilla luciferase (REN) was determined using a GLO-MAX 20/20 luminometer (Promega) after culturing the protoplasts under low-light conditions for 16 h (Guo et al., 2017). The LUC : REN ratio indicates transcriptional activity.

Quantification and statistical analysis

The protein quantification was performed with ImageJ software. Statistical analysis was performed with SPSS statistics software (https://www.ibm.com/products/spss-statistics). Data were analyzed by one-way ANOVA with Tukey’s Honest Significant Difference post-hoc tests. P-values < 0.05 are noted in the figure legends, and significant differences are indicated by different letters. Statistical analysis results are provided in Table S5.

Results

UBC1 and -2 positively regulate salt tolerance

In order to determine whether the E2 conjugated enzymes UBC1 and -2 are involved in responses to salt stress, WT Arabidopsis and T-DNA insertion mutants of UBC1 and UBC2 were used in this study. We first observed the salt-sensitive phenotype of the WT, and the ubc1-1, ubc2-1 and ubc1,2 mutants. The ubc mutants were much more sensitive than the WT to salt treatment (Fig. 1a). The ubc1,2 double mutant showed significantly reduced relative root lengths compared with the WT and ubc1-1 after 5 d of a 100-mM NaCl treatment (Fig. 1b). Moreover, the seedling survival rates of ubc mutants were markedly lower than that of the WT during salt stress. Specifically, c. 30% of ubc1,2

**Fig. 1** Phenotypes of ubc mutants under salt-stress conditions. (a) The ubc mutants display sensitive phenotypes in response to salt stress. Seedlings (5-d-old) of wild-type (WT, Col-0) and ubc mutants were transferred from ½ Murashige & Skoog medium to ½MS medium with or without 175 mM NaCl. Photographs were taken 4 d after transfer. Bar, 1.5 cm. (b) Quantification of the relative root lengths of mutants and the WT. Primary root lengths were measured 5 d after transfer to medium supplemented with 100 mM NaCl. The relative root lengths were calculated by comparing seedlings treated with NaCl to seedlings treated without NaCl. (c) Seeding survival for WT and ubc mutants treated with NaCl. Seedlings (5-d-old) of WT and ubc mutants were transferred from ½MS medium to ½MS medium supplemented with 175 mM NaCl. Survival was measured 4 d after transfer. (d) Relative expression levels of UBC genes in WT plants (7-d-old) after treatment with 175 mM NaCl. Total RNA was extracted after different of treatment times for quantitative reverse transcription (qRT)-PCR analysis. (e), (f) and (g) Na⁺ contents, K⁺ contents, Na⁺ : K⁺ ratios, respectively, in the WT and mutants after treatment with 100 mM NaCl. Seedlings (5-d-old) of WT and ubc mutants were transferred from ½MS medium to ½MS medium with or without NaCl. In (b) and (c), each bar represents the mean ± SD of three independent experiments, where each genotypic replicate had 30 seedlings. In (d)-(g), each bar represents the mean ± SD of three technical replicates. Different letters represent significant differences at P < 0.05 by one-way ANOVA followed by Tukey’s Honest Significant Difference (HSD) post-hoc test. UBC, UBQUITIN CARRIER PROTEIN.
seedlings survived, whereas >70% of WT seedlings survived (Fig. 1c).

In order to examine whether the expression levels of \textit{UBC1} and \textit{UBC2} were induced by salt stress, their expression levels were analyzed using qRT-PCR in the WT after a NaCl treatment. The expression levels of \textit{UBC1} and \textit{UBC2} were induced and increased along with the NaCl treatment time (Fig. 1d).
In order to confirm that disruptions of the UBC1 and UBC2 genes were responsible for the salt-sensitive phenotype, complementary lines UBC1/ubc1-1 and UBC2/ubc2-1 were used to analyze the phenotypes. There were no significant differences in salt-sensitivity between complementary lines and the WT after treatment with NaCl (Fig. S1). To provide additional support, 35S:UBC1 and 35S:UBC2 lines were generated by transforming the WT with a vector expressing the UBC1 and UBC2 cDNA, respectively, driven by the strong 35S promoter of Cauliflower mosaic virus. The 35S:UBC1 and 35S:UBC2 lines had enhanced tolerance to salt stress (Fig. S2).

Then, we examined the Na+ and K+ contents in the WT and ubc mutants. The Na+ contents were significantly increased and the K+ contents were significantly decreased in the mutants compared with in the WT; therefore, the Na+:K+ ratios were significantly increased in the ubc mutants compared with the WT under the same salt conditions (Fig. 1c–g). These results suggest that UBC1 and -2 play positive roles in regulating plant salt tolerance.

**UBC1 and -2 regulate the expression of MYB42 during salt stress**

In order to explore the target genes of H2Bub1 in response to salt stress, we performed transcriptome profiling of the WT and ubc1,2 mutant after a NaCl treatment. The transcription factor MYB42 was selected from differentially expressed genes (Table S6), and the expression of MYB42 was confirmed by qRT–PCR. The expression of MYB42 increased significantly after salt stress in the WT but was decreased in the ubc1,2 mutant in comparison with the WT (Fig. 2a).

In order to further investigate how UBC1 and -2 influence the transcription of MYB42, we performed a ChIP assay using an anti-H2Bub1 antibody against chromatin derived from 2-wk-old WT and the ubc1,2 mutant, and designed three fragments in the chromatin of MYB42 for ChIP-qPCR, including the promoter (P1), transcriptional start site (P2) and gene body (P3) regions (Fig. 2b). qRT-PCR was performed to detect the relative H2Bub1 enrichment in the chromatin of MYB42. The result showed that H2Bub1 was enriched intensively on the P2 region on the chromatin of the MYB42 locus of the WT, whereas it was enriched weakly in the ubc1,2 mutant. Moreover, H2Bub1 was significantly increased in the chromatin of MYB42 of the WT after NaCl treatment, whereas it was not increased in the ubc1,2 mutant (Fig. 2c).

Histone modifications are associated with the activation and repression of transcription. H2Bub1 is required for H3K4 and H3K79 methylation in yeast (Lee et al., 2007). H2Bub1 is required for the enhancement of H3K4me3 in the chromatin of FLOWERING LOCUS C (FLC) and some other FLC clade genes in Arabidopsis (Cao et al., 2008). Therefore, we performed ChIP assays using an anti-H3K4me3 antibody to detect the level of H3K4me3 enrichment on the chromatin of MYB42. The H3K4me3 was enriched intensively on the P2 region of the chromatin of MYB42 in WT plants, whereas it was enriched weakly in the ubc1,2 mutant. In addition, H3K4me3 was significantly increased in the chromatin of MYB42 of the WT after NaCl treatment; however, it was lower in the ubc1,2 mutant (Fig. 2d). Thus, H2Bub1 appears to regulate the expression of MYB42 by enhancing the level of H3K4me3 on the MYB42 locus under salt-stressed conditions.

Furthermore, we examined whether the expression of MYB42 was induced in hub1-4, hub2-2, hub1-4hub2-2, ubc1-1 and ubc2-1 mutants after the NaCl treatment. The transcription level of MYB42 was notably increased after the NaCl treatment in the WT and ubc1-1 and ubc2-1 single mutants, whereas it was lower in the hub1-4, hub2-2 and hub1-4hub2-2 mutants (Fig. S3). These data suggest that both HUB1/HUB2 and UBC1/UBC2 may regulate the NaCl-induced expression of MYB42 through H2Bub1 modifications.

Next, we generated the 35S:MYB42-GFP construct and transformed it into WT protoplasts to examine the subcellular localization of MYB42 in Arabidopsis. We also performed a transcriptional activation assay for MYB42 in yeast cells. The result showed that the MYB42-GFP was localized in the nuclei of Arabidopsis protoplasts, and MYB42 was a transcriptional activator in yeast (Fig. 2e,f), suggesting that it functions likewise in plants.

**MYB42 positively modulates salt tolerance**

In order to further dissect the effect of MYB42 in salt-stress responses, we generated complementation lines (Com 1# and Com 2#) and MYB42-overexpression lines (35S:MYB42 2# and 35S:MYB42 10#). Next, the salt-sensitive phenotype was analyzed. The myb42-1 mutant enhanced salt sensitivity compared with the WT. However, MYB42-overexpression lines exhibited decreased salt-sensitivity levels compared with the WT (Fig. 3a). Additionally, the survival rate of myb42-1 mutant seedlings was significantly lower than that of the WT during salt stress, whereas MYB42-overexpression lines showed a greater seedling survival rate (Fig. 3b). The complementation lines restored the salt sensitivity to the WT. qRT-PCR revealed that the expression of MYB42 was enhanced in the overexpression lines and no longer reduced in the complementation lines compared with myb42-1 (Fig. 3c).

We also compared the Na+ content and Na+/K+ ratio among the myb42-1 mutant, overexpression lines and WT plants. The myb42-1 mutant’s Na+ content and Na+/K+ ratio were significantly increased, whereas the Na+ content and Na+/K+ ratio of the overexpression lines were dramatically decreased compared with the WT under saline conditions (Fig. 3d,e). In addition, we used the CRISPR/Cas9 technique to generate a myb42 mutant (cas9#16), which showed a salt-sensitive phenotype similar to that of the myb42-1 mutant (Fig. S4). These data demonstrate that MYB42 plays a positive role in regulating salt tolerance.

**MYB42 is a downstream target gene of H2Bub1 in salt response**

In order to determine whether MYB42 and UBC1/UBC2 function in the same pathway, a myb42-1ubc1-1ubc2-1 triple mutant
UBIQUITIN CARRIER PROTEIN1 (UBC1) and -2-mediated histone H2B monoubiquitination (H2Bub1) regulate the expression of MYB42 during salt stress and the characterization of the MYB42 protein. (a) Expression of MYB42 in wild-type (WT) and ubc1,2 mutant plants treated with NaCl. The 7-d-old seedlings of WT and mutants were treated with 200 mM NaCl for 3 h and then total RNA was extracted for quantitative reverse transcription (qRT)-PCR analysis. Error bars indicate SDs. (b) Diagram of the MYB42 gene. P1 to P3 represent the primers used to assess the level of H2Bub1 and histone H3 trimethylated on K4 (H3K4me3) by chromatin-immunoprecipitation (ChIP). +1, the translational initiation point. (c) and (d) Relative enrichments of H2Bub1 and H3K4me3 at the MYB42 locus after treatment with NaCl in the WT and ubc1,2 mutant. Chromatin was extracted 1 h after NaCl treatment and immunoprecipitated DNA was analyzed using qPCR. Data were calculated by H2Bub1: H3 and H3K4me3: H3 for each individual gene position. Error bars indicate SDs; n = 3. Different letters represent significant differences at P < 0.05 by one-way ANOVA followed by Tukey’s Honest Significant Difference (HSD) post-hoc test. (e) Localization of MYB42 in Arabidopsis protoplasts. MYB42-GFP was transformed into Arabidopsis protoplasts. The green fluorescent protein (GFP) signals were visualized under confocal microscopy after a 16-h incubation. GFP was used as a control. Bar, 20 μm. (f) Assay of MYB42 transcriptional activation activity in yeast cells. The MYB42 coding sequence was cloned into the pGBKT7 vector. The plasmid was introduced into yeast strain AH109. The pGBKT7 vector was used as a negative control. MYB42 transcriptional activation activity was assessed on SD-/Trp-His.
Fig. 3 MYB42 positively regulates salt tolerance. (a) Salt-sensitivity analysis of the myb42-1 mutant and MYB42-overexpression plants. Wild-type (WT), myb42-1, complementation and MYB42-overexpression lines were grown on ½ Murashige & Skoog medium for 5 d and transferred to ½MS medium with or without 175 mM NaCl for 4 d, after which they were photographed. Bar, 1.5 cm. (b) Seedling survival rates of WT, myb42-1 mutant, complementation and MYB42-overexpression lines treated with NaCl. Seedlings were grown on ½MS medium for 5 d and transferred to ½MS medium supplemented with 175 mM NaCl for 4 d. The data are shown as the means ± SDs of three independent experiments, where each genotypic replicate had 30 seedlings. (c) Expression levels of MYB42 in myb42-1, complementation lines and MYB42-overexpression lines. (d, e) Na⁺ contents (d) and Na⁺:K⁺ ratios (e) of salt-treated WT, myb42-1 and MYB42-overexpression lines. In (c)–(e), each bar represents the mean ± SD of three technical replicates. Different letters represent significant differences at \( P < 0.05 \) by one-way ANOVA followed by Tukey’s Honest Significant Difference (HSD) post-hoc test.
MYB42 acts downstream of UBIQUITIN CARRIER PROTEIN1 (UBC1)/-2 in salt responses. (a, b) Salt-sensitivity analysis and seedling survival rates of the myb42-1 ubc1-1 ubc2-1 triple mutant. Wild-type (WT), ubc1,2, myb42-1 and myb42-1 ubc1-1 ubc2-1 were grown on ½MS medium for 5 d and transferred to ½MS medium with or without 175 mM NaCl for 3 d, after which they were photographed. Bar, 1.5 cm. The survival rates are shown as the means ± SDs of three independent experiments, in which each genotypic replicate had 24 seedlings. (c) Immunoblot analysis of the MYB42-MYC protein in 35S:MYB42/ubc1,2 seedlings. ACTIN was used as a loading control. (d) Expression levels of MYB42 in 35S:MYB42/ubc1,2 seedlings. (e, f) Salt-sensitive phenotype analysis and seedling survival rates of 35S:MYB42/ubc1,2 seedlings. Wild-type, ubc1,2 and 35S:MYB42/ubc1,2 were grown on ½MS medium for 5 d and transferred to ½MS medium with or without 175 mM NaCl for 4 d, after which they were photographed. Bar, 1.5 cm. The survival rates are shown as the means ± SDs of three independent experiments, in which each genotypic replicate had 24 seedlings. Different letters represent significant differences at $P < 0.05$ by one-way ANOVA followed by Tukey’s Honest Significant Difference (HSD) post-hoc test.
was generated. Salt-sensitive phenotype analyses showed that the triple mutant displayed similar levels of salt sensitivity compared to those of myb42-1 and ubc1, 2 (Fig. 4a). In addition, the seedling survival rate of the triple mutant was similar to those of myb42-1 and ubc1, 2, and was lower than that of the WT (Fig. 4b). Thus, MYB42 functions in the same pathway as UBC1/UBC2 to mediate the salt-stress response in Arabidopsis.

In order to further explore the genetic interactions between MYB42 and UBC1/UBC2, we constructed the transgenic plants 35S:MYB42:ubc1, 2. An immunoblot analysis revealed that the MYB42-MYC protein was readily detected in transgenic plants (Fig. 4c). Additionally, the MYB42 transcriptions was increased in transgenic plants compared with the WT (Fig. 4d). Phenotypic analyses revealed that the overexpression of MYB42 could totally complement the salt-sensitive phenotype of ubc1, 2 (Fig. 4e). Additionally, the seedling survival rate of 35S:MYB42:ubc1, 2 was significantly increased compared with that of the ubc1,2 mutant (Fig. 4f). Thus, MYB42 acts downstream of UBC1/UBC2 to regulate salt tolerance.

MYB42 protein interacts with MPK4 and the interaction is enhanced by NaCl treatments

MPK4 and MPK6 are activated by salt stress in plants (Ichimura et al., 2000), and MPK3/6 participate in the salt-stress response (Li et al., 2014; Zhou et al., 2017). We investigated whether MYB42 is associated with MAPks. We performed a Y2H assay to determine whether MYB42 interacted with MAPks. The result revealed that MYB42 specifically interacted with MPK4 in yeast (Fig. 5a). To determine whether MYB42 interacted with MPK4 in vivo, we performed a co-immunoprecipitation assay using transgenic 35S:MYB42:Myc plants. Protein extracts were immunoprecipitated with anti-Myc magnetic beads, and the precipitated proteins were analyzed by immunoblotting with anti-MPK4 antibody. As expected, a band mobility consistent with MPK4 was detected in the anti-Myc immunoprecipitate (Fig. 5b). In addition, a LCI assay was performed with tobacco leaves, and it verified the MYB42–MPK4 interaction in plants (Fig. 5c). Thus, MYB42 interacts with MPK4 in vitro and in vivo.

In order to determine whether the MYB42–MPK4 interaction was associated with salt tolerance, we detected the intensity of their interaction under salt-treatment conditions. A co-immunoprecipitation assay was performed in Arabidopsis seedlings. The interaction was enhanced by the NaCl treatment, although the level of MYB42 protein decreased under salt-treatment conditions (Fig. 5d). Furthermore, a LCI assay showed that the MYB42–MPK4 interaction obviously increased after the NaCl treatment compared with the control (Fig. 5e). Thus, the MYB42–MPK4 interaction is required for salt tolerance.

MPK4 phosphorylates MYB42, which facilitates MYB42 protein stability under salt-stress conditions

Because MPK4 interacted with MYB42, we hypothesized that MYB42 was a substrate of MPK4. Therefore, we purified recombinant His-tagged MYB42 and MPK4 for phosphorylation assays. MKK2 is required for activating MPK4, but we failed to purify the activated MKK2-His protein. However, constitutively active MPK4D198G/E202A-His protein (MPK4DE), which has MAP2K-independent activity (Berriri et al., 2012), was purified and used to perform in vitro phosphorylation assays. The result indicated that MPK4DE phosphorylated MYB42 in vitro as indicated by a nonradioactive kinase assay and subsequent Pro-Q diamond phosphoprotein staining (Fig. 6a) (Sheikh et al., 2016). By contrast, MPK4 failed to phosphorylate MYB42 in the absence of MAP2K (Fig. 6a).

We further assessed the biological significance of MPK4-mediated MYB42 phosphorylation. When the MYB42 protein was characterized, it was unstable under salt-treatment conditions. An immunoblot analysis indicated that the MYB42-Myc level in 35S:MYB42-Myc transgenic seedlings decreased after the NaCl treatment (Fig. 6b). Consistently, under confocal microscopy, MYB42-GFP fluorescence significantly decreased in WT overexpressing MYB42-GFP (35S:MYB42-GFP) root epidermal cells in the presence of NaCl (Fig. 6c). Furthermore, we performed transient transformation assays in tobacco leaves using the 35S:MYB42-GFP vector. The MYB42-GFP fluorescence also was notably reduced after the NaCl treatment (Fig. 6d). However, when 35S:MYB42-GFP and pBI-HA-MPK4DE (a constitutively active form of MPK4) constructs were co-transformed into tobacco leaves, the MYB42 protein had a dramatically enhanced stability compared with transformations without pBI-HA-MPK4DE under salt-stress conditions (Fig. 6d). Thus, MPK4-mediated MYB42 phosphorylation may facilitate MYB42 protein stability under salt-stress conditions.

Next, we examined MYB42 degradation in the WT and pAI-HA-MPK4DE/Col-0 (an active mutant of MPK4 driven by a steroid-inducible promoter) plants using a cell-free protein degradation assay. First, the MPK4DE seedlings were treated with 15 μM dexamethasone for 2 h. Recombinant MYB42-His protein was incubated with total protein extracts from WT and MPK4DE plants in the presence of ATP. The increasing ATP incubation time gradually increased MYB42-His degradation in WT plants; however, the degradation of MYB42-His was largely suppressed in pAI-HA-MPK4DE/Col-0 plants (Fig. 6e). These data provide further evidence that MYB42 stability is positively regulated by MPK4.

UBC1 and -2 regulate the transcription of MPK4 during salt stress

We determined whether the expression of MPK4 was induced in the WT and ubc1,2 double mutant after NaCl treatment using qRT-PCR. The transcription level of MPK4 was notably increased after the salt treatment in the WT, but was not increased in the ubc1,2 double mutant (Fig. 7a). ChIP assays were performed to examine the levels of H2Bub1 and H3K4me3 enrichment in the chromatin of MPK4 in the WT and ubc1,2 double mutant. Two fragments were on the chromatin of MPK4 were targeted for ChIP-qPCR (Fig. 7b). The H2Bub1 enrichment was strongly detected on the P1 region of the MPK4 locus.
in the WT, but only a weak enrichment was detected in the ubc1,2 mutant. Moreover, H2Bub1 was significantly increased in the chromatin of MPK4 of the WT after NaCl treatment, whereas it was not increased in the ubc1,2 mutant (Fig. 7c). Consistently, the level of H3K4me3 was greater in the chromatin of MPK4 after the NaCl treatment in the WT than in the ubc1,2

Fig. 5 MYB42 interacts with Mitogen-Activated Protein Kinase 4 (MPK4), and the interaction is enhanced by a NaCl treatment. (a) MYB42 interacts with MPK4 in yeast. Yeast was grown on SD/-Trp-Leu, SD/-Trp-Leu-His-Ade and SD/-Trp-Leu-His-Ade + X-gal media. Empty pGADT7 and pGBK7T were used as negative controls. pGADT7-T + pGBK7T-53 was used as a positive control. (b) Co-immunoprecipitation of MYB42 with MPK4 in plants. Total proteins were extracted from 7-d-old transgenic plants expressing 35S:MYB42:Myc, and immunoprecipitated with anti-Myc magnetic beads. Input and immunoprecipitated proteins were analyzed by immunoblotting with anti-MYC and anti-MPK4 antibodies, respectively. (c) Luciferase complementation imaging (LCI) assay showing the interaction of MYB42 with MPK4 in tobacco leaves. nLuc-SGT1 + RAR1-cLuc was used as a positive control. nLuc + MYB42-cluc and nLuc-MPK4 + cLuc were used as negative controls. (d, e) The interaction of MYB42 with MPK4 was enhanced by NaCl. (d) Coimmunoprecipitation of MYB42 with MPK4 in plants after treatment with 200 mM NaCl for 1 h. (e) Infiltrated tobacco leaves were sprayed with water or saline solution in the LCI assays.
mutant (Fig. 7d). These results suggest that H2Bub1 regulates the expression of MPK4 by enhancing the enrichment of H3K4me3 during salt stress.

MYB42 mediates the salt-induced expression of SOSs and binds to the SOS2 promoter

The SOS pathway is crucial for the regulation of plant Na⁺:K⁺ homeostasis under salt stress (Zhu, 2016). To further explore the function of MYB42 in salt tolerance, WT and myb42-1 mutant Arabidopsis seedlings were used. The relative expression levels of SOS1, SOS2 and SOS3 in response to salt stress were monitored by qRT-PCR. The expression levels of SOS2 and SOS3 were significantly increased in the WT after the NaCl treatment; however, their transcript levels were barely increased in the myb42-1 mutant (Fig. 8a; Fig. S5). The transcription of SOS1 was not significantly different in the WT and myb42-1 mutant. The results demonstrate that MYB42 positively regulates the expression of SOS2 and SOS3 under salt conditions. In addition, we examined the expression of SOS2 and SOS3 in the WT and ubc1,2 double mutant. Consistently, the transcript levels of SOS2 and SOS3 were lower in ubc1, 2 double-mutant compared with that of the WT after the salt treatment (Fig. S6). These data indicate that MYB42 mediates the transcription of SOS2 and SOS3 in response to salt stress.
Next, we investigated whether MYB42 interacted directly with the promoters of \textit{SOS2} and \textit{SOS3}. Yeast-one-hybrid assays were performed to examine the interactions between MYB42 and the promoter fragments. A 1.5-kb promoter fragment of \textit{SOS2} (from $/C0$1463 to the transcriptional start site) and a 2.7-kb promoter fragment of \textit{SOS3} (from $/C0$2721 to the transcriptional start site) were used to generate bait vectors. MYB42 was used as prey and co-transformed with the \textit{LacZ} reporter driven by respective promoter fragments of \textit{SOS2} and \textit{SOS3} into the yeast strain EGY48. All of the yeast cells grew well on SD/-Ura-Leu medium, whereas only yeast cells co-transformed with the prey and bait harboring promoter fragments of \textit{SOS2} turned blue on SD/-Ura-Leu dropout plates containing X-gal (Fig. 8b). The results suggest that MYB42 binds directly to the \textit{SOS2} promoter but does not interact with the \textit{SOS3} promoter. Moreover, we further confirmed that MYB42 bound the S2 fragment (−863 to −263) of the \textit{SOS2} promoter using Y1H assays (Fig. S7).

In order to assess whether MYB42 directly bound the \textit{SOS2} promoter to regulate its transcription in vivo, we performed ChIP assays using \textit{Pro35S:MYB42-Myc} \textit{Col-0} plants. A 132-bp fragment (−1 to +132, P1), and two 100-bp fragments (−364 to −464, P2 and −1263 to −1363, P3) of the \textit{SOS2} promoter (Fig. 8c) were examined using ChIP qPCR. Significant enrichments of MYB42 were revealed in the −1 to −132 and −364 to −464 regions of the \textit{SOS2} promoter, particularly the P2 fragment (Fig. 8d), indicating that MYB42 binds to the \textit{SOS2} promoter in vivo.
Fig. 8 MYB42 mediates the salt-induced expression of SALT OVERLY SENSITIVE genes (SOSs) and binds to the SOS2 promoter. (a) Expression levels of SOS2 in the wild-type (WT) and myb42-1 mutant plants treated with NaCl. The 7-d-old seedlings of the WT and mutant were treated with 200 mM NaCl for 3 h, and total RNA was extracted for quantitative reverse transcription (qRT)-PCR analysis. (b) Yeast-one-hybrid assays showing that MYB42 bound to the promoter of SOS2 in vitro. (c) Structure of the SOS2 promoter. P1 to P3 represent the primers used to assess the enrichment level by chromatin immunoprecipitation (ChiP). (d) ChiP-qPCR analysis of the relative MYB42 binding to the promoter of SOS2. An anti-Myc antibody was used for DNA immunoprecipitation from 15-d-old 35S:MYB42-Myc transgenic plants. (e) Schematics of all constructs used for β-glucuronidase (GUS) transactivational assays in Nicotiana benthamiana leaves. The SOS2 promoter was fused to the GUS reporter gene. The 35S promoter was fused to the luciferase (LUC) gene as an internal control. The effector constructs were 35S:MYB42-Myc and pBI:HA-MPK4DE. (f) MYB42 activated the expression of SOS2 in the GUS assay. SOS2:GUS was co-transformed with other constructs into tobacco leaves. 35S:LUC was used as an internal control. (g) Schematic diagrams of the effector and reporter constructs used for dual-luciferase transient expression assays. LUC and REN activity levels were measured after culturing the protoplasts under low-light conditions for 16 h. The ProSOS2:LUC:Pro35S:REN ratio represents the relative activity of SOS2 transcription. (i) Salt-sensitive phenotypes of the myb42-1/sos2-2 mutant. Seedlings were grown on ½ Murashige & Skoog medium for 5 d and transferred to ½MS medium with or without 175 mM NaCl for 4 d before being photographed. Bars, 1.5 cm. (j) Seedling survival rates of myb42-1/sos2-2, myb42-1, sos2-2 and WT treated with NaCl. Seedlings were grown on ½MS medium for 5 d and transferred to ½MS medium with 250 mM NaCl for 4 d. The data are shown as the means ± SDs of three independent experiments, in which each genotypic replicate included 30 seedlings. Different letters represent significant differences at P < 0.05 by one-way ANOVA followed by Tukey’s Honest Significant Difference (HSD) post-hoc test. (k) Model for the mechanisms of UBIQUITIN CARRIER PROTEIN1 (UBC1) and -2 in the regulation of salt-stress responses. UBC1/UBC2-mediated histone H2B monoubiquitination (H2Bub1) modulates the salt-induced transcription of MYB42 and MPK4 by enhancing the level of histone H3 tri-methylated on K4 (H3K4me3) on their chromatin. In addition, activated MPK4 phosphorylates MYB42 and facilitates MYB42’s binding to the SOS2 promoter, thereby promoting the expression of SOS2 and salt tolerance.
In order to explore how MYB42 regulates SOS2 expression, we performed transient transactivation assays in tobacco leaves using the SOS2 promoter fused to GUS (SOS2-GUS) (Fig. 8e). A MYB42 effector construct was expressed under the control of the 35S promoter and was co-transfected with the SOS2:GUS reporter construct and the internal control (35S:LUC) into N. benthamiana leaves. Consistent with previous results, MYB42 activated the SOS2 promoter (Fig. 8f). Furthermore, to determine whether MPK4-mediated MYB42 phosphorylation affects MYB42's function, 35S:MYB42-Myc and pBI-HA-MPK4DE constructs were co-transfomed with the SOS2:GUS reporter construct and the internal control (35S: LUC) into tobacco leaves, and there was an obvious enhancement of MYB42-activated SOS2 expression compared with MYB42 alone (Fig. 8f). MPK4-mediated MYB42 phosphorylation may positively regulate MYB42 transcriptional activity and SOS2 expression.

We further investigated the positive regulation of MYB42 on SOS2 transcription using a dual-LUC reporter assay. The SOS2 promoter-driven LUC reporter (ProSOS2: LUC) and 35S promoter-driven REN (Pro35S: REN; as an internal control) were introduced into the same plasmid and transiently expressed in Arabidopsis protoplasts (Hellens et al., 2005; Guo et al., 2017) (Fig. 8g). Another plasmid, with or without the MYB42 coding region, was co-expressed in protoplasts and the LUC:REN ratio was determined. It reflects the transcriptional activity of the SOS2 promoter in vivo. The co-expression of MYB42 and ProSOS2:LUC/Pro35S:REN increased the LUC:REN ratio (Fig. 8h), suggesting that MYB42 is capable of activating SOS2 promoter-driven transcription. In addition, the co-expression of MYB42 and ProSOS2:LUC/Pro35S:REN in pAI-HA-MPK4DE/Col-0 protoplasts (an active mutant of MPK4 driven by a steroid-inducible promoter) markedly enhanced the LUC:REN ratio compared with in the WT protoplasts (Fig. 8h). These data also indicate that phosphorylation promotes the MYB42 transcriptional activity.

We also generated a myb42-1 sos2-2 double mutant by crossing the myb42-1 with sos2-2 mutants to observe the salt-sensitive phenotype. The myb42-1 and sos2-2 mutants are both sensitive to salt stress. The myb42-1 sos2-2 double mutant displayed a similar level of salt sensitivity as that of sos2-2 and a greater sensitivity than both the WT and myb42-1 (Fig. 8i). The seedling survival rate of the double mutant was similar to that of the sos2-2 mutant and was lower than those of the WT and myb42-1 (Fig. 8j). These results reveal that MYB42 functions in the same pathway as SOS2 to mediate plant salt tolerance.

Discussion

In Arabidopsis, E3 ligases (HUB1 and HUB2, HISTONE MONOUBQUITINATION1 and -2) and E2 conjugated enzymes (UBC1 and UBC2, UBIQUITIN CARRIER PROTEIN1 and -2) are responsible for ubiquitination of histone H2B. Histone H2B monoubiquitination (H2Bub1) regulates multiple developmental processes and is involved in biotic and abiotic stress responses (Fleury et al., 2007; Cao et al., 2008; Dhawan et al., 2009; Bourbousses et al., 2012; Hu et al., 2014; Zou et al., 2014; Zhou et al., 2017; Chen et al., 2019). HUB1 and HUB2 loss-of-function mutants (hub1-4, hub2-2 and the hub1-4 hub2-2 double mutant) exhibit salt-sensitive phenotypes, and H2Bub1 regulates salt-induced microtubule depolymerization and the PTP-MPK3/6 signaling module in response to salt stress (Zhou et al., 2017) (MPK, Mitogen-Activated Protein Kinase). However, the functions and mechanisms of UBC1 and UBC2 in response to salt stress are unknown.

In this study, we proposed a working model of UBC1 and -2’s positive regulation of MYB42-mediated SALT OVERLY SENSITIVE 2 (SOS2) expression to enhance plant salt tolerance (Fig. 8k). UBC1- and -2-mediated H2Bub1 regulated the expression of MYB42 and MPK4 by enhancing the enrichment of histone H3 tri-methylated on K4 (H3K4me3) on the chromatin of MYB42 and MPK4 under salt-stress conditions. Activated MPK4 phosphorylated MYB42. MPK4-mediated MYB42 phosphorylation enhanced MYB42 protein stability and transcriptional activity under salt-stress conditions, which contributed to the increased expression of SOS2 and salt tolerance.

In general, H2Bub1 is considered to represent an active chromatin marker broadly involved in transcriptional regulation, including activation and elongation (Pavri et al., 2006; Weake & Workman, 2008; Feng & Shen, 2014). H2Bub1 is required for H3K4 and H3K79 methylation in yeast (Lee et al., 2007). However, hub mutants exhibit reductions in H3K4me3 only at specific chromatin regions of some genes in Arabidopsis (Cao et al., 2008; Himanen et al., 2012). For example, H2Bub1 is required for the enhancement of H3K4me3 in the chromatin of FLC and some other FLOWERING LOCUS C (FLC) clade genes, and, in turn, it controls flowering time in Arabidopsis (Cao et al., 2008). In addition to flowering, H2Bub1 also regulates the expression levels of some circadian clock genes by increasing the H3K4me3 deposition in their chromatin (Himanen et al., 2012; Malapeira et al., 2012). Moreover, the overexpression of AtHUB2 enhances the level of H2Bub1 and H3K4me3 modifications at the GhDREB locus, leading the GhDREB gene to respond quickly to drought stress, which improves transgenic cotton’s drought resistance (Chen et al., 2019). However, how H2Bub1 regulates transcription induction in plant responses to salt stress remains unclear.

Our results showed that UBC1-mediated H2Bub1 was enriched intensively on the chromatin loci of MYB42 and MPK4 in the wild-type (WT), whereas it was enriched weakly in the ubc1-2 mutant. In addition, we revealed that the level of H3K4me3 was greater in the chromatin of MYB42 and MPK4 after a NaCl treatment in the WT than in the ubc1-2 mutant. Therefore, H2Bub1 activated the salt-induced expression of MYB42 and MPK4 by enhancing the level of H3K4me3. Furthermore, the salt-induced expression of MYB42 was impaired in hub mutants, indicating that both HUB1/HUB2 and UBC1/UBC2 regulate the salt-induced expression of MYB42 through H2Bub1 modification.

MYB42 is a R2R3-MYB transcription factor. R2R3-MYB proteins regulate many physiological and biochemical processes (Dubos et al., 2010). Several R2R3-MYB transcription factors, including MYB20, MYB30 and MYB96, are involved in...
responding to abscisic acid (ABA) and other stresses, such as osmotic, drought and salinity (Seo et al., 2009; Zheng et al., 2012; Cui et al., 2013). Moreover, the AtMYB44 promoter is activated to induce the accumulation of gene transcripts in response to salt stress (Nguyen & Cheong, 2018). Nevertheless, the function of MYB42 in response to salt stress has not been reported. Our results indicated that MYB42 was localized in the nucleus and was a transcriptional activator. In this study, the myb42-1 mutant was more sensitive to salt stress than the WT. Additionally, MYB42 acted downstream of UBC1/UBC2 to mediate salt tolerance in Arabidopsis. Further studies demonstrated that MYB42 interacted with MPK4 and that their interaction was enhanced by the salt treatment.

MPK4 and MPK6 are activated by salt stress in Arabidopsis (Ichimura et al., 2000); however, it is unclear how MPK4 regulates downstream target proteins in response to salt stress. Here, MPK4 phosphorylated MYB42, which contributed to the increased MYB42 protein stability under salt-stress conditions. Moreover, MPK4-mediated MYB42 phosphorylation enhanced MYB42’s transcriptional activity in response to salt stress. These results indicate that activated MPK4 is important to the function of MYB42 in salt-stress responses. It also provides new evidence that the MAPK signaling pathway is involved in responding to salt stress.

The SOS pathway plays a crucial role in regulating Na\(^+\): K\(^+\) homeostasis in response to salt stress (Zhu, 2016). There are three important factors in the SOS pathway, SOS3 (a Ca\(^-\)-binding protein), SOS2 (a serine/threonine protein kinase) and SOS1 (a Na\(^+\): H\(^+\) exchanger). SOS2 has a central role in the SOS pathway (Yang & Guo, 2018). In Arabidopsis, the SOS2 kinase is activated by NaCl and SOS2 phosphorylates SCaBP8, which stabilizes the SCaBP8–SOS2 interaction on the plasma membrane and, in turn, enhances the SOS1 activity under salt-stress conditions (Lin et al., 2009). Furthermore, SOS2 is a key node connecting H\(_2\)O\(_2\) signaling and salt-stress responses by interacting with CATALASE 2 and 3 (Verslues et al., 2007). In addition, SOS2 phosphorylates ethylene-insensitive 3 to increase target gene expression, which links ethylene signaling with salt-stress responses (Quan et al., 2017). These findings demonstrate that SOS2 has a crucial role in the SOS pathway.

Although the SOS pathway has been investigated extensively, upstream transcriptional regulators of the SOS genes remain poorly characterized. In this study, we revealed that MYB42 modulated the salt-induced transcription of SOS2 and SOS3. Further studies indicated that MYB42 binds directly to the promoter of SOS2, but not to the SOS3 promoter. Therefore, SOS2 is a direct target gene of MYB42, and SOS3 is regulated indirectly by MYB42. The elevations in the SOS genes’ activity levels are crucial for determining intracellular Na\(^+\) accumulation and plant salt tolerance. These findings indicate that MYB42-mediated salt tolerance depends on its positive regulation of the SOS genes.

In conclusion, our results demonstrate that H2Bub1 is required for mediating the expression of MYB42 and MPK4 during salt stress and that MPK4-mediated MYB42 phosphorylation is crucial for responses to salt stress. This study expands our understanding of the molecular mechanisms of H2Bub1 in response to salt stress.

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**Author contributions**

YS and YL designed the research and wrote the article; YS, JZ and XL performed specific experiments and analyzed the data; and YL revised and edited the article.

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**References**


**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Phenotypes of complementary lines *UBC1/ubc1-1* and *UBC2/ubc2-1* in salt-stress responses.

**Fig. S2** Phenotypes of *UBC*-overexpression lines in salt-stress responses.

**Fig. S3** Expression levels of *MYB42* in *hub* mutants treated with NaCl.

**Fig. S4** Salt-sensitive phenotype of the *crispr#16* mutant.

**Fig. S5** Expression levels of *SOS3* in WT and *myb42-1* mutant plants treated with NaCl.

**Fig. S6** Expression levels of (a) *SOS2* and (b) *SOS3* in WT and *ubc1,2* mutant plants during salt-stress responses.

**Fig. S7** Yeast-one-hybrid assays showing that MYB42 binds to the –863 to –263 region of the *SOS2* promoter.

**Table S1** Primers used for genotyping the mutants.

**Table S2** Primers used for qRT-PCR.

**Table S3** Primers used for ChIP-qPCR.

**Table S4** Primers used for plasmid construction.

**Table S5** Summary of statistical tests.

**Table S6** Salt-stress induced genes with lower expression levels in *ubc1,2* than in the WT.

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