Planta (2023) 257:32
https://doi.org/10.1007/s00425-022-04064-1

ORIGINAL ARTICLE

A *Medicago truncatula* lncRNA *MtCIR1* negatively regulates response to salt stress

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Received: 14 September 2022 / Accepted: 29 December 2022 / Published online: 5 January 2023

Abstract

Main conclusion **A lncRNA *MtCIR1* negatively regulates the response to salt stress in *Medicago truncatula* seed germination by modulating seedling growth and ABA metabolism and signaling by enhancing Na^+ accumulation.**

Abstract Increasing evidence suggests that long non-coding RNAs (lncRNAs) are involved in the regulation of plant tolerance to varying abiotic stresses. A large number of lncRNAs that are responsive to abiotic stress have been identified in plants; however, the mechanisms underlying the regulation of plant responses to abiotic stress by lncRNAs are largely unclear. Here, we functionally characterized a salt stress-responsive lncRNA derived from the leguminous model plant *M. truncatula*, referred to as *MtCIR1*, by expressing *MtCIR1* in *Arabidopsis thaliana* in which no such homologous sequence was observed. Expression of *MtCIR1* rendered seed germination more sensitive to salt stress by enhanced accumulation of abscisic acid (ABA) due to suppressing the expression of the ABA catabolic enzyme CYP707A2. Expression of *MtCIR1* also suppressed the expression of genes associated with ABA receptors and signaling. The ABA-responsive gene *AtPGIP2* that was involved in degradation of cell wall during seed germination was up-regulated by expressing *MtCIR1*. On the other hand, expression of *MtCIR1* in *Arabidopsis thaliana* enhanced foliar Na^+ accumulation by down-regulating genes encoding Na^+ transporters, thus rendering the transgenic plants more sensitive to salt stress. These results demonstrate that the *M. truncatula* lncRNA *MtCIR1* negatively regulates salt stress response by targeting ABA metabolism and signaling during seed germination and foliar Na^+ accumulation by affecting Na^+ transport under salt stress during seedling growth. These novel findings would advance our knowledge on the regulatory roles of lncRNAs in response of plants to salt stress.

Keywords ABA signaling · *Arabidopsis thaliana* · LncRNA · *Medicago truncatula* · *MtCIR1* · Salt stress · Seed germination

Communicated by Dorothea Bartels.

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Introduction

Non-coding RNAs with a length greater than 200 nucleotides are defined as long non-coding RNAs (lncRNAs), which have little or no capacity of protein-coding potential (Ponting et al. 2009; Ariel et al. 2015). LncRNAs can be classified into intronic non-coding RNAs, intergenic non-coding RNAs, and natural antisense non-coding RNAs (NATs) according to their genomic localization (Ponting et al. 2009; Wang et al. 2014a, b; Ariel et al. 2015). Numerous lncRNAs in plants have been identified by high-throughput sequencing technology (Zhu et al. 2013; Li et al. 2014; Deng et al. 2018; Huanca-Mamani et al. 2018; Zhao et al. 2018). Plant lncRNAs regulate gene expression via a series of complex mechanisms (Kornienko et al. 2013; Liu et al. 2015). The involvement of lncRNAs in regulation

of physiological processes in response of plants to abiotic stress is emerging. For example, cabbage lncRNA *BoNR8* negatively regulates *Arabidopsis* tolerance to salt stress by affecting seed germination, primary root, and silique growth (Wu et al. 2019). Salt stress-related *LncRNA973* is reported to enhance tolerance of cotton to salt stress by modulating the expression of a series of genes involved in response to salt stress (Zhang et al. 2019). Nonetheless, the molecular mechanisms underlying the gene regulation by lncRNAs remain largely unknown. Several lncRNAs have been identified to be responsive to cold, salt, and osmotic stresses in *M. truncatula* (Wang et al. 2015a, b; Zhao et al. 2020). However, the functional characterization of *M. truncatula* lncRNAs involved in salt stress is still in its infancy.

Salt stress adversely affects seed germination, root growth, and seedling development (Zhu 2002, 2016; Ketehouli et al. 2019). Compared with other developmental stages of plants, seed germination is more sensitive to salt stress (Manz et al. 2005; Finch-Savage and Leubner-Metzger 2006; Nonogaki et al. 2010; Rajjou et al. 2012). Seed germination starts with the uptake of water by the mature dry seeds, and ends with the radicle protruding the endosperm and seed coat. This process is regulated by both environmental and hormonal cues (Alboresi et al. 2005; Cheng et al. 2016; Liu et al. 2016). Plants can quickly sense and respond to external cues by complex mechanisms, resulting in a set of changes in metabolism, physiology and development (Yamaguchi-Shinozaki and Shinozaki 2006; Shinozaki and Yamaguchi-Shinozaki 2007). The biosynthesis and accumulation of ABA are the most common phenomenon in response to abiotic stress including salt, drought and cold stress (Wilkinson and Davies 2002; Xu et al. 2013). ABA plays a pivotal role in the regulation of response and adaption of plants to abiotic stress by sensing and transducing stress signals, as well as modulating numerous gene expressions (Cutler et al. 2010). Many developmental processes, such as seed germination, early seedlings growth, lateral root formation, leaf senescence, and stomatal closure, are regulated by ABA (Wang et al. 2020). In addition, ABA is also involved in the control of plant water status by regulating stomata under abiotic stress, e.g., salt stress (Vishwakarma et al. 2017). Recent studies have reported that the lncRNA *DRIR* regulates ABA-mediated drought and salt stress responses (Qin et al. 2017).

ABA metabolism is sensitive to salt stress, such that ABA concentration is enhanced under salt stress (Wang et al. 2015a, b). Seed germination is inhibited under salt stress by suppressing water uptake and endosperm rupture (Koornneef et al. 2002; Yuan et al. 2011). ABA concentration is a key regulator in the control of seed germination and dormancy. For example, in Arabidopsis, the catabolic enzyme CYP707A2 is involved in down-regulation of ABA levels during seed imbibition (Kushiro et al. 2004). Moreover, genes involved in ABA biosynthesis are often up-regulated, thereby leading to an increase in planta ABA level, which in turn activates the ABA-responsive target genes through ABA-responsive elements (ABREs) in their promoters (Guo et al. 2011). ABA signaling is perceived by a group of receptors that belong to the PYR/ PYL/RCAR family and ABA co-receptors of the PP2C family (Miyazono et al. 2009; Park et al. 2009). ABA binding to the receptors activates the subsequent kinase activity of SnRKs. SnRKs phosphorylate downstream components, such as ABA-responsive element-binding factors (AREBs/ABFs), to regulate the expression of ABA-responsive genes (Miyazono et al. 2009). *ABI1* and *ABI2*, which encode protein phosphatases, have been reported to negatively regulate ABA signaling during seed dormancy and germination (Nakashima and Yamaguchi-Shinozaki 2013). *ABI3*, *ABI4*, and *ABI5* genes encode transcription factors that regulate ABA responses during seed germination and vegetative growth (Lopez-Molina et al. 2002; Finkelstein et al. 2005; Zhang et al. 2005; Khandelwal et al. 2010).

Arabidopsis polygalacturonase inhibitors of PGIP1 and PGIP2 are associated with seed germination (Kanai et al. 2010). Pectin is a main mucilage in seed coat (Ferrari et al. 2002). Breakdown of the seed pectin can soften seed coat and promote seed coat rupture, thus facilitating seed germination (Kanai et al. 2010). Overexpression of *PGIP1* and *PGIP2* prevents mucilage degradation due to disruption of pectin degradation (Kanai et al. 2010). Given that PGIP1 and PGIP2 are direct targets of ABI5 (Kumar et al. 2019), it is likely that ABI5 acts as a master regulator of seed germination through the ABA signaling pathway. However, there have been no studies linking lncRNAs to ABA-dependent, PGIPs-mediated seed germination under salt stress.

The excessive accumulation of Na^+ by plants is a key factor to adversely affect cell division, photosynthesis and development (Horie and Schroeder 2004; Julkowska and Testerink 2015). Therefore, maintaining a low Na^+ concentration in the cytoplasm is critical to improve tolerance to salt stress. Plants have evolved mechanisms to maintain the low cytoplasmic Na^+ under salt stress. Among them, salt overly sensitive genes (*SOSs*) pathways play important roles in excluding excess Na^+ out of the cell via Na^+/H^+ antiporters in the plasma membrane (Zhu et al. 1998). Under salt stress, excessive Na^+ in the cytoplasm initiates a calcium signal that activates the SOS3–SOS2 protein kinase complex, which in turn stimulates the Na^+/H^+ activity of SOS1, leading to export of Na^+ from the plant cells (Ishitani et al. 2000; Liu et al. 2000; Shi et al. 2000; Zhu 2001; Qiu et al. 2002). SOS3–SOS2 complex may also positively regulate the activities of vacuolar Na^+/H^+ exchangers NHX to maintain a low cytoplasmic Na^+ by sequestrating Na^+ into the vacuoles (Blumwald 2000; Batelli et al. 2007). In *Arabidopsis*, NHX is a potential Na^+/H^+ exchanger, which

transports Na^+ from the cytoplasm to the vacuole (Yu et al. 2008). Overexpression of *AtNHX1* enhances the tolerance of *Arabidopsis* plants to salt stress (Apse et al. 1999). In addition, HKT1 is another membrane transport protein mediating Na^+ influx into plant cells. The first member of HKT family was cloned in wheat (Rus et al. 2001; Laurie et al. 2002). AtHKT1 plays a key role in plant tolerance to salt stress by regulating Na^+ entry from xylem vessels to xylem parenchyma cells, leading to a decrease of Na^+ in xylem vessels and leaves (Sunarpi et al. 2005).

A cold-responsive lncRNA *MtCIR1* in the legume model plant *M. truncatula* was identified in our previous studies (Zhao et al. 2020). The *MtCIR1* target genes were predicted to be involved in varying biological processes including defense response and regulation of transcription under abiotic stress (Zhao et al. 2020). However, the physiological functions of *MtCIR1* remain to be characterized. In the present study, we further investigated the roles of *MtCIR1* in regulation of response to salt stress by expressing the *MtCIR1* in *A. thaliana*, and functionally characterized its role in response to salt stress during seed germination and seedling growth. We found that expression of *MtCIR1* in *Arabidopsis* rendered the transgenic plants sensitive to salt stress during seed germination and seedlings growth, and explored the mechanisms by which *MtCIR1* negatively regulates responses to salt stress.

Materials and methods

Plasmid construction and transgenic plant generation of *A. thaliana*

The full-length cDNA sequence of *MtCIR1* was amplified using primers as follows:

5′-TCGGCGCGCCGGTGTAGCTAAACCCTATGA-3′ (AscI site underlined) and 5′-GTTAATTAATGTCCTCTT TTATTGCTATCA-3′ (PacI site underlined). The sequencing confirmed that PCR fragments were directionally cloned into a pMDC32 vector driven by the two constitutive cauliflower mosaic virus (CaMV) 35S promoters to create the pMDC32-*MtCIR1* construct (Supplementary Fig. S1) (Zhang et al. 2016). These vectors were introduced into the *Agrobacterium tumefaciens* EHA105 strain for transformation of *A. thaliana* (ecotype Columbia 0) using the *Agrobacterium tumefaciens*-mediated floral dip method (Clough and Bent 1998). *MtCIR1* transgenic lines were identified by DNA-PCR using primers designed on both side sequences of *MtCIR1* insertion point in pMDC32. pMDC32-up: 5′-AGG ACCTCGACTCTAGAGGATC-3′. pMDC32-Down: 5′-TTG CCAAATGT TTGAAGCATCG-3′. Two independent homozygous lines (L-2 and L-4) of the T3 generation were randomly selected and were used for subsequent salt-stress tolerance tests (Supplementary Fig. S2).

Plant growth and stress treatments

Seeds of *A. thaliana* WT and *MtCIR1* transgenic lines were surface-sterilized in 75% (v/v) ethanol for 0.5 min and 10% (v/v) sodium hypochlorite for 15 min in sequence. After rinsing several times with sterilized distilled water, the seeds were grown on 1/2 Murashige and Skoog (MS) medium (1% sucrose, 0.7% agar, pH 5.8) in the green house with a 14 h light, 22 °C/10 h, 20 °C dark cycle, light intensity is 120 μmol m^{-2} s^{-1}. Seed germination was defined as the emergence of the radicle break through the seed coat. The seed germination rate was continuously counted for a week. Approximately 100 seeds per sample in each treatment were tested for the determination of seed germination rate and each treatment had at least five replicates.

For the analysis of root growth responding to salt stress and ABA treatment, seeds of *A. thaliana* WT and *MtCIR1* transgenic lines were surface-sterilized in 75% (v/v) ethanol and 10% (v/v) sodium hypochlorite in sequence. After rinsing several times with sterilized distilled water, the seeds were grown on 1/2 MS medium (1% sucrose, 0.7% agar, pH 5.8) with or without 100 mM NaCl. The primary root length was monitored after the seedlings vertically grown for 10 days in the green house with a 14 h light, 120 μmol m^{-2} s^{-1}, 22 °C/10 h, 20 °C dark cycle.

To analyze stress responses in seedling development stage, we examined salt stress and ABA treatment on seedlings, respectively. For the NaCl treatment, surface-sterilized seeds were sown onto 1/2MS medium (1% sucrose, 0.7% agar, pH 5.8) and were stratified at 4 °C for 2 d. After growth in the green house for 5 days, *Arabidopsis* seedlings were transferred to 1/2 MS medium (1% sucrose, 0.7% agar, pH 5.8) with or without 75 mM NaCl for an additional 4 days. The growth conditions are 14 h light, 22 °C/10 h, 20 °C dark cycle, and light intensity of 120 μmol m^{-2} s^{-1}. Survival seedling was referred to that with one green leaf at least. For NaCl treatment of soil-grown seedlings, 4-week-old seedlings were irrigated with 100 mM NaCl for every two weeks and total of 4 times. After 2 months of treatment, they were watered with 1/2 MS solution. Plants were considered dead if all leaves were yellow and did not restore growth after recovery for 7 days.

To evaluate the effect of ABA on seedling growth, surface-sterilized seeds were sown onto 1/2MS medium (1% sucrose, 0.7% agar, pH 5.8) and were placed at 4 °C for 2 d.

After that, the plates were move to the green house with a 14 h light, 22 °C/10 h, 20 °C dark cycle, and light intensity of 120 μmol m^{-2} s^{-1}. Five-d-old seedlings grown on 1/2 MS medium (1% sucrose, 0.7% agar, pH 5.8) were transferred to 1/2 MS medium (1% sucrose, 0.7% agar, pH 5.8)

supplemented with different concentrations of ABA. Green leaves were scored from third to sixth day, and seedling survival rates were scored after treatment for 6 days.

Analysis of GUS activity

The *MtCIR1* promoter fragment (1.76 kb, − 1760 to − 1 upstream of *MtCIR1* sequence) was amplified and cloned into pEASY-T3 vector, after sequencing verification, the *MtCIR1* promoter on the pEASY-T3 vector was transferred to pENTR4 through the EcoRI site. Finally, *MtCIR1* recombined with pHGWFS7.0 (Supplemental Fig. S3). After sequence confirmation, the terminal construct p^{MtCIR1}-GUS was transformed into *Arabidopsis* using *Agrobacterium tumefaciens* (strain EHA105)-mediated floral dip method (Clough and Bent 1998). For analysis of the *β*-glucuronidase (GUS), plant samples were first fixed in 90% acetone for 10 min, then washed thoroughly with GUS staining buffer and vacuumed for 20 min. After that, plant materials were stained in GUS staining buffer at 37 °C overnight followed by decoloring with 30%, 50%, 70%, 85% ethanol for 30 min, respectively. Finally, 95% ethanol was used to decolorize until the chlorophyll was completely removed. Samples were observed and photographed using Leica Smart 3D Digital Microscope.

Analysis of gene expression during seed imbibition

Seeds of *A. thaliana* WT and *MtCIR1* transgenic lines were surface-sterilized in 75% (v/v) ethanol for 0.5 min and 10% (v/v) sodium hypochlorite for 15 min in sequence. After rinsing several times with sterilized distilled water, about one-hundred seeds were placed on filter paper soaked with different treatment solutions. After the imbibition for 0, 3, 6, and 12 h, seeds were collected and stored in – 80 °C after quick freezing in liquid nitrogen. RNA was extracted and quantitative real-time PCR was used to detect the expression level of genes under different treatments.

Determination of the endogenous ABA level

For estimation of endogenous ABA levels of germinating seeds, *Arabidopsis* WT and transgenic L-2 and L-4 seeds were surface-sterilized and spread on sterilized filter paper, soaked with 1/2 MS medium (1% sucrose, pH 5.8) with or without 150 mM NaCl. The germinated seeds treated with or without 150 mM NaCl were collected on the 5th and 2nd day after imbibition, respectively. Approximately 200 mg fresh weight (FW) germinating seeds were homogenized under liquid nitrogen, weighted and extracted for 24 h with methanol and $^2H_6$-ABA. Endogenous ABA was purified and measured as previously described (Fu et al. 2012) with some modifications in detection conditions. Briefly, LC–MS/MS analysis was performed on a UPLC system (Waters) coupled to the 6500 Qtrap system (AB SCIEX). LC separation used a BEH C18 column (1.7 µm, 100 × 2.1 mm; Waters) with mobile phase A, 0.05% (v/v) acetic acid in water and B, 0.05% (v/v) acetic acid in acetonitrile. The gradient was set with initial 20% B and increased to 70% B within 6 min. ABA was detected in multiple reaction monitoring (MRM) mode with transition. The MRM transitions for ABA and [$^2H_6$]-ABA are 263.0 > 153.1 and 269.2 > 159.2. Three biological replicates were analyzed for each treatment.

RNA isolation and quantitative real-time PCR analysis

For gene expression analysis, total RNA was isolated using Omini Plant RNA Kit (with DNase I) (CW2598S). About 1 µg RNA was reverse-transcribed into first-strand cDNA with Evo M-MLV RT kit (AG11711). Real-time quantitative PCR (qRT-PCR) was performed using ABI Stepone Plus Instrument. We reverse-transcribed 30 µL cDNA, diluted 10 times with ddH_2O, and took 4.2 µL of the diluted cDNA for Q-PCR. Each reaction contained 5.0 µL of SYBR Green Master Mix reagent (AG11701), 4.2 µL cDNA samples, 0.2 µL ROX Reference Dye, and 0.6 µL of 10 µM gene-specific primers in a final volume of 10.0 µL. The reaction was performed using a two-step method. The first step was 95 °C for 1 min as the initial denaturation, the second step was 40 cycles of 95 °C for 20 s, 56 °C for 20 s and 72 °C for 25 s for amplification. The relative expression level was analyzed by normalized with *Arabidopsis AtACTIN7* gene. For each sample, the mean value from three qRT-PCR data was adapted to calculate the transcript abundance. Gene-specific primers required for the experiment are shown in Supplementary Table S1. All the primers are specific and have no extra products in the melting curve.

Measurement of Na^+ and K^+ concentrations

Four-week-old seedlings of WT and transgenic lines were irrigated with 1/2 MS solution with or without 300 mM NaCl for 7 days. The leaves were harvested, and washed with ddH_2O for several times, dried and weighed. Fifty milligrams of dry materials were placed in a digestion tube, and suspended in 5 mL of premium pure concentrated nitric acid for overnight. One milliliter of 30% hydrogen peroxide was added to the digested tissue

powder, and microwave system (MARS, CEM) was used to completely digest for 1.5 h. The digested solution was diluted with double distilled water to 50 mL. The K^+ and Na^+ contents were detected by ICP-AES (Thermo). The expressions of *AtSOS1*, *AtSOS2*, *AtSOS3*, *AtNHX1*, and *AtHKT1* in leaves were detected after four-week-old seedlings of WT, and transgenic lines were irrigated with 1/2 MS solution with or without 300 mM NaCl for 7 days.

Statistical analysis

All experiments in this study were repeated independently at least three times. The results are given means ± SE. The statistical analysis was performed using IBM SPSS Statistics software.

Results

Expression patterns of *MtCIR1* in *Arabidopsis* transgenic lines

MtCIR1 was identified as a cold-responsive long non-coding RNA (lncRNA) in our previous study (Zhao et al. 2020). Based on the low conservation characteristics of lncRNAs, *MtCIR1* gene sequence was analyzed by NCBI BLAST, and no homologous sequence was found in *A. thaliana*. To functionally characterize *MtCIR1*, we expressed the *MtCIR1* in *Arabidopsis* under the control of two CaMV35S promoters. Genome PCR analysis results showed that the *MtCIR1* gene was integrated into the *Arabidopsis* genome. A number of transgenic lines were obtained, and two independent transgenic lines (L-2 and L-4) were randomly selected for further research (Fig. 1a and Supplementary Fig. S2). Real-time RT-PCR of RNA extracted from transgenic L-2 and L-4 showed that *MtCIR1* gene was expressed in all of the organs examined, with the highest expression in roots and lowest in leaves, inflorescence and silique, respectively (Fig. 1b). To confirm these results, P_{MtCIR1}: GUS *Arabidopsis* transgenic lines were obtained, in which the GUS reporter gene was placed under the control of a 1761 bp *MtCIR1* promoter.

During seed germination, *MtCIR1* was first expressed in cotyledon vascular tissues, and gradually expanded to cotyledon mesophyll and radicle vascular tissues (Fig. 1c). In 3- to 7-day-old seedlings, GUS activity was strong in all organs except root tips (Fig. 1d). In 14-day-old seedling, GUS staining signal was also strong in the root and cotyledon, but only weak GUS signal was detected at the apical point of leaf (Fig. 1d). Consistent with the real-time RT-PCR results, the strongest GUS signals were detected in primary roots of 7-d old seedlings (Fig. 1d). While GUS signal was strong in stamens of adult plants, none GUS signal was detected in pedicel, calyx, and silique (Fig. 1d). These results suggest that the expression and tissue localization of *MtCIR1* in *Arabidopsis* are closely related to the developmental stage.

Expression of *MtCIR1* was suppressed by salt stress and ABA treatment

To functionally characterize *MtCIR1* in response to salt stress, we first determined its transcriptome profiles under salt stress. A reduction in the *MtCIR1* transcript was observed in the two transgenic lines when treated with NaCl for 2 h and 5 h (Fig. 2a, b). Moreover, we examined the effects of several plant hormones on the transcript level of *MtCIR1*. Exogenous application of plant hormones to the *Arabidopsis* seedlings led to a down-regulation of *MtCIR1* expression to varying degrees. Among the plant hormones, ABA suppressed expression of *MtCIR1*, and the ABA-induced down-regulation of *MtCIR1* exhibited similar patterns to that of NaCl treatment (Fig. 2c, d). The identical sensitivity of *MtCIR1* to ABA and NaCl treatment may imply that ABA is involved in the regulation of responses to salt stress by *MtCIR1*.

MtCIR1 negatively regulates salt tolerance

The function of *MtCIR1* in responses to salt stress was characterized by measuring seed germination, primary root elongation and seedling growth. Seed germination rates of the transgenic line L-2 were significantly lower than WT after imbibition for 2 days in the absence of NaCl (Fig. 3a). Treatment with NaCl suppressed germination rates for both WT and two transgenic lines, and the NaCl-induced reduction in seed germination rate in the transgenic lines was significantly higher than WT (Fig. 3b). The suppression of seed germination rate by NaCl was specific to Na^+ as the identical concentration of KCl did not affect seed germination for both WT and transgenic lines (Fig. 3c).

In addition to seed germination, we also evaluated the effects of salt stress on root elongation. Root length of WT and the two transgenic lines was comparable under control conditions, and treatment with NaCl equally suppressed root elongation for WT and transgenic plants (Fig. 3e, f). We further monitored the effects of NaCl on survival of seedling growth, and found that the two transgenic lines were more sensitive to NaCl treatment in terms of survival rate (Fig. 3g–j).

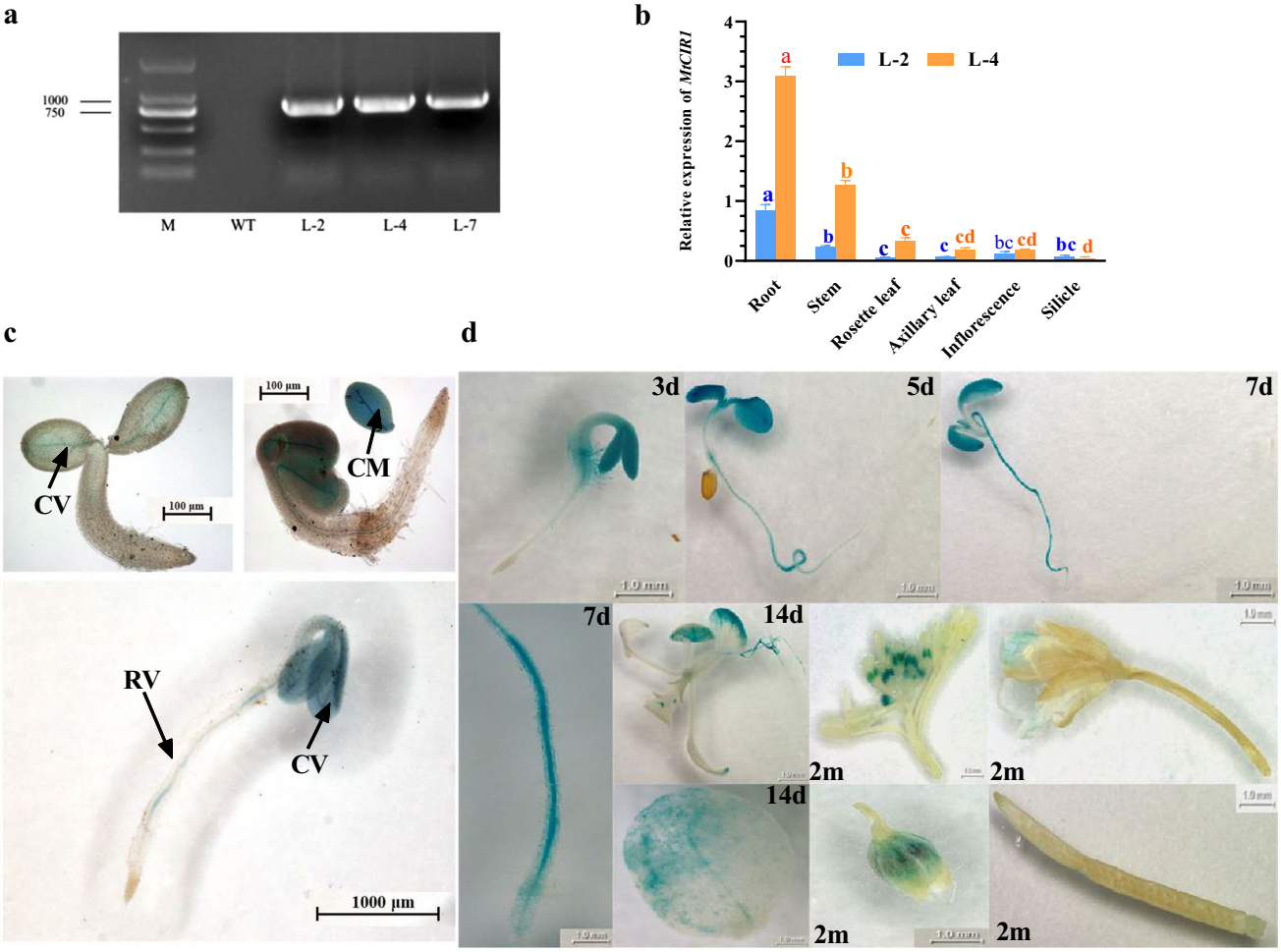


Fig. 1 Expression patterns of *MtCIR1* in *Arabidopsis* transgenic lines. **a** *Arabidopsis MtCIR1* transgenic lines were identified by DNA-PCR using primers designed on both side sequences of *MtCIR1* insertion point in plasmid pMDC32. The marker used for electrophoresis detection is DL2000. **b** *MtCIR1* transcript abundance in different organs of two transgenic lines was determined by qRT-PCR. Values are means ± SD ($n = 4$, biological repeats) and the letter indicates a significant difference at $P < 0.05$ according to the student's t test. **c** *MtCIR1* promoter-GUS activities in cotyledon vascular (CV), cotyledon mesophyll (CM), cotyledon and radicle vascular (CV and RV) of *Arabidopsis* seedlings. Bars = 100 μm and 1000 μm, respectively. **d** *MtCIR1* promoter-GUS activities in organs of seedlings at different developmental stages from 3 days to 2 months. d, day; m, month. Bars = 1 mm

MtCIR1-mediated seed germination under salt stress occurred in an ABA-dependent manner

To test whether ABA is involved in the *MtCIR1*-mediated seed germination to salt stress, the effects of ABA on seed germination rates of the two transgenic lines and WT were examined. No differences in seed germination rates between *MtCIR1* transgenic lines and WT were detected in the absence of ABA during 7 days except the second day, which the germination rate of transgenic line L-2 was significantly lower than that of the WT (Fig. 4a). Seed germination of the WT and *MtCIR1* transgenic lines was delayed significantly upon exposure to ABA, and seed germination in the transgenic lines was more sensitive to ABA than that of WT (Fig. 4b). Furthermore, we determined ABA concentrations in seeds of WT and *MtCIR1* transgenic lines treated with or without NaCl. ABA concentrations in seeds of *MtCIR1* transgenic line L-4 were higher than in seeds of WT and L-2 line after imbibition in the control condition without NaCl treatment. Exposure to NaCl led to significant increases in ABA concentrations in seeds of both WT and transgenic lines, with ABA concentrations in seeds of the transgenic lines higher than that in WT (Fig. 4d). The ABA concentrations were negatively correlated with seed germination rates

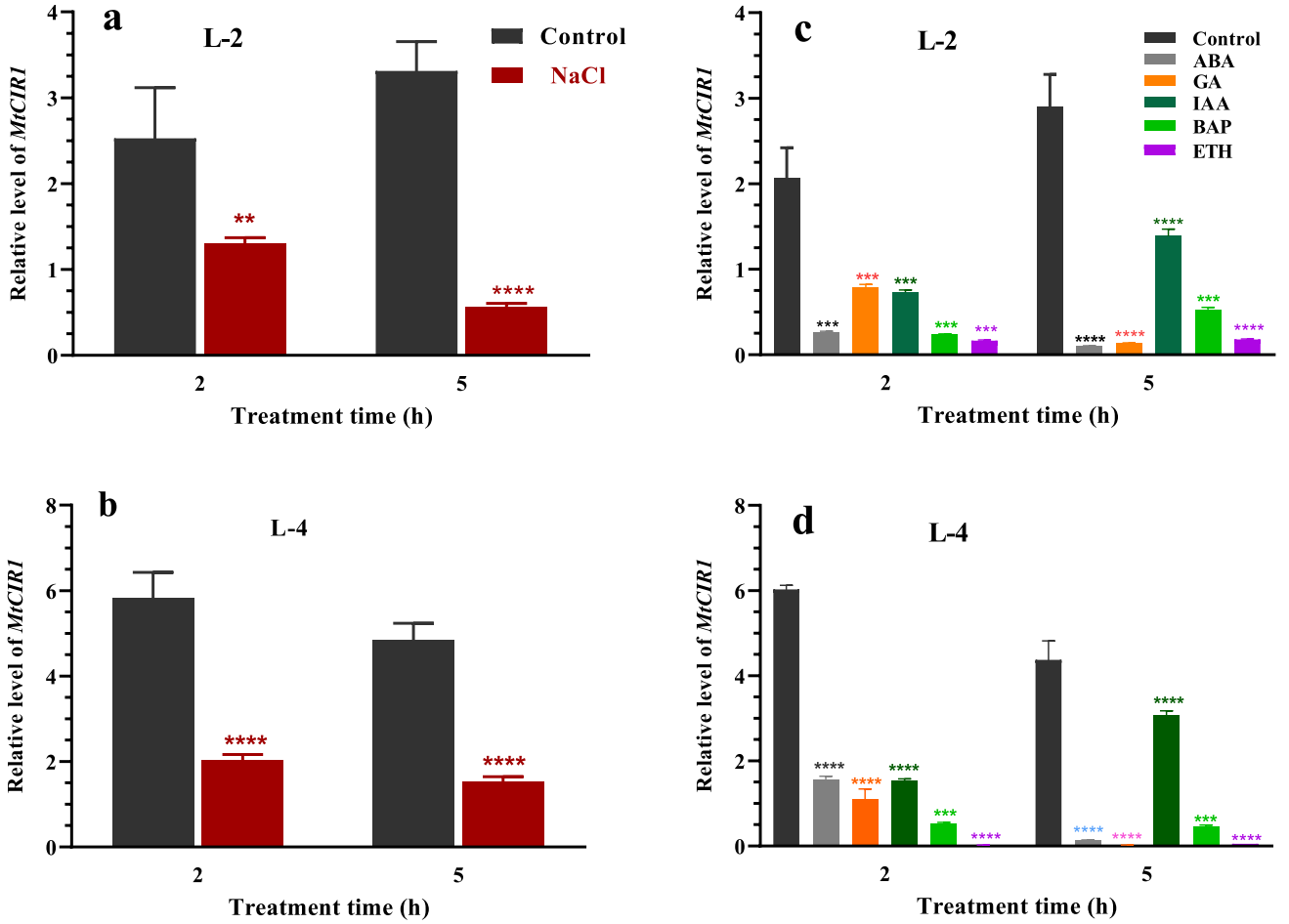
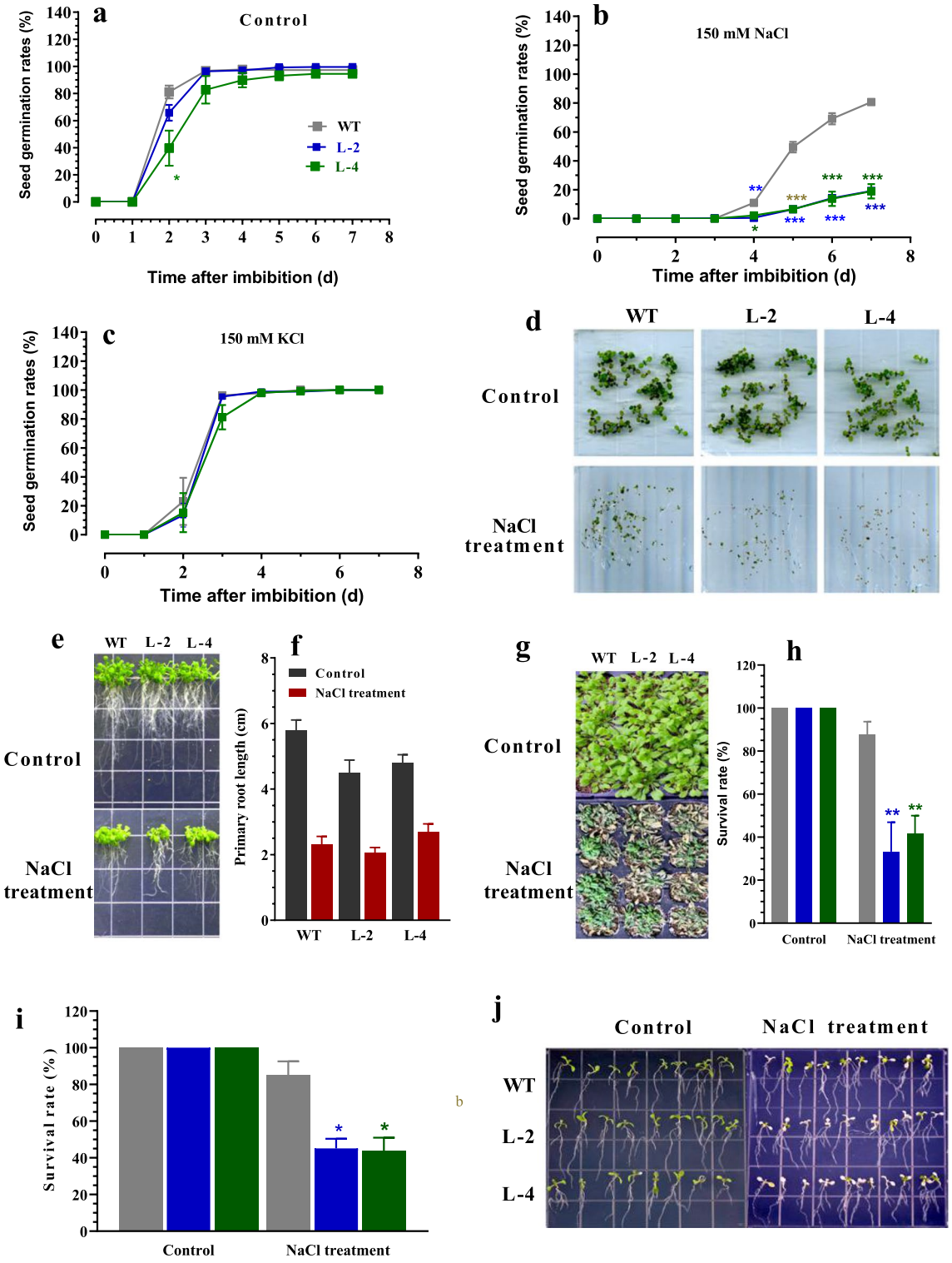


Fig. 2 The expression of *MtCIR1* in *Arabidopsis* transgenic lines treated with NaCl and phytohormones. The transcript levels of *MtCIR1* in 2-week-old seedlings exposed to 100 mM NaCl for 2 h and 5 h of L-2 (**a**) and L-4 (**b**) compared with control conditions. The expression of *MtCIR1* in L-2 (**c**) and L-4 (**d**) leaves of 14 d seedlings, respectively, treated with 1 μM ABA, GA, IAA, BAP and ETH for 2 h and 5 h. Gene expression level was measured by qRT-PCR. The expression of *MtCIR1* was normalized against the *AtACTIN7* gene. Data are means ± SE for four biological replicates and each replicate contained three seedlings. Asterisks indicate a statistically significant differences (**$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ determined by the Student's *t* test) between treated and untreated (Control) samples at the same time point

(Fig. 4c). In addition, we analyzed the expression of several genes involved in ABA catabolism, including *CYP707A1*, *CYP707A2*, *CYP707A3*, and *CYP707A4*. Expression of *AtCYP707A2* was the highest, followed by *AtCYP707A1* and *AtCYP707A3*, and the expression of *AtCYP707A4* was not detected during *Arabidopsis* seed germination (Fig. 4e, Supplementary Fig. S4). Expression of *AtCYP707A2* in *Arabidopsis* WT seeds rapidly increased and peaked after 3 h imbibed on medium without NaCl, exposure to NaCl led to greater up-regulation of *AtCYP707A2*. The NaCl-induced up-regulation of *AtCYP707A2* was almost abolished in the two transgenic lines (Fig. 4e).

To clarify whether the *MtCIR1* may also affect ABA signaling in response to salt stress during seed germination, we compared expression of 14 *AtRCARs* and 5 *AtABIs* genes associated with ABA signal transduction between WT and the transgenic lines in the absence and presence of NaCl in the medium (Figs. 5 and 6, Supplementary Figs. S5, S6, and S7). During seed germination, the expressions of five *AtRCARs*, such as *RCAR1*, *RCAR5*, *RCAR8*, *RCAR9*, and *RCAR10* (Fig. 5a–f), five *ABI*s including *ABI1*, *ABI2*, *ABI3*, *ABI4*, and *ABI5* (Fig. 6a–e) in WT seeds were induced after 6 h imbibition under ABA and salt stress, while the expression of these genes in *MtCIR1* transgenic lines was little changed in response to ABA and salt stress.

The accumulation of PGIPs may lead to impaired germination by the inhibition of pectin degradation (Ferrari et al. 2002; Kanai et al. 2010). The expression of two *PGIPs*



was monitored in response to ABA and salt stress (Fig. 7, Supplementary Fig. S8). Both the expressions of *AtPGIP1* and *AtPGIP2* in WT seeds were decreased by these two treatments during imbibition. By contrast, transcripts of *AtPGIP1* and *AtPGIP2* in seeds of transgenic lines were up-regulated when treated by NaCl and ABA (Fig. 7a, b).

◂**Fig. 3** Expression of *MtCIR1* in *Arabidopsis* rendered the transgenic seedlings sensitive to salt stress. Seed germination rates of WT, *MtCIR1* transgenic lines L-2 and L-4 on 1/2 MS medium supplemented with 0 (**a**), 150 mM NaCl (**b**) and 150 mM KCl (**c**) for 7 days. Data are means ± SE for three biological replicates and each replicate contained 50 seeds at least. **d** Photograph of seeds germinated on 1/2 MS medium with 0 or 150 mM NaCl for 7 days. **e** Photographs of primary root length treated with or without 150 mM NaCl for 10 days. **f** Primary root length of WT, L-2 and L-4 after treated with or without 100 mM NaCl for 10 days. Data are means ± SE for four biological replicates and each replicate contained 5 seedlings at least. **g** Photographs of seedlings treated with or without 100 mM NaCl for 2 months. **h** Survival rate of seedlings treated with 100 mM NaCl for 2 months. Data are means ± SE for five biological replicates and each replicate contained 20 seedlings. **i** Survival rate of 5-day-old seedlings of the WT, L-2 and L-4 treated with 75 mM NaCl for 4 days. Data are means ± SE for three biological replicates and each replicate contained 10 seedlings. Asterisks indicate significant differences between WT and transgenic lines under the same treatment (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). **j** Photograph were taken after NaCl treatment for 4 days

These results suggest that suppression of seed germination by *MtCIR1* may occur by positively modulating the expressions of *AtPGIPs* during salt stress and ABA treatment.

MtCIR1-mediated response to salt stress involves Na^+ and K^+ accumulation by plants

To further unveil the mechanisms by which *MtCIR1* transgenic plants became more sensitive to salt stress than WT, effects of salt stress on Na^+ and K^+ concentrations as well as Na^+/K^+ ratios in leaves of WT and transgenic plants were investigated. There were marked increases in Na^+ concentrations both in WT and transgenic plants upon exposure to NaCl, but the magnitude of increase was greater in transgenic plant than in WT plants, thus leading to a higher Na^+ concentration in transgenic plants than in WT plants (Fig. 8a). In contrast to Na^+, K^+ concentrations in leaves of transgenic lines were lower than WT plants when treated with NaCl (Fig. 8b). The higher Na^+ and lower K^+ concentrations in the transgenic lines led to higher Na^+/K^+ ratio in transgenic lines than in WT plants under salt stress (Fig. 8c).

We further monitored the effects of salt stress on expression patterns of *AtSOS1*, *AtSOS2*, *AtSOS3*, *AtNHX1* and *AtHKT1* that are key components involved in Na^+ accumulation in *Arabidopsis* plants. Expression of *MtCIR1* in *Arabidopsis* led to down-regulation of *AtSOS1* and *AtSOS2* in transgenic plants under salt stress (Fig. 9a, b). However, *AtSOS3* expression in transgenic plants was significantly lower than in WT plants under both control and salt stress (Fig. 9c). In addition, salt stress significantly increased *AtNHX1* expression in both WT and transgenic plants, with the *AtNHX1* expression in transgenic plant being lower than that of WT plants, indicating that *MtCIR1* may suppress Na^+ flux into the vacuole by regulating NHX transporter (Fig. 9d). On the contrary, expression of *AtHKT1* in both WT and transgenic plants was equally suppressed upon exposure to NaCl (Fig. 9e), suggesting that the HKT may not be involved in regulation of *MtCIR1*-depedent Na^+ acquisition.

Discussion

We identified a cold-responsive lncRNA, *MtCIR1,* in *M. truncatula* previously (Zhao et al. 2020). In the present study, we functionally characterized *MtCIR1* by expressing this gene in *Arabidopsis* that does not contain the homologous sequence, and compared the response of the transgenic plants to salt stress with that of WT plants. One important finding is that the *MtCIR1* negatively regulates the response of *Arabidopsis* to salt stress during seed germination and seedling growth (cf. Fig. 3). We further explored the physiological mechanisms underlying the negative regulation of salt stress response by *MtCIR1*. Our results revealed that the effect of *MtCIR1* on seed germination under salt stress was achieved in an ABA-dependent manner (Figs. 4, 5, and 6), while the effect of *MtCIR1* on seedling growth under salt stress was mainly accounted for by excessive accumulation of Na^+ due to down-regulation of SOS systems (Figs. 8 and 9). These findings provide experimental evidence in support of the involvement of lncRNAs in the regulation of response to salt stress.

Despite identification of numerous abiotic stress-related lncRNAs in higher plants, only a small number of lncRNAs have been functionally characterized in response to abiotic stresses (Wang et al. 2015a, b; Chen et al. 2016). For example, the lncRNA *At5NC056820* improves drought tolerance in *Arabidopsis* by enhancing accumulation of free proline (Wu et al. 2017). A nuclear-located *lncRNA973* was found to act as a positive regulator in response to salt stress in cotton (Zhang et al. 2019). Inconsistent with the function of *MtCIR1*, *lncRNA354* and *BoNR8* have been reported to act as negative factors to regulate responses to salt stress. Overexpression of *lncRNA354* inhibited plant growth and reduced tolerance to salt stress in cotton (Zhang et al. 2021). Overexpression of cabbage lncRNA *BoNR8* in *Arabidopsis* made seed germination more sensitive to salt stress (Wu et al. 2019). Our results that the lncRNA *MtCIR1* negatively

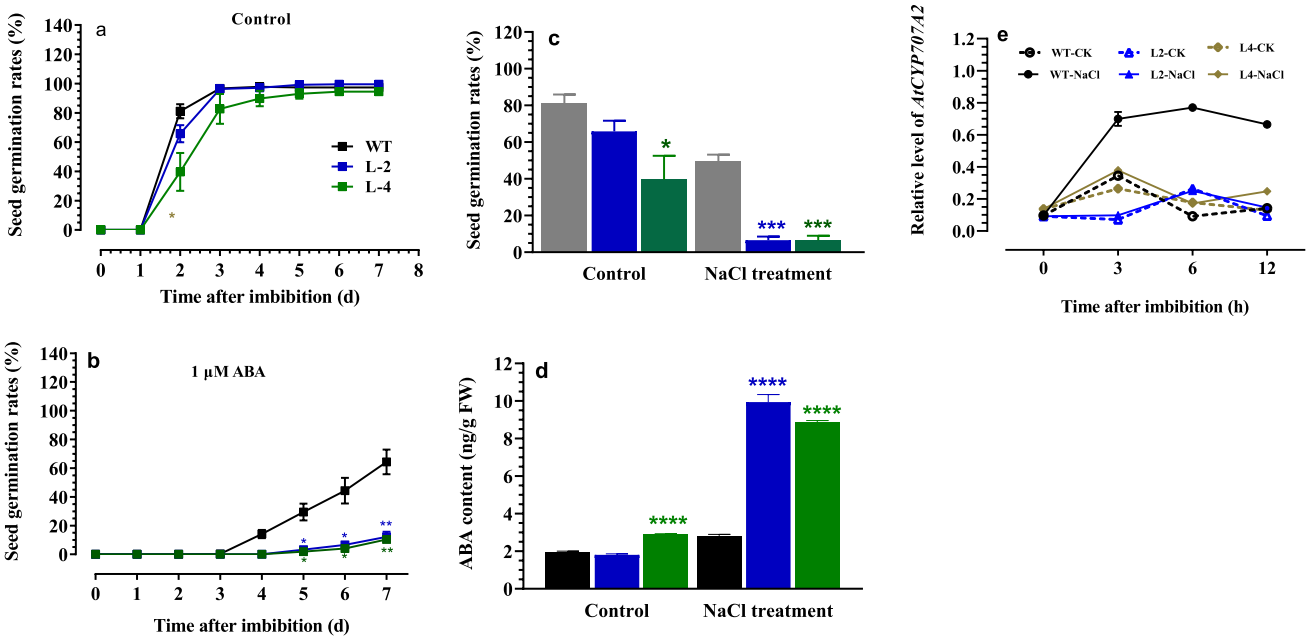


Fig. 4 Expression of *MtCIR1* rendered the transgenic lines sensitive to ABA during seed germination. Seed germination rates of WT and transgenic lines under the condition of 0 (**a**) and 1 μM ABA (**b**) for 7 days. Data are means ± SE for five biological replicates and each replicate contained 100 seeds at least. Asterisks indicate a statistically significant differences between WT and transgenic lines under the same treatment time point (*$P < 0.05$; **$P < 0.01$). **c** Seed germination of WT and *MtCIR1* transgenic lines with 150 mM NaCl treatment for 5 days (NaCl) or without 150 mM NaCl treatment for 2 days (Control), respectively. **d** Changes of ABA content during seed germination of WT and *MtCIR1* transgenic lines with or without 150 mM NaCl treatment. Seeds were collected after imbibed on filter paper soaked with deionized water for 2 days or with 150 mM NaCl for 5 days. Values are means ± SE of three biological replicates. Asterisks indicate a significant difference between WT and transgenic lines under the same treatment according to the Student's *t* test (*$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$). **e** Transcript analysis of ABA catabolism gene *AtCYP707A2* in imbibed seeds. Total RNAs were prepared from seeds of WT, L-2 and L-4 lines imbibed on filter paper soaked with deionized water, 150 mM NaCl for 0, 3, 6 and 12 h. The amount of *AtCYP707A2* transcripts was measured by qRT-PCR. The expression of *AtCYP707A2* was normalized against the *AtACTIN7* gene. Values are means ± SE of three biological replicates

regulated seed germination under salt stress would extend our knowledge on the physiological function of lncRNAs in the regulation of salt response in plants. In addition, we found that ABA was involved in the regulation of seed germination by *MtCIR1* under salt stress.

The plant hormone ABA plays a critical role in regulating responses of plants to abiotic stress (Holbrook et al. 2002; Verslues and Zhu 2007; Luo et al. 2013). The endogenous ABA content was significantly increased upon exposure to drought and salt stress (Yamaguchi-Shinozaki and Shinozaki 2006; Cutler et al. 2010; Kim et al. 2010). ABA has also been reported to regulate stomatal closure, ion accumulation, and ROS scavenging under abiotic stress (Nakashima and Yamaguchi-Shinozaki 2013; Munemasa et al. 2015; Sah et al. 2016). Similar to our results, recent studies also showed the involvement of lncRNAs in phytohormone-mediated responses to the environmental stress. For example, some lncRNAs positively responded to salt stress by targeting ABA signaling cascades in tomato (Li et al. 2022). A lncRNA *DRIR* in *Arabidopsis* was reported to regulate plant tolerance to salt and drought stress in an ABA-dependent manner (Qin et al. 2017).

In the present study, we demonstrated that *MtCIR1* regulated ABA-mediated salt stress response. The expression of *MtCIR1* was suppressed by salt stress and exogenous application of ABA (Fig. 2). Further analysis revealed that expression of *MtCIR1* in *Arabidopsis* led to greater ABA accumulation under salt stress by negatively regulating expression of ABA catabolic gene *CYP707A2* (Fig. 4). These findings provide experimental evidence in support of involvement of ABA in the lncRNA *MtCIR1*-mediated seed germination under salt stress. We further dissected the mechanisms underlying the ABA-dependent seed germination under salt stress. We found that expression of

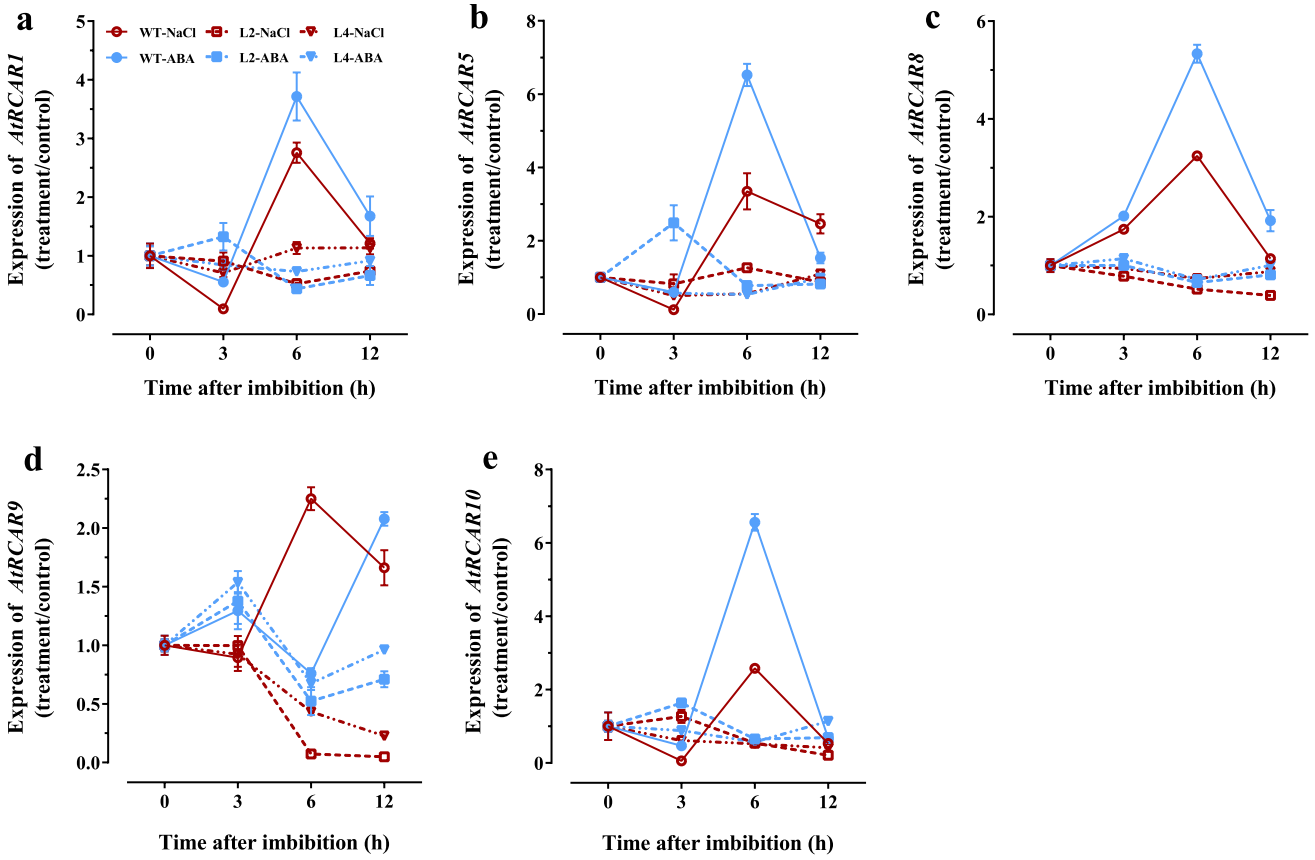


Fig. 5 Transcriptional responses of *AtRCARs* to salt and ABA treatment in *MtCIR1* transgenic lines. Expression of *AtRCAR1* (**a**), *AtRCAR5* (**b**), *AtRCAR8* (**c**), *AtRCAR9* (**d**) and *AtRCAR10* (**e**) in imbibed seeds of *Arabidopsis* WT and *MtCIR1* transgenic lines. Total RNAs were prepared from seeds of WT, L-2 and L-4 lines imbibed on filter paper soaked with deionized water, 150 mM NaCl and 1 μM ABA for 0, 3, 6, and 12 h. The gene transcripts were measured by qRT-PCR. The expression of gene was normalized against the *AtACTIN7* gene. Values are means ± SE of three biological replicates

genes associated with ABA receptors and signal transduction was suppressed in response to salt stress and ABA treatment in *MtCIR1* transgenic *Arabidopsis* (Figs. 5 and 6), suggesting that *MtCIR1* may inhibit seed germination and post-germination plant growth by negatively regulating ABA signaling. These results are supported by a large number of studies that the ABA receptors PYR/PYL/RCAR and many ABA signal transduction genes affect seed germination and early seedling growth in *Arabidopsis* (Park et al. 2009; Huang et al. 2016; Miao et al. 2018). In addition to ABA signal transduction-related genes, the expression of polygalacturonase inhibitory protein-encoding genes *PGIPs* was investigated in this study. PGIPs are polygalacturonase inhibitor involved in the cell wall pectin-depolymerizing (Kalunke et al. 2015; Rathinam et al. 2020). Kanai et al. (2010) reported that PED3, a peroxisomal ABC transporter, promoted seed germination by suppressing PGIPs via an *ABI5*-dependent manner. Our results showed that *MtCIR1* up-regulated *AtPGIPs* expression under salt stress, implying that pectin degradation may also be involved in the suppression of seed germination by *MtCIR1* under salt stress (Fig. 7 and Supplementary Fig. S8).

ABA has been reported to enhance Na^+ exclusion from the cytosol and sequestration into the vacuoles via modifying the plasma membrane ATPase and vacuolar ATPase, thereby alleviating Na^+ toxicity to plants (Mansour et al. 2003). Na^+ sequestration into the vacuoles by the vacuolar Na^+/H^+ antiporter NHX1 is an effective cellular mechanism to reduce Na^+ toxicity of under salt stress (Yokoi et al. 2002). In this study, we found that salt stress significantly increased *AtNHX1* expression in both WT and transgenic

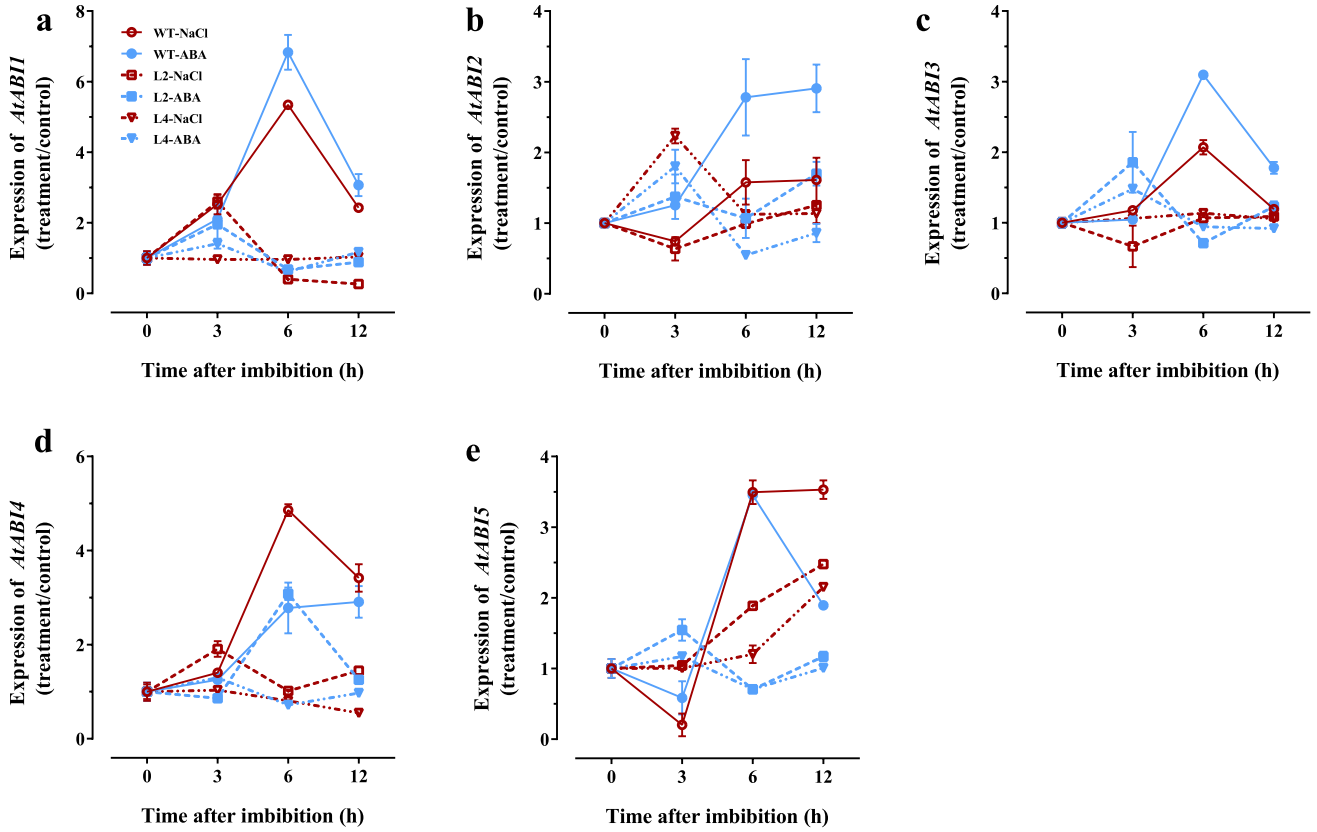


Fig. 6 Transcriptional responses of *AtABIs* to salt and ABA treatment in *MtCIR1* transgenic lines. Expression of *AtABI1* (**a**), *AtABI2* (**b**), *AtABI3* (**c**), *AtABI4* (**d**), and *AtABI5* (**e**) in imbibed seeds of *Arabidopsis* WT and *MtCIR1* transgenic lines. Total RNA was prepared from seeds of WT, L-2 and L-4 lines imbibed on filter paper soaked with deionized water, 150 mM NaCl and 1 μM ABA for 0, 3, 6 and 12 h. The gene transcripts were measured by qRT-PCR. The expression of gene was normalized against the *AtACTIN7* gene. Values are means ± SE of three biological replicates

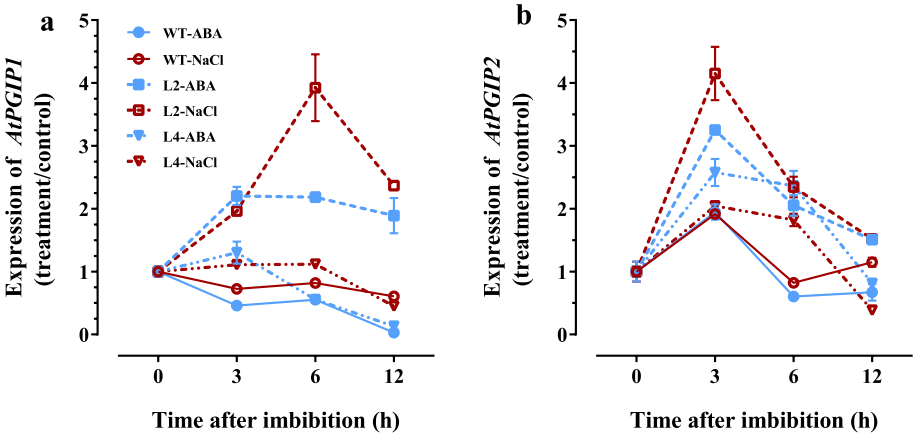


Fig. 7 Transcriptional responses of *AtPGIPs* to salt and ABA treatment in *MtCIR1* transgenic lines. Expression of *AtPGIP1* (**a**) and *AtPGIP2* (**b**) in imbibed seeds of *Arabidopsis* WT and *MtCIR1* transgenic lines. Total RNAs were prepared from seeds of WT, L-2 and L-4 lines imbibed on filter paper soaked with 150 mM NaCl and 1 μM ABA for 0, 3, 6, and 12 h. The gene transcripts were measured by qRT-PCR. The expression of gene was normalized against the *AtACTIN7* gene. Values are means ± SE of three biological replicates

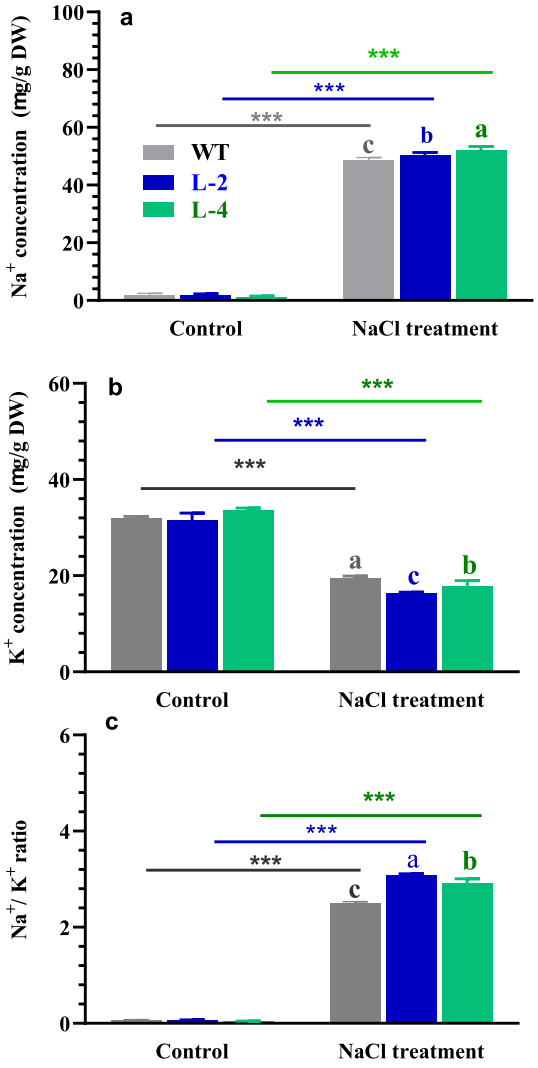


Fig. 8 *MtCIR1* regulated Na^+ accumulation in *Arabidopsis* leaves under salt stress. Concentrations of Na^+ (**a**), K^+ (**b**) and Na^+/K^+ ratio (**c**) in leaves of *Arabidopsis* WT, L-2 and L-4 treated with or without 300 mM NaCl for 7 days. Experiments are conducted in nine biological replicates. Data are means ± SE. Different letters indicate significant differences ($P < 0.05$) among different genotypes under the same treatment. Asterisks represent significant differences between NaCl treatment and control of the same genotypes (***$P < 0.001$) according to the Student's *t* test

plants with the *AtNHX1* expression in transgenic plant being lower than that of WT plants, indicating that *MtCIR1* may suppress Na^+ flux into the vacuole by regulating NHX transporter (Fig. 9d). Expression of *MtCIR1* did not alter the sensitivity of root elongation, chlorosis and survival rate to ABA (Supplementary Fig. S9 and Supplementary Fig. S10). The regulation of *MtCIR1* on salt sensitivity at seedling stage appears to be independent of the ABA pathway. The *MOCA1* gene encodes an inositol phosphorylceramide glucuronosyltransferase (IPUT1), an enzyme catalyzing the biosynthesis of the sphingolipid glycosyl inositol phosphorylceramide (GIPC) (Jiang et al. 2019). Like the *MtCIR1* transgenic plants, mutation of *MOCA1* led to increased sensitivity of *moca1* mutant seedlings to treatment with NaCl, but the mutation did not affect the response of *moca1* mutant seedlings to ABA, indicating that the *MOCA1* functions specifically in mediating Na^+ signaling. The similar response to salt stress and ABA treatment between *MtCIR1* transgenic plants and *moca1* mutant suggests that *MtCIR1* and *MOCA1* may have some correlations in response to salt stress, which warrants further investigation.

In summary, we functionally characterized an lncRNA *MtCIR1* from legume *M. truncatula* by expressing it in *Arabidopsis*. We demonstrated that expression of *MtCIR1* rendered the transgenic lines more sensitive to NaCl treatments during seed germination and seedlings development. We further discovered that the *MtCIR1*-mediated seed germination under salt stress was dependent on ABA metabolism and signaling, while expression of *MtCIR1* resulted in greater foliar accumulation of Na^+ via SOS-dependent manners, thus making the transgenic lines more sensitive to salt stress. These novel findings could shed new light on the regulatory roles of lncRNAs in response to salt stress, they may also contribute to development of crops tolerant to salt stress by manipulating the expression of lncRNAs.

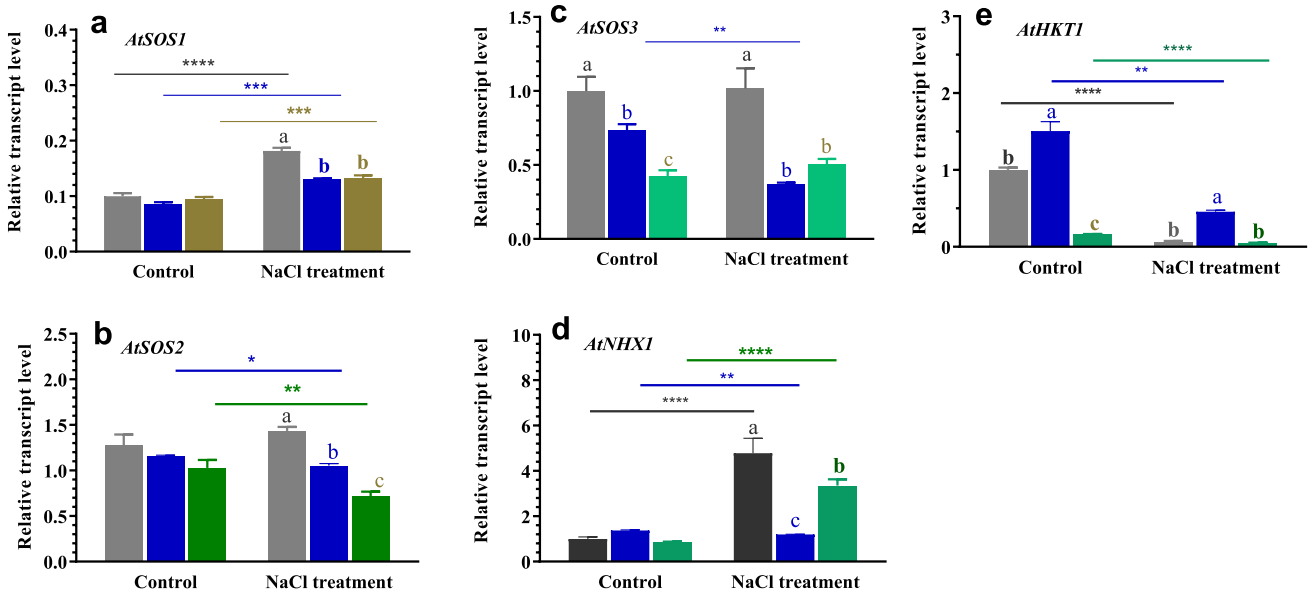


Fig. 9 Expression levels of Na^+ transporter genes were regulated by *MtCIR1* under salt stress. The expression levels of *AtSOS1* (**a**), *AtSOS2* (**b**), *AtSOS3* (**c**), *AtNHX1* (**d**) and *AtHKT1* (**e**) in *Arabidopsis* WT, L-2 and L-4 leaves treated with 300 mM NaCl for 7 days. Experiments are conducted in nine biological replicates. Data are means ± SE. Different letters indicate significant differences ($P < 0.05$) among different genotypes under the same treatment. Asterisks represent significant differences between NaCl treatment and control of the same genotypes (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$) according to the Student's *t* test

Author contribution statement The authors have made the following declarations about their contributions: MZ and WZ designed the experiments. MZ, RT and XS, CL and JC performed the experiments and analyzed data. MZ, RT and WZ wrote the manuscript.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00425-022-04064-1.

Acknowledgements We thank the technical support from the Test Centre of State Key Laboratory of Vegetation and Environmental Change.

Funding Science and Technology Program of Inner Mongolia, China (2021ZD004502; 2021GG0372), the National Natural Science Foundation of China (31671270).

Data availability All data generated or analyzed Science and Technology Department of Inner Mongolia Autonomous Region (grant number 2021ZD004502; 2021GG0372) during this study are included in this published article and its supplementary information files.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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