



MsDIUP1 encoding a putative novel LEA protein positively modulates salt tolerance in alfalfa (*Medicago sativa* L.)

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Abstract

Background Salt stress seriously impacts the plantation and productivity of global alfalfa (*Medicago sativa*). Previously, we genetically defined the *M. sativa* DROUGHT-INDUCED UNKNOWN PROTEIN 1 (*MsDIUP1*) as a drought tolerance gene of alfalfa. However, whether it is associated with salt tolerance needs to be explored.

Results In this study, we re-classified *MsDIUP1* as a putative novel late embryogenesis abundant protein, and found its promoter activity was strongly induced by salt and ABA treatment. Heterologous

overexpression of *MsDIUP1* in *Arabidopsis* led to enhanced salt tolerance and ABA insensitivity, with a higher fresh weight, lateral root number, primary root length, and survival rate compared to the wild-type (WT) plants. Similarly, homologous overexpression of *MsDIUP1* also enhanced the salt tolerance of alfalfa over-expression (OE) plants, accompanied by reduced oxidative damage, higher K^+/Na^+ ratio, increased osmoprotectant accumulation, and improved photosynthetic capacity relative to the WT plants. In reverse, disruption of *MsDIUP1* expression caused a salt-hypersensitive phenotype of alfalfa RNA interference (RNAi) plants, with higher oxidative damage, less osmoprotectant accumulation, and lower photosynthetic pigment content than that of the WT. These physiological discoveries were in accordance with differential transcript profiles of the

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relevant genes associated with stress signaling, antioxidant defense, and osmotic adjustment in the salt-treated alfalfa WT, OE, and RNAi lines. Furthermore, we found that alfalfa OE plants showed no apparent alteration in their forage quality compared with the WT plants.

Conclusions Overall, these results reveal that *MsDIUPI* improves plant salt tolerance via regulating stress signaling, antioxidant defense, ion homeostasis, osmotic adjustment, and photosynthesis.

Keywords Alfalfa · *MsDIUPI* · Salt stress · Osmoprotectant · Photosynthesis

Introduction

Plants are often affected by various adverse environmental stimuli during their life cycles, with salt stress representing one of the leading impediments that limits plant growth and productivity (Morton et al. 2019). Salt impacts plant growth by mainly imposing two types of stresses on plants: ion toxicity arising from the disruption of the ionic equilibrium of the cell, including excess toxic Na^+ in the cytoplasm and a deficiency of essential mineral nutrients such as K^+ , and physiological drought, resulting from low water availability due to the high osmotic pressure in the soil (Zhu 2002). Upon perceiving the two stresses, plants will develop intricate defense mechanisms at the physiological level, by reinstating the cellular ionic equilibrium as well as by activating antioxidant enzymes and accumulating osmoprotectants (Arif et al. 2020). The physiological responses of plants for adapting to salt stress are triggered upon the activation of several core stress signaling pathways (Luo et al. 2019a). For ionic stress signaling, molecular and genetic analyses have demonstrated that plants use the salt overly sensitive (SOS) pathway, with SOS3-SOS2-SOS1 as the core module, in regulating Na^+ transport and hence salt tolerance (Zhu 2016). For osmotic stress signaling, it has been documented that the existence of abscisic acid (ABA)-independent and ABA-dependent signal transduction cascades occur between an initial stimulating osmotic signal and the expression of target stress-responsive genes in plants (Chen et al. 2018). The downstream stress-responsive genes encode antioxidative enzymes, osmoprotectants, and protective proteins, which

ultimately contribute to tolerating or combating salt-elicited osmotic damage.

The protective proteins of particular interest in this context are late embryogenesis abundant (LEA) proteins, which are extremely hydrophilic polypeptides (Park et al. 2011). LEA proteins were originally discovered to accumulate abundantly during seed maturation, and then were also found in vegetative tissues in plants exposed to ABA and abiotic stress conditions such as cold, drought, and salt (Amara et al. 2014). LEA proteins have been widely identified in many plant species, including 34 OsLEAs in rice (*Oryza sativa*) (Wang et al. 2007), 51 AtLEAs in *Arabidopsis* (Hundertmark and Hinch 2008), and 108 BnLEAs in *Brassica napus* (Liang et al. 2016). They have been categorized into at least eight distinct groups, based on their conserved protein domains in the Pfam database: LEA1–LEA6, dehydrin (DHN), and seed maturation protein (SMP) (Muvunyi et al. 2018). To date, the role of LEA proteins in salt stress tolerance has been well studied through functional analyses. A previous study reported that the ectopic expression of a durum wheat (*Triticum durum*) group 2 LEA gene, known also as *DHN-5*, in *Arabidopsis* plants improved tolerance to salt stress through osmotic adjustment (Brini et al. 2007). Transgenic *Salvia miltiorrhiza* plants overexpressing their endogenous gene *SmLEA2* exhibited a significantly increased salt tolerance, possibly due to the protection of cell membrane and the maintenance of cellular ion homeostasis (Wang et al. 2017). It has also been shown that the overexpression of *OsLEA5* in rice leads to tolerance to salt stress by modulating reactive oxygen species (ROS) homeostasis (Huang et al. 2018). Thus, LEA proteins have a wide effect on plant salt responses by functioning as protectants of cell membranes, scavenging ROS, sequestering ions, and maintaining osmotic homeostasis.

Alfalfa (*Medicago sativa* L.), commonly referred to as the “Queen of forage”, is the most widely grown perennial forage legume crop in the world (Luo et al. 2014; Luo et al. 2019b). It has also been of critical importance in ecological management and the bio-fuel industry for hundreds of years (Fernandez et al. 2019). Nowadays, about 20% of the world’s arable land and 33% of irrigated land is salinized, which seriously impacts the global plantation and production of alfalfa (Ashrafi et al. 2014; Devkota et al. 2022). Hence, breeding alfalfa varieties tolerant to salt

represents the goal and task of modern breeders. With advanced genetic engineering approaches, numerous important core genetic determinants for salt tolerance have been genetically defined in alfalfa, including the genes encoding protein kinase *AtNDPK2* (Wang et al. 2014a), cation/H⁺ antiporter *ZxNHX* (Bao et al. 2016), transcription factor *GsWRKY20* (Tang et al. 2014), antioxidative enzyme *OsAPX2* (Zhang et al. 2014), and protective protein *CsLEA* (Zhang et al. 2016). These genes have great potential for optimizing the agronomic trait of salt tolerance in future alfalfa molecular breeding programs. However, genetic engineering studies of the alfalfa endogenous genes in alfalfa itself are relatively scarce, and the function and mechanism of action of these genes remain largely unclear.

Our previous study identified a *M. sativa* DROUGHT-INDUCED UNKNOWN PROTEIN 1 (*MsDIUPI*), and confirmed that it improved the drought tolerance of transgenic *Arabidopsis* and alfalfa (Luo et al. 2022a). Since *MsDIUPI* could also be strongly induced by salt and ABA exposure at a transcriptional level, it remains to be explored whether it is associated with salt tolerance and ABA sensitivity. In this study, *MsDIUPI* was reclassified as a putative novel LEA protein and studied its characteristics of salt tolerance and ABA sensitivity in *Arabidopsis* plants overexpressing *MsDIUPI*. Then, transgenic alfalfa plants overexpressing or RNA-interfering *MsDIUPI* were comprehensively investigated by monitoring the physiological shift and transcriptional regulation under salt stress. The goal of our study was to generate high-quality alfalfa transformation events that were tolerant to salt stress.

Materials and methods

Sequence analysis of *MsDIUPI*

The number and proportion of the amino acid residues of *MsDIUPI* were calculated using the IPC database (<http://isoelectric.org/calculate.php>). Hydropathy analysis was performed via the Kyte–Doolittle algorithm by using the ProtScale database (<https://web.expasy.org/protscale/>). The intrinsically disordered protein region was predicted by using the IUPred2A database (<https://iupred2a.elte.hu/>). The tertiary protein structure was predicted by using the I-TASSER

database (<https://seq2fun.dcmdb.med.umich.edu/I-TASSER/>). The phylogenetic tree was constructed via the neighbor-joining method, using the MEGA 6.06 software, and gaps were treated by pairwise deletion.

Transactivation assay of *MsDIUPI* in yeast

The coding region of *MsDIUPI* was introduced into the pLexA expression vector using restriction enzymes *EcoRI* and *XhoI* and transformed into the yeast (*Saccharomyces cerevisiae*) strain EGY48. The empty pLexA vector was introduced into yeast as the negative control, and yeast harboring *pLexA-MsMYB144* (*MS.gene005488*) that is a transcription factor was used as the positive control. Three types of yeast colonies in SD/-Ura/-His medium were incubated at 30 °C for 3 days. Subsequently, the positive clones were moved to the SD/-Ura/-His medium supplemented with X-gal and incubated at 30 °C for 3 days. β-galactosidase activity of the fused proteins was determined based on the growth status (blue/white) of the transformants (Wang et al. 2021; Li et al. 2022).

Plant materials and growth conditions

All the transgenic plants used in this study were created in a previous drought tolerance assay (Luo et al. 2022a), including one *Arabidopsis MsDIUPI* promoter-driven β-glucuronidase (GUS) line; three *Arabidopsis MsDIUPI* over-expression (OE) lines: OE11, OE13, and OE19; three alfalfa OE lines: OE6, OE31, and OE40; and three alfalfa *MsDIUPI* RNA interference (RNAi) lines: RNAi1, RNAi7, and RNAi33. The *Arabidopsis* plants were grown in a growth chamber at 22 °C under a 16 h/8 h light/dark photoperiod, whereas the alfalfa plants were grown in a greenhouse at 26±2 °C under a 16-h photoperiod. In each independent experiment, selected transgenic plants were propagated synchronously with at least three biological replicates.

Promoter activity analysis of *MsDIUPI* under ABA and salt treatment conditions

Seeds of *Arabidopsis* WT and *MsDIUPI* promoter-driven GUS line were germinated and grown on Murashige and Skoog (MS) medium at 22 °C for one week. Then, the seedlings were moved to MS

medium containing different concentrations of NaCl (150 and 200 mM) or ABA (60 and 80 μ M) for 0, 6, 12, and 24 h. The seedlings were stained with X-Gluc solution (Cat. No. G3061, Solarbio, Shanghai, China) at 37 °C for 24 h, and dipped in 75% ethanol for chlorophyll removal under room temperature conditions. Images of the seedlings were taken with a stereo microscope (SteREO Discovery V20, Carl Zeiss, Jena, Germany).

Evaluation of ABA sensitivity and salt tolerance of *Arabidopsis* overexpressing *MsDIUPI*

To estimate the effects of ABA and salt stress on root growth, seeds of *Arabidopsis* WT and OE lines were germinated and grown on MS medium. After one week, seedlings were moved to MS medium containing 150 mM NaCl, 30 μ M ABA, 150 mM NaCl +30 μ M ABA, 200 mM NaCl, or 200 mM NaCl +60 μ M ABA. All the seedlings were grown on MS medium with a vertical orientation. On the 12th day after seedling transfer, the fresh weight, lateral root number, and primary root length were measured. Three independent experiments were performed.

The salt tolerance of the *Arabidopsis* plants was further tested in soil. Seedlings of *Arabidopsis* WT and OE lines were germinated and grown on MS medium for one week, and then were planted into artificial soil comprising a 3:1 (v/v) mixture of moss peat (Pindstrup Mosebrug A/S, Pindstrup, Denmark) and vermiculite, which has high water- and nutrient-holding capacity and aeration (Gao et al. 2006). These plants were grown under well-watered conditions until the imposition of stress treatment. For the comparative salt stress experiment, two-week-old *Arabidopsis* plants were divided into three sets: 1) WT and OE11 lines; 2) WT and OE13 lines; and 3) WT and OE19 line. Each set had two lines with four plants for each line. These plants were subjected to salt treatment by irrigating with 200 mM NaCl for 20 and 30 days. For the salt stress assay on the survivability of adult plants, the three-week-old *Arabidopsis* plants were exposed to salt treatment by irrigating with 200 mM NaCl solution for 12 and 22 days. The experiments were repeated three times, and the survival rate was recorded.

Evaluation of salt tolerance of alfalfa on medium and in the greenhouse

The transgenic alfalfa callus overexpressing *MsDIUPI* was created by transforming a previously described *pEarleyGate100-Pro35S:MsDIUPI* construct into the alfalfa genotype SY-4D following the established protocol (Fu et al. 2015; Luo et al. 2022a). The one-month-old alfalfa callus was transferred to selection medium 4 (SM4 medium) containing 0, 100, 150, or 200 mM NaCl. Fresh weight was measured on the 14th day after callus transfer.

The alfalfa WT, OE, and RNAi lines were vegetatively propagated by means of stem cuttings (Bao et al. 2009). Two-week-old alfalfa seedlings were planted in vermiculite and grown in a greenhouse, watered 1/2 MS solution every two days for six weeks. For the salt stress experiment, the two-month-old alfalfa plants were irrigated with 1/2 MS+200 mM NaCl solution every two days for 17 days. Three independent experiments were performed, and the survival rate was recorded.

Determination of physiological indicators

The leaves of alfalfa WT, OE, and RNAi lines were collected before salt stress and after salt stress for 15 days: each line involved three biological replicates. Leaf samples were used to assess six physiological indicators. The H₂O₂, malondialdehyde (MDA), soluble sugar, and proline contents were ascertained using four Comin Biochemical Test Kits (Cat. Nos. H₂O₂-2-Y, MDA-2-Y, KT-2-Y, and PRO-2-Y, respectively; Cominbio, Suzhou, Jiangsu, China). The concentrations of K⁺ and Na⁺ were measured using a flame spectrophotometer (2655-00, ColeParmer Instrument Co., Vernon Hills, IL, USA), according to the method described previously (Zhang et al. 2016).

To determine the photosynthetic parameters, leaf samples were used to measure the chlorophyll content using a Comin Biochemical Test Kit (Cat. No. CPL-2-G, Cominbio) and the net photosynthetic rate using a portable photosynthesis system (LI-6800, LI-COR Inc., Lincoln, NE, USA). The measurements were conducted before stress and after stress for 15 days from 9:00 to 11:00 am.

RNA-Seq assay

Two-month-old alfalfa plants in the vermiculite were irrigated with 1/2 MS + 200 mM NaCl solution for 24 h. Leaves were collected from three WT plants (three biological replicates), three OE lines (OE6, OE31, and OE40, each transgenic line serving as a biological replicate), and three RNAi lines (RNAi1, RNAi7, and RNAi33, each transgenic line serving as a biological replicate) before salt and after salt stress, resulting in a total of 18 samples. Thereafter, the 18 alfalfa samples were separately sequenced on an Illumina HiSeq 2000 platform (Biomarker Technologies, Beijing, China).

For the data analysis, all sequenced reads were mapped to the alfalfa (Xinjiang Daye cultivar) reference genome sequence (<http://47.92.172.28:12088/alfalfa/download/downloadPage>), using Hisat2 software with the default parameters. The differentially expressed genes (DEGs) between the non-stress control and salt stress groups were identified using the DESeq2 package, with a P value ≤ 0.01 and a fold-change cutoff of ≥ 1.5 . The function enrichment analysis of DEGs annotated by the GO database was performed using OmicShare (Q -value < 0.05 ; <https://www.omicshare.com/tools/Home/Soft/gogseasenior>). Real-time quantitative polymerase chain reaction (RT-qPCR) analysis was carried out as previously described (Luo et al. 2022a), and the primers for RT-qPCR are listed in Table S1. The regulatory networks were predicted using GeneMANIA (<http://genemania.org/>) and visualized using the Cytoscape 3.9.0 software (<https://cytoscape.org/>).

Forage quality assay

Shoot tissue from the two-month-old alfalfa WT, OE, and RNAi lines was harvested. All shoot samples were pulverized to powder and passed through a sieve with 0.5-mm holes. Then, the crude protein, crude ash, dry matter, calcium ion, 30-h digestible neutral detergent fiber (DNDF30), and relative feed value (RFV) of all shoot samples were determined by a near infrared spectrometer (SpectraStar 1400XTR, Unity Scientific, Milford, MA, USA), based on the constructed prediction model of alfalfa (Arnold et al. 2019; Lin et al. 2020).

Results

MsDIUP1 is a putative novel late embryogenesis abundant protein

In this study, amino acid sequence analysis revealed that the MsDIUP1 putative protein is rich in polar amino acids (66.67%), including Ser (15.07%), Lys (11.11%), Glu (7.94%), and Thr (7.14%) residues (Fig. 1a). Hydropathy plots predicted that MsDIUP1 is predominantly hydrophilic, with few hydrophobic amino acid residues (Fig. 1b). The disorder prediction showed that MsDIUP1 has a large number of disordered residues and has no recognized protein binding region (Fig. 1c), indicating that MsDIUP1 is an intrinsically disordered protein. Supporting this hypothesis, protein structure analysis further revealed that MsDIUP1 does not exhibit well-defined tertiary structures throughout the entire sequence (Fig. S1). Moreover, the transactivation assay exhibited that pLexA-MsDIUP1 does not have any β -galactosidase activity (Fig. 1d), indicating that MsDIUP1 is a functional protein without transactivation activity. These features are in agreement with the characteristics of most LEA proteins, suggesting that MsDIUP1 might be an LEA protein. However, Pfam analysis revealed that MsDIUP1 lacks the eight LEA-conserved domains including LEA1–LEA6, DHN, and SMP (E -value < 0.05); additionally, phylogenetic analysis showed that MsDIUP1 has low posterior probability values for all of the proteins from the eight known LEA groups in alfalfa (Fig. S2). Thus, we speculated that MsDIUP1 is a novel LEA protein.

MsDIUP1 promoter response to salt or ABA treatment

The expression patterns of *MsDIUP1* in response to salt or ABA treatment were investigated using *Arabidopsis* seedlings expressing *ProMsDIUP1:GUS*. It was shown that the GUS activity of the *MsDIUP1* promoter was strongly enhanced in both shoots and roots during the 24-h NaCl or ABA treatment (Fig. 2). However, the time course of induction differed between the two treatments. NaCl induced GUS activity of the *MsDIUP1* promoter more quickly in leaves and relatively more slowly in roots compared to ABA during the 24-h treatment (Fig. 2). These results suggest that there are common and distinct

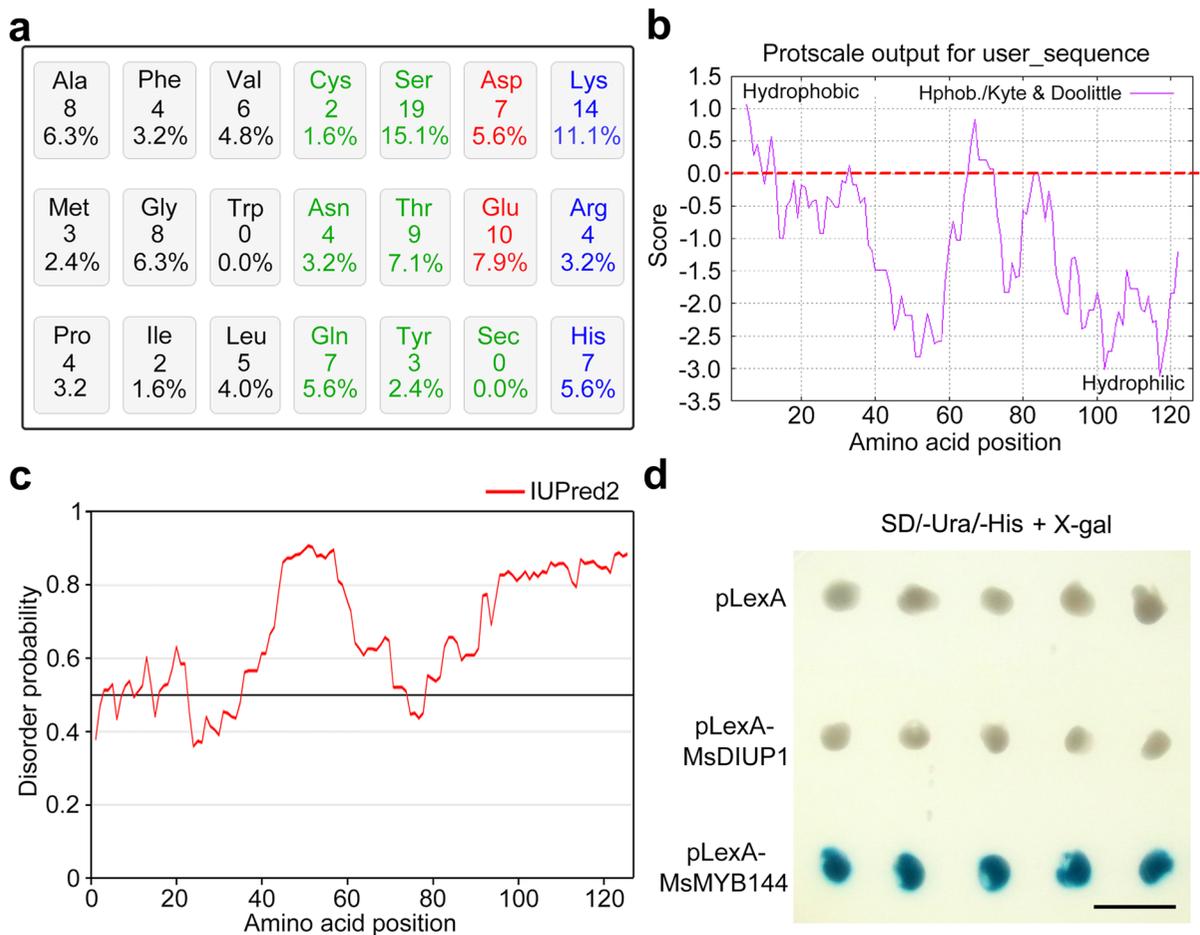


Fig. 1 Sequence analysis and auto-transactivation assay of MsDIUP1. **(a)** Statistics of number and proportion of the amino acid residues of MsDIUP1. Black, green, red, and blue colors indicate non-polar amino acids, neutral polar amino acids, acidic amino acids, and alkaline amino acids, respectively. **(b)** Hydropathy analysis of the amino acid sequence of MsDIUP1. The values below the zero line indicate predicted hydrophilicity. **(c)** Prediction of intrinsically disordered protein

regions of MsDIUP1. The values above the 0.5 threshold indicate predictions of disorder. **(d)** Auto-transactivation assay of MsDIUP1 in yeast. The pLexA empty vector is set as the negative control and the pLexA-MsMYB144 is set as the positive control. Blue color colonies indicate that transformants have β -galactosidase activity, whereas white color colonies indicate that transformants has no β -galactosidase activity. Scale bar: 1 cm in (d)

molecular mechanisms of *MsDIUP1* underlying the response to NaCl and ABA.

Overexpression of *MsDIUP1* in *Arabidopsis* improved salt tolerance and ABA insensitivity

Three typical *Arabidopsis* OE lines (OE11, OE13, and OE19) were used for salt tolerance and ABA sensitivity evaluation at the seedling stage (Figs. 3a, S3). There were no significant differences in the fresh weight, lateral root number, or primary root

length when cultured on MS medium (Figs. 3b, c, S4). However, the three indicators of the *Arabidopsis* OE lines at 150 mM or 200 mM NaCl, as well as at 30 μ M ABA, were higher than those of the WT plants (Figs. 3b, c, S4). When the ABA was put into the MS medium containing 150 mM or 200 mM NaCl, all the three indicators decreased compared to those under salt stress treatment alone (Figs. 3b, c, S4). However, in the *Arabidopsis* OE plants compared with the WT plants, the application of ABA expanded the difference in the fresh weight, enhancing it by 7.78%

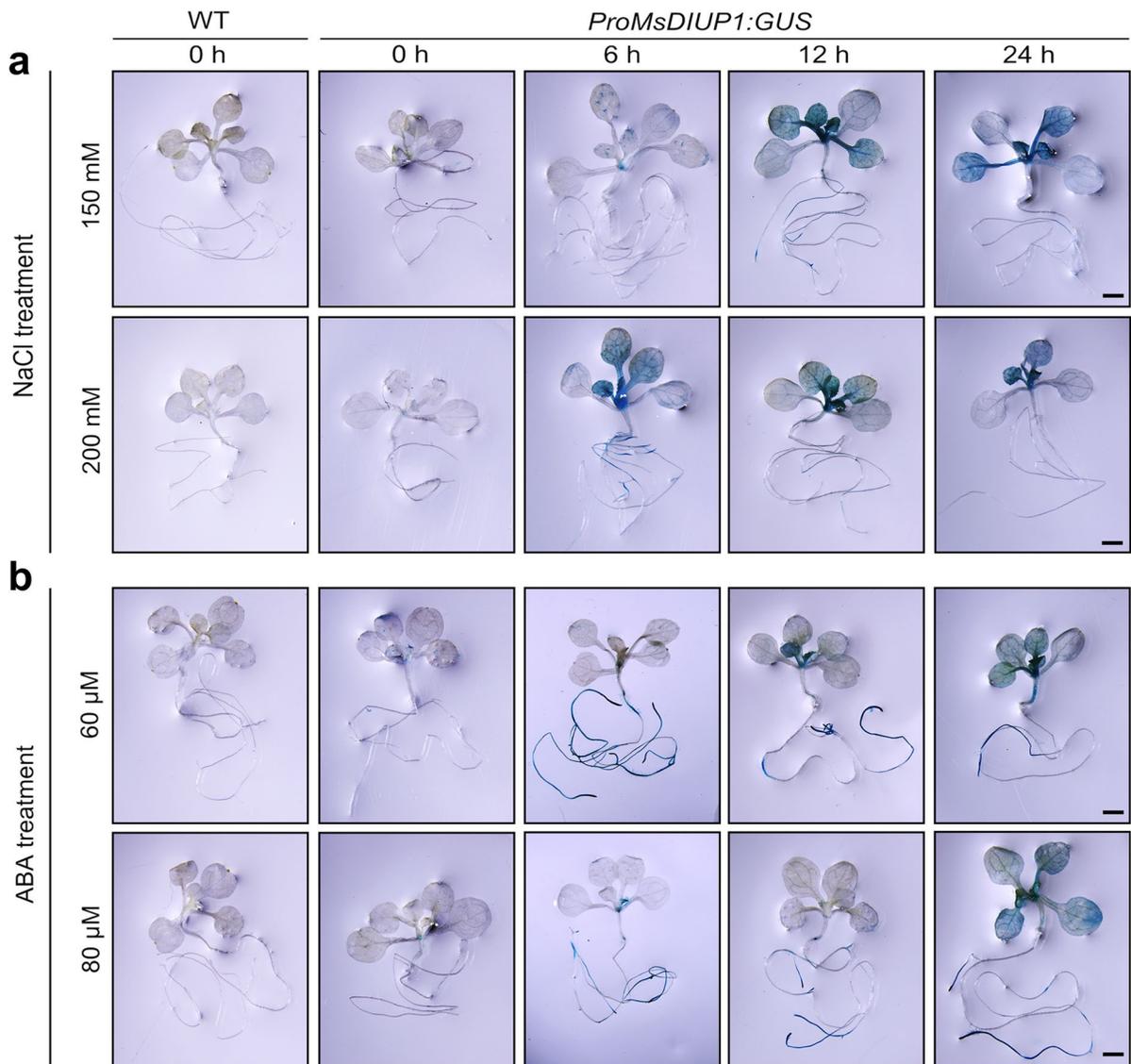


Fig. 2 Promoter analysis of *MsDIUP1* gene in *Arabidopsis*. (a, b) The promoter from *MsDIUP1* was used to drive GUS expression in *Arabidopsis* plant under NaCl treatment (a) and ABA treatment (b). Scale bars: 2 mm in (a) and (b)

and 26.64% at 150 and 200 mM NaCl, respectively, and the lateral root number, enhancing it by 56.67% and 303.70% at 150 and 200 mM NaCl, respectively (Fig. 3b, c), whereas it shrank the difference in the primary root length, reducing it by 24.14% and 18.40% at 150 and 200 mM NaCl, respectively (Fig. S4).

We carried out a comparison test for the two-week-old *Arabidopsis* WT and OE plants in the same pot under salt stress. The OE plants showed more

tolerance to salt stress than the WT plants in terms of growth status, and the growth differences become more obvious as the time of salt treatment increased (Fig. 3d). The tolerances of the three-week-old *Arabidopsis* WT and OE plants under salt stress were further examined in different pots. When the salt treatment was carried out for 12 days, the WT leaves began to turn yellow, whereas the OE lines showed a relatively more normal phenotype than the WT plants (Fig. 3e). After 22 days of salt treatment, almost all

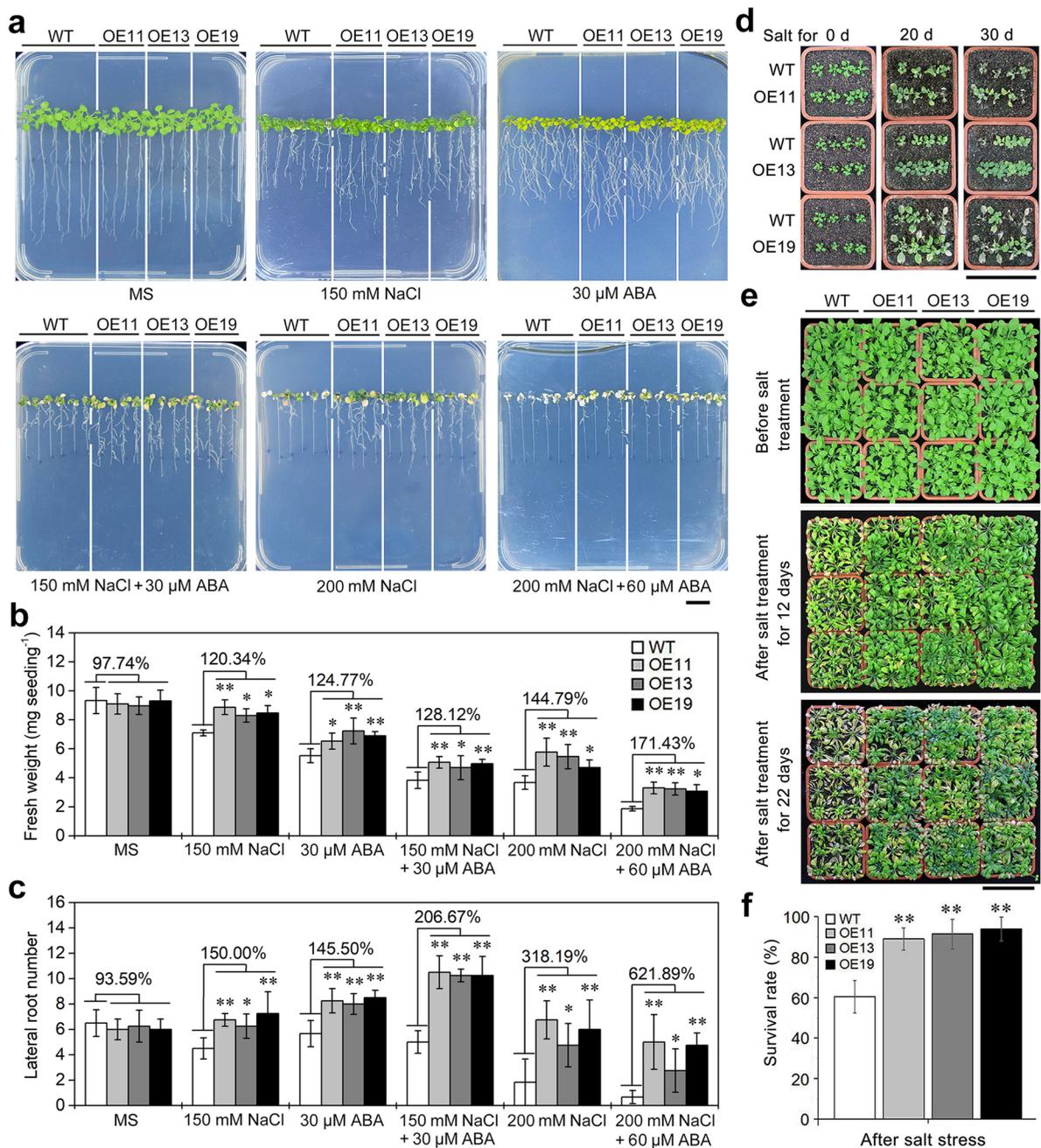


Fig. 3 Overexpression of *MsDIUPI* in *Arabidopsis* enhanced salt tolerance and ABA insensitivity. **(a)** The phenotype of one-week-old *Arabidopsis* WT and OE plants grown on MS medium or MS medium containing 150 mM NaCl, 30 μ M ABA, 150 mM NaCl + 30 μ M ABA, 200 mM NaCl, or 200 mM NaCl + 60 μ M ABA for 12 days. **(b, c)** The fresh weight **(b)** and lateral root number **(c)** of *Arabidopsis* WT and OE plants from **(a)**. The percentages above each bar indicate

the proportion of fresh weight or lateral root number in three OE lines relative to the WT plants. **(d)** The phenotype of two-week-old *Arabidopsis* plant sets including WT and OE11, WT and OE13, and WT and OE19 under salt stress for 20 and 30 days. **(e)** The phenotype of one-month-old *Arabidopsis* WT and OE plants under salt stress for 12 and 22 days. **(f)** The survival rate of *Arabidopsis* WT and OE plants from **(d)**. Scale bars: 1 mm in **(a)**, 9 cm in **(d)** and **(e)**

the WT plants were severely damaged and the leaves become withered, whereas most of the OE plants were less damaged and the leaves remained green (Fig. 3e). The survival rate of the three salt-treated OE lines ranged from 88.89 to 93.83%, which was significantly higher than the survival rate of 60.49% of salt-treated WT plants (Fig. 3f). Together, these phenotype discoveries strongly imply *MsDIUPI* could increase salt tolerance and ABA insensitivity in *Arabidopsis*.

MsDIUPI is a positive regulator for salt tolerance in alfalfa

To assess whether *MsDIUPI* is involved in regulating salt tolerance in alfalfa, we carried out a comparison salt assay for alfalfa WT and OE callus on SM4 medium upon treatment with different concentrations of NaCl. Under normal conditions, both alfalfa WT and OE calluses exhibited a healthy growth status and a light-yellow color. However, the OE callus remained light yellow, whereas the WT callus became brownish and the color deepened with the increase in NaCl concentrations (Fig. 4a). In agreement with the phenotype presentation, both the gene expression of *MsDIUPI* and the fresh weight of the OE callus at 100, 150, and 200 mM NaCl were significantly higher than those of the WT callus (Fig. 4b, c).

Thereafter, we compared the growth status of alfalfa plants for the WT, OE lines (OE6, OE31, and OE40), and RNAi lines (RNAi1, RNAi7, and RNAi33) in vermiculite, which were irrigated with 200 mM NaCl solution for 0 and 17 days. Under normal conditions, alfalfa WT, OE, and RNAi lines showed no obvious differences in growth status, exhibiting healthy and green leaves (Fig. 4d). After salt stress, most of the leaves of the OE plants kept spreading and remained green, whereas some leaves of the WT plants turned curly and yellow (Fig. 4d). In contrast, most leaves of the RNAi lines exhibited severe chlorosis (Fig. 4d). Importantly, OE lines displayed obviously higher survival rates ranging from 77.78–94.44% and RNAi lines exhibited evidently lower survival rates ranging from 27.78–38.89%, when compared to the survival rate of 61.11% of WT plants under salt stress (Fig. 4e). These phenotype observations repeatedly confirmed that *MsDIUPI* is a positive regulator of salt tolerance in alfalfa.

MsDIUPI regulates salt tolerance in alfalfa mainly by affecting physiological balance

To evaluate the role of *MsDIUPI* in salt-elicited oxidative stress, H_2O_2 and MDA contents were measured. Before salt stress, no apparent differences in the H_2O_2 content were detected among alfalfa WT, OE, or RNAi lines (Fig. 4f). After salt stress, the H_2O_2 content of all the alfalfa WT, OE, and RNAi lines was largely increased. This indicator in two of the OE lines (OE31 and OE40) was significantly lower, whereas in all three of the RNAi lines it was significantly higher than that of the WT plants under salt stress (Fig. 4f). There were no obvious differences in MDA content among the alfalfa WT, OE, and RNAi lines under normal conditions (Fig. 4g). However, when they were subjected to salt stress, the MDA content increased less in all three OE lines but more in all three RNAi lines than in the WT plants (Fig. 4g). The accumulation of soluble sugar and proline was also monitored to assess whether *MsDIUPI* is associated with osmotic adjustment during salt stress. Before salt stress, the contents of soluble sugar and proline showed no obvious differences among the alfalfa WT, OE, and RNAi lines (Fig. 4h, i). However, the contents of soluble sugar and proline were increased more in all three OE lines and were increased less in all three RNAi lines as compared to the WT plants after salt stress (Fig. 4h, i). These results suggest *MsDIUPI* reduced oxidative damage and enhanced osmoprotectant accumulation to copy salt stress.

To ascertain whether *MsDIUPI* altered ion homeostasis during salt stress, the contents of K^+ and Na^+ were determined. It was shown that salt stress strongly decreased the cellular K^+ content but increased the cellular Na^+ content in the alfalfa WT, OE, and RNAi lines (Fig. 5a, b). Although the K^+ content showed no obvious differences among the salt-treated alfalfa WT, OE, and RNAi lines, the Na^+ content in all three OE lines was significantly lower, and in two of RNAi lines (RNAi1 and RNAi33), it was higher than that of the WT plants after salt stress (Fig. 5a, b). These data resulted in a significantly higher K^+/Na^+ ratio in salt-treated OE plants when compared with salt-treated WT or RNAi plants (Fig. 5c), implying the involvement of *MsDIUPI* in salt tolerance through the regulation of ion homeostasis.

Furthermore, two key photosynthetic indicators of the chlorophyll content and net photosynthetic rate

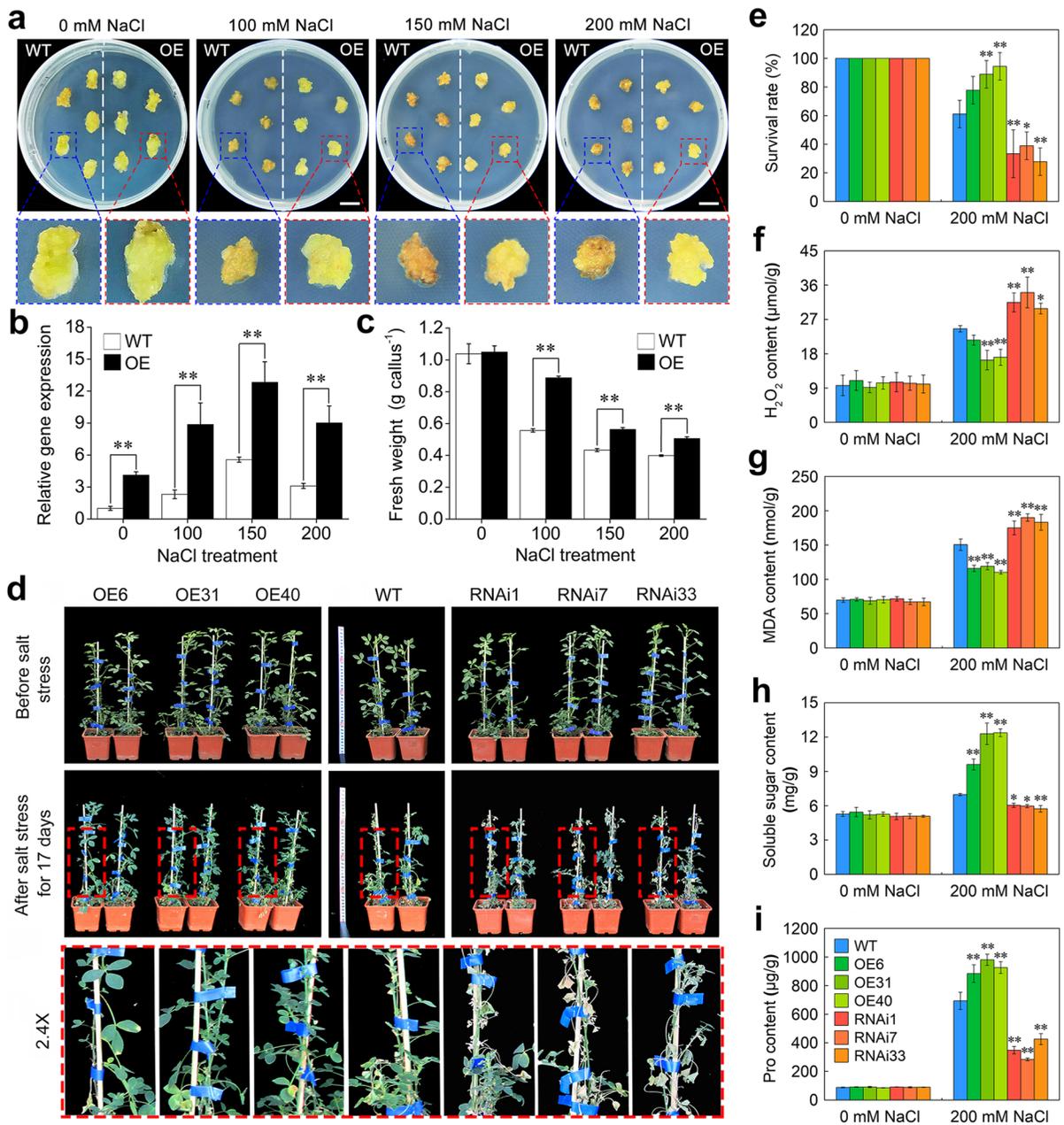


Fig. 4 Investigation of salt tolerance of alfalfa WT, OE, and RNAi lines. **(a)** The phenotype of one-month-old alfalfa WT and OE calluses under different concentrations of NaCl (0, 100, 150, or 200 mM) for 14 days. **(b, c)** The *MsDIU1* gene expression **(b)** and fresh weight **(c)** of salt-treated alfalfa WT and OE calluses from **(a)**. **(d)** The phenotype of two-month-old alfalfa WT, OE, and RNAi plants under salt stress for 17 days.

(e–i) Comparison of the survival rate **(e)** and four physiological indexes, including H₂O₂ content **(f)**, MDA content **(g)**, soluble sugar content **(h)**, and proline content **(i)**, in alfalfa WT, OE, and RNAi plants from **(d)** before salt stress and after salt stress for 15 days. **P* < 0.05; ***P* < 0.01. Scale bars: 1 cm in **(a)** and 40 cm in **(d)**

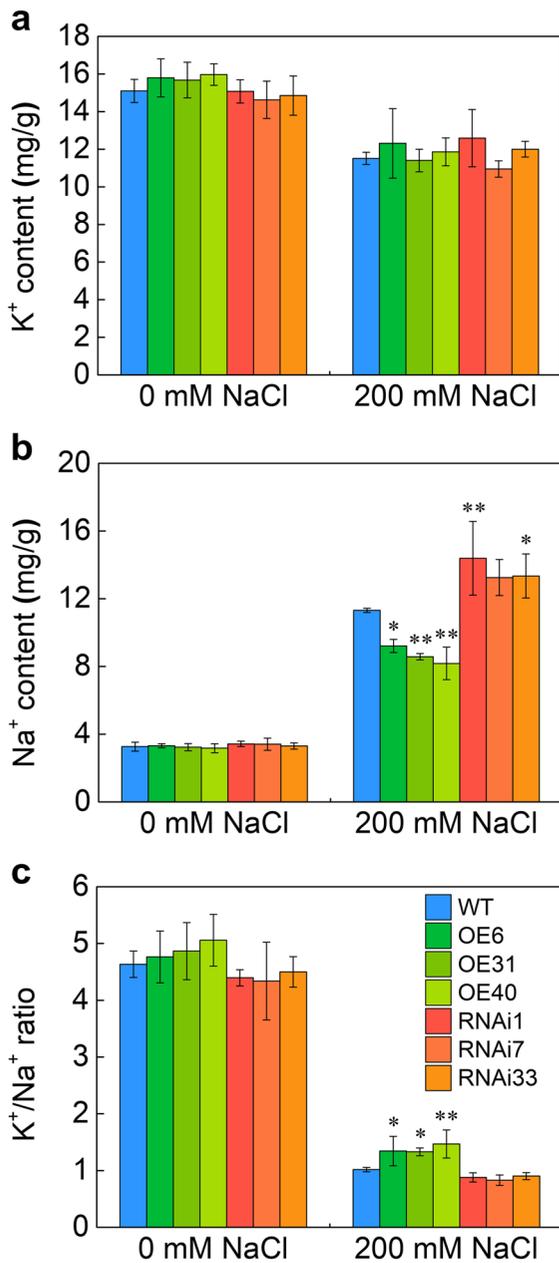


Fig. 5 Accumulation of K⁺ and Na⁺ in alfalfa WT, OE, and RNAi lines during salt stress. **(a, b)** K⁺ content (a) and Na⁺ content (b) in two-month-old alfalfa WT, OE, and RNAi plants were determined before salt stress and after salt stress for 15 days. **(c)** K⁺/Na⁺ ratio. **P* < 0.05; ***P* < 0.01

were measured. Under normal conditions, the chlorophyll content was comparable among the alfalfa WT, OE, and RNAi lines, whereas the net photosynthetic rate in the OE lines was remarkably higher than that

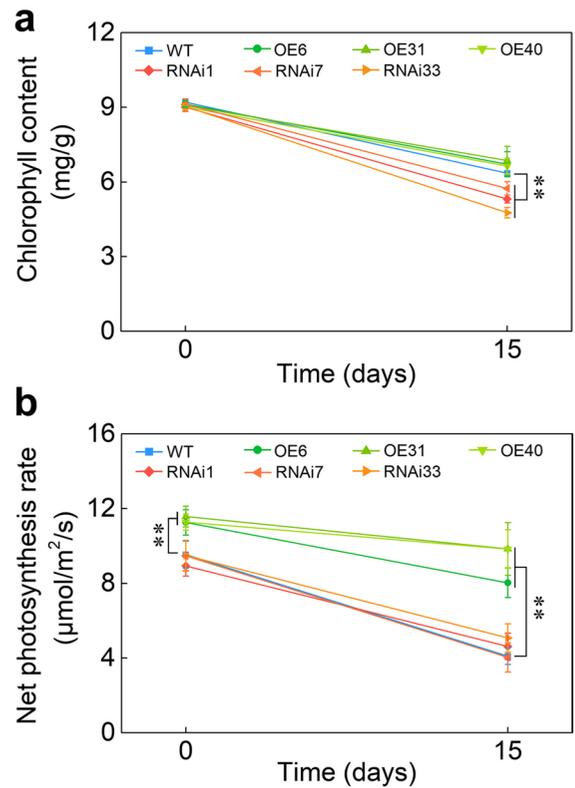


Fig. 6 Change of photosynthetic indicators in alfalfa WT, OE, and RNAi lines during salt stress. **(a, b)** Chlorophyll content (a) and net photosynthetic rate (b) in two-month-old alfalfa WT, OE, and RNAi plants were determined before salt stress and after salt stress for 15 days. ***P* < 0.01

of the WT or RNAi lines (Fig. 6). When they were exposed to salt stress, both the chlorophyll content and net photosynthetic rate in the alfalfa WT, OE, and RNAi lines largely declined (Fig. 6). However, the chlorophyll content was significantly lower in the salt-treated RNAi plants than in the salt-treated WT or OE plants, whereas the net photosynthesis rate was remarkably greater in the alfalfa OE plants when compared with the WT or RNAi lines during salt stress (Fig. 6). These results suggest that *MsDIUPI* is somewhat responsible for maintaining the photosynthetic capacity in alfalfa under salt stress.

MsDIUPI remodels transcriptional responses in alfalfa under salt stress

To explore the potential functions of *MsDIUPI*-regulated salt tolerance, an RNA-seq of the alfalfa WT,

OE, and RNAi lines before and after salt stress was performed. Two-way comparisons of gene expression profiles showed that gene regulation differed in each comparison of RNAi-S/RNAi (salt-treated RNAi compared to RNAi), WT-S/WT (salt-treated WT compared to WT), and OE-S/OE (salt-treated OE compared to OE). A total of 304, 518, and 177 DEGs were found in RNAi-S/RNAi, WT-S/WT, and OE-S/OE, respectively (Fig. 7a). Venn diagrams displayed that 45 DEGs overlapped in all three comparisons, whereas 141, 340, and 105 DEGs were exclusively modulated in RNAi-S/RNAi, WT-S/WT, and OE-S/OE, respectively (Fig. 7b).

We carried out a functional enrichment analysis of all 770 DEGs to reveal the characteristics of the different responses. A total of 83 GO terms were identified, which were mainly associated with processes of stress signaling, such as “intracellular receptor signaling pathway” and “protein serine/threonine phosphatase activity”; antioxidant defense, such as “response to oxygen-containing compound” and “glutathione transferase activity”; and osmotic adjustment, such as “carbon fixation” and “carbohydrate catabolic process” (Fig. 7c, Table S2).

Then, the expression patterns of DEGs that were functionally enriched in the three processes mentioned above were analyzed. For the stress signaling components, a wide range of DEGs exhibited a differential expression among the alfalfa WT, OE, and RNAi lines during salt stress, including two *PROLINE-RICH RECEPTOR-LIKE PROTEIN KINASEs* (*PERK9* and *PERK12*), one *RUST RESISTANCE KINASE LR10* (*LRK10*), one *SHEWANELLA-LIKE PROTEIN PHOSPHATASE 1* (*SLP1*), one *SERINE/THREONINE PROTEIN PHOSPHATASE 2A* (*PP2AB2*), and two *PROTEIN PHOSPHATASE 2Cs* (*PP2C51* and *PP2C12*) that were identified (Fig. 7d). In the antioxidant defense process, many DEGs showed differential induction among the alfalfa WT, OE, and RNAi lines during salt stress, including three *GLUTATHIONE S-TRANSFERASEs* (*GSTU4*, *GSTU8*, and *GSTU25*) and two *S-ADENOSYL-L-HOMOCYSTEINE HYDROLASEs* (*SAHH1* and *SAHH2*) (Fig. 7d). In the osmotic adjustment process, various types of DEGs were affected in the alfalfa WT, OE, and RNAi lines during salt stress, including two *DHNs* (*ERD14* and *RAB18*), two *BETA-GLUCOSIDASEs* (*BGLU11* and *BGLU2x*), one *BETA-AMYLASE 3* (*BAM3*), and one *CHAPERONE PROTEIN DnaJ 11* (*DnaJ11*) (Fig. 7d). Additionally,

RT-qPCR validation of six DEGs randomly selected from the identified 18 DEGs mentioned above revealed an accordant expression pattern (Fig. S5a) and a high correlation coefficient ($R^2 = 0.8249$) (Fig. S5b) between the RNA-Seq and RT-qPCR data, confirming the reliability of the RNA-Seq data.

Furthermore, *MsDIUPI*-associated networks were constructed based on the 18 DEGs mentioned above, revealing the potential interaction relationship. As expected, except for *LRK10* and *PP2C13*, all of the other genes formed a co-expression network (Fig. 7e). Among the network, the antioxidant defense-related *GSTU4* and *GSTU8* and the osmotic adjustment-related *RAB18* were co-expressed with five, five, and five DEGs, respectively; and they were located in the center of the co-regulatory network. In contrast, the stress signaling-related *PERK12*, *PP2C51*, *PERK9*, and *SLP1* were co-expressed with three, two, two, and one DEGs, respectively; and they were located in the bound of the co-regulatory network. *MsDIUPI* were co-expressed with *GSTU4* and *RAB18*, indicating that *MsDIUPI* plays a key role in reprogramming transcriptional responses in alfalfa under salt stress, especially in antioxidant defense and osmotic adjustment processes.

MsDIUPI has no obvious effect on the forage quality of alfalfa

Before developing a potential forage breeding material into a new cultivar, it is essential to assess its forage quality. Thus, we determined the six nutritive indicators of the alfalfa OE and RNAi lines and compared them to that of the WT controls. There were no apparent differences in the contents of crude protein, crude ash, dry matter, or calcium ions between the WT and OE lines or the WT and RNAi lines (Fig. S6a–d). Although the content of DNDF30 and the RFV were obviously reduced in the alfalfa RNAi lines, they were not apparently changed in the alfalfa OE lines in comparison to the WT plants (Fig. S6e, f). These results suggest that the alfalfa OE plants have a comparable forage quality with the WT plants.

Discussion

Accumulating genetic evidence suggests that many stress-inducible *LEA* genes may play critical roles

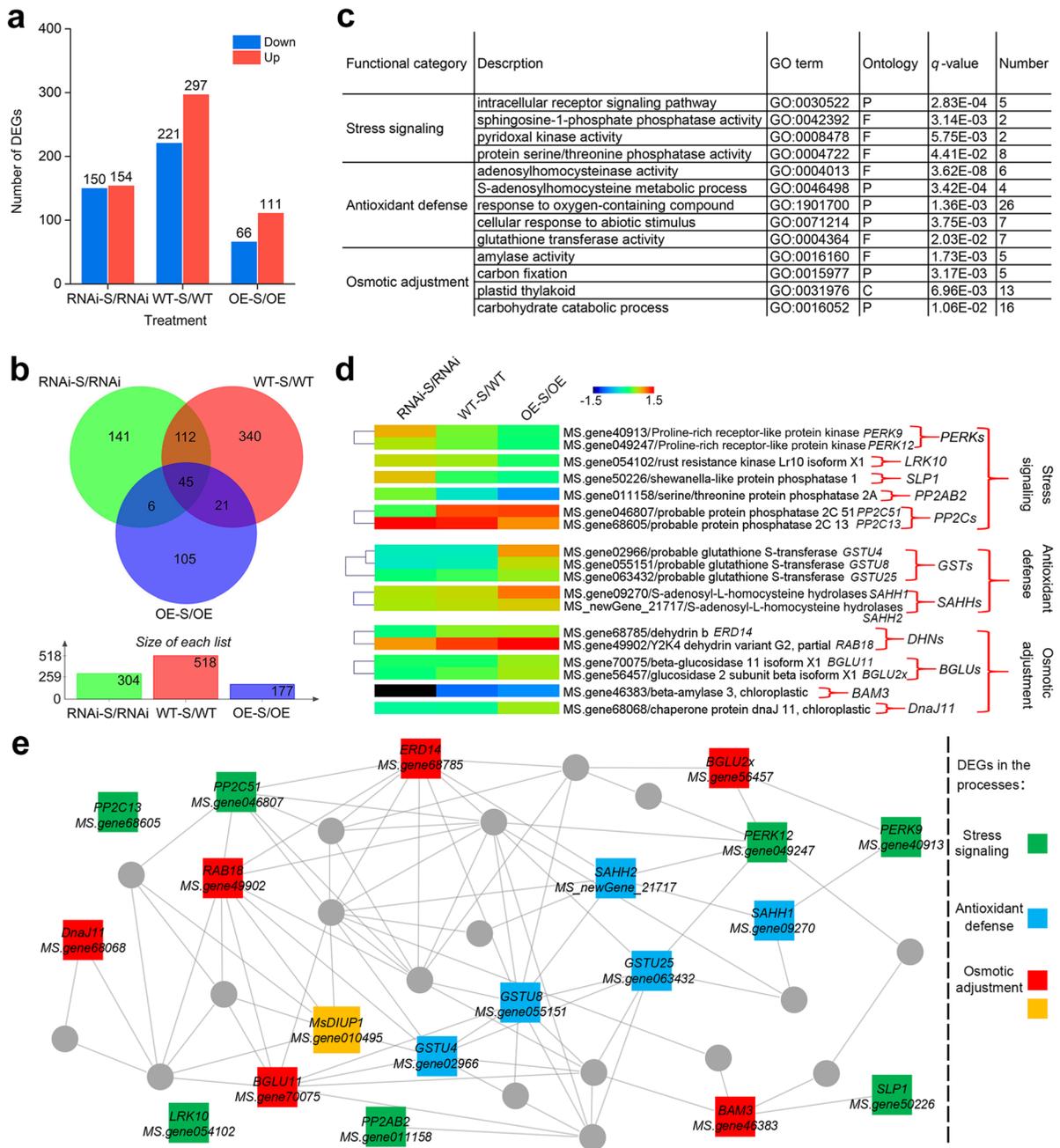


Fig. 7 Analysis of gene expression profiles of the DEGs associated with stress signaling, antioxidant defense, and osmotic adjustment in the three comparisons of RNAi-S/RNAi, WT-S/WT, and OE-S/OE in alfalfa. **(a)** The DEGs showing upregulation or downregulation in the three comparisons. **(b)** The DEGs showing overlapped or specific expressions in the three comparisons. **(c)** The involvement of significant enrichment GO categories in the processes of stress signaling, antioxidant response, and osmotic adjustment. **(d)** Heat pots of the expression changes (log₂-fold change) of the stress signaling-,

antioxidant defense-, and osmotic adjustment-related DEGs in the three comparisons. The colors from blue to red indicate the expression changes from low to high. **(e)** *MsDIUP1* transcriptional co-regulatory network. The network was constructed based on *MsDIUP1* and the 18 DEGs identified in the processes of stress signaling, antioxidant defense, and osmotic adjustment. Big boxes represent *MsDIUP1* plus the 18 identified DEGs, whereas their co-expressed genes are depicted as small grey circles. The grey line represents gene co-expression

in plant tolerance against salt stress, such as *Cleistogenes songorica* *CsLEA* (Zhang et al. 2016), *Arabidopsis* *AtEDT1* (Zheng et al. 2017), and alfalfa *MsLEA3-1* and *MsLEA4-4* (Bai et al. 2012; Jia et al. 2020). We previously found that *MsDIUP1* is exclusively expressed in mature seeds during plant's life cycle (Luo et al. 2022a), which is reminiscent of that of LEA proteins. In this study, although *MsDIUP1* lacks the eight LEA-conserved domains, we found that alfalfa *MsDIUP1* is rich in polar amino acids, is hydrophilic, is predicted to be an intrinsically disordered protein, and has no transactivation activity (Fig. 1), which is consistent with the characteristics of most LEA proteins (Park et al. 2011; Pantelić et al. 2022). These results imply that *MsDIUP1* might be a novel LEA protein. However, this hypothesis was mainly supported by bioinformatics data and lacks substantial experimental evidence. Previous reports showed that the circular dichroism spectra of intrinsically disordered LEA proteins usually have a large negative peak at approximately 200 nm (Lv et al. 2021a, b), and they often act as molecular chaperones to prevent protein aggregation during abiotic stress conditions (Kovacs et al. 2008). Thus, it is critical to investigate the two characteristics of *MsDIUP1* via experimental tests in a future study.

The power of *Arabidopsis* genetics has been widely recognized in dissecting developmental programs and hormonal, biotic, and abiotic responses (Zhu 2000). Using the *Arabidopsis* ectopic expression system, the promoter activity of *MsDIUP1* showed a large increase in *Arabidopsis* after exposure to salt stress (Fig. 2a), which inspired our interest in exploring its role in salt stress tolerance. Then, we found that *Arabidopsis* overexpressing *MsDIUP1* exhibited dramatically enhanced salt tolerance in comparison to the WT plants, with a higher fresh weight, lateral root number, and primary root length at the early seedling stage as well as a better growth status and higher survival rate of plants at the late developmental stage (Fig. 3). These results are similar to the growth performance of *Arabidopsis* ectopically expressing two *PpLEA3s* of *Physcomitrella patens* under salt stress (Du et al. 2016), suggesting that *MsDIUP1* confers *Arabidopsis* salt tolerance through the remodeling of plant growth. Due to the material and methodological limitations of the heterologous system, it is better to comprehensively investigate the functional roles of an uncharacterized gene in its native systems

(Wang et al. 2017). As expected, when the expression of *MsDIUP1* was modified in an alfalfa system, it showed that the overexpression of *MsDIUP1* strongly improved the salt tolerance of the alfalfa callus and plants, slowing down the plant surface lesions and discoloration, whereas the knockdown of *MsDIUP1* markedly reduced the salt tolerance of alfalfa plants, accelerating the plant tissue breakdown and senescence (Fig. 4a, d). Similar results have appeared in previous reports on a salt tolerance phenotype positively regulated by *OsbZIP71* in rice (Liu et al. 2014) and *AtSIZ1* in *Arabidopsis* (Han et al. 2019). Thus, we concluded that *MsDIUP1* functions as a positive regulator in improving plant tolerance to salt stress.

Numerous studies have shown that ABA modulates the expression of many LEA proteins in different plant species, such as *CaLEA6* in hot pepper (*Capsicum annuum*) (Kim et al. 2005), *SIER5* in tomato (*Solanum lycopersicum*) (Zegzouti et al. 1997), and *BnLEA4-1* in *Brassica napus* (Dalal et al. 2009). Our study showed that *MsDIUP1* promoter-driven GUS expression was dramatically increased under ABA treatment at the seedling stage (Fig. 2b), implying that *MsDIUP1* might be associated with modulating plant growth and development under ABA conditions. Supporting this hypothesis, we found that *Arabidopsis* seedlings overexpressing *MsDIUP1* exhibited an enhanced insensitivity to ABA, and had a higher fresh weight, lateral root number, and primary root length than did the WT plants (Figs. 3a–c, S4). Interestingly, unlike transgenic *Arabidopsis* overexpressing *AtLEA3-3* or pepper *CaLEA1* showing enhanced salt tolerance with increased sensitivity to ABA (Zhao et al. 2011; Lim et al. 2015), our study found that *Arabidopsis* OE plants showed stronger ABA insensitivity compared to the WT plants, suggesting that members of the LEA family have become diverse in their response to ABA during adaptive evolution. ABA functions as an integrative signal and played a critical role in plant responses to adverse environmental conditions such as salt stress (Zhu 2016). A previous study on *C. songorica* showed that an exogenous ABA application on plants overexpressing *CsSAMDC2* induced salt tolerance, promoting lateral and primary root growth (Wu et al. 2022). However, our study showed that the exogenous ABA application did not improve salt tolerance but enlarged the differences in salt tolerance between the *Arabidopsis* WT and OE plants by enhancing the

differences in the fresh weight and lateral root number (Fig. 3b, c). Notably, the exogenous ABA application reduced the difference in the primary root length between salt-treated *Arabidopsis* WT and OE plants (Fig. S4), which might be due to the mutual inhibition of growth between the lateral and primary roots (Duan et al. 2013). The two contrasting results could be explained by the fact that genes have specific mechanisms to regulate salt tolerance under ABA treatment. In the context of the previous study, ABA induced salt tolerance by enhancing the expression of the polyamine genes *AtADC2* and *AtSAMDC2*, which are involved in promoting cell differentiation and division for roots during salt stress (Couée et al. 2004; Wang and Liu 2016). Here, we showed that there may be crosstalk between *MsDIUPI*-mediated ABA and the salt stress response, as proved by two *PP2Cs*, which encode core regulatory components of the ABA signaling module (Chan 2012), that were differentially expressed in the salt-treated alfalfa WT, OE, and RNAi plants (Fig. 7d). Thus, *MsDIUPI* improves plant salt tolerance possibly through modulating an ABA-signaling pathway.

It is well known that a high salt level often triggers the excessive generation of ROS (including H_2O_2), as by-products, which can cause severe oxidative damage (Taïbi et al. 2016; Luo et al. 2022b). MDA, a product of lipid peroxidation, is widely used as a marker for reflecting the extent of the membrane oxidative damage in plants (Zhu et al. 2010). Our results showed that the content of H_2O_2 and MDA increased less in the alfalfa OE plants but increased more in the alfalfa RNAi plants when compared with the alfalfa WT plants under salt stress (Fig. 4f, g), implying that *MsDIUPI* might contribute to scavenging ROS and mitigating lipid peroxidation in alfalfa under salt stress. Substantial evidence supports the claim that plants often combat and eliminate adverse oxidative effects by deploying an antioxidant defense system that constitutes both enzymatic components and non-enzymatic components (Luo et al. 2022b). Indeed, our transcriptome data showed that the majority of antioxidant defense genes including three *GSTs* and two *SAHs* were highly activated in the alfalfa WT, OE, and RNAi plants under salt stress (Fig. 7d). *GSTs* constitute an ancient and highly diverse group of proteins that quench reactive molecules (such as free radicals and ROS) with the addition of glutathione (GSH) and protect the cell from oxidative

damage (Kumar and Trivedi 2018). There were extensive reports showing that the overexpression of gene encoding *GST* family proteins enhances the antioxidant defense and improves abiotic stress tolerance in various plant models (Chan and Lam 2014). *SAHs* are responsible for the regeneration of S-adenosylmethionine, which serves as the methyl donor for the biosynthesis of antioxidants, such as GSH (Zhou et al. 2011; Mäkinen and De 2019). In tobacco, *SAHH* silencing caused a profound reduction in the GSH concentration in *Potato virus A*-infected plants, which was accompanied by severe oxidative damage symptoms (De et al. 2018). Thus, a greater induction of these genes occurred in the alfalfa OE plants and a lesser induction occurred in the RNAi plants compared to the WT plants under salt stress (Fig. 7d), strongly suggesting that *MsDIUPI* could participate in strengthening the antioxidant defense system to alleviate oxidative damage in alfalfa under salt stress.

Salt stress mainly imposes ionic and osmotic stress on plants (Zhu 2016). Therefore, a key adaptive response of plants to salt-triggered ionic stress is the maintenance of ion homeostasis, by limiting the cellular absorption of Na^+ and export of K^+ (Huang et al. 2012). In transgenic alfalfa plants, the overexpression of *CsLEA* from *C. songorica* resulted in significantly greater Na^+ accumulation and K^+/Na^+ ratios in the leaves (Zhang et al. 2016). In agreement with this report, we found that salt-treated alfalfa OE plants had comparable K^+ but decreased Na^+ levels, leading to a higher K^+/Na^+ ratio than that in the WT or RNAi plants (Fig. 5). These results suggest that *MsDIUPI* is associated with maintaining high cytosolic K^+/Na^+ ratios to extenuate the ion toxicity caused by salt stress. Another key adaptive response of plants to salt-triggered osmotic stress is osmotic adjustment, which occurs by accumulating osmoprotectants inside the cell (Alzahrani 2021). Two major osmoprotectants, soluble sugar and proline, could raise the osmotic pressure in the cytoplasm and preserve the cellular apparatus from injury, thus acclimating the plants to tolerate salt stress (Rahimi and Biglarifard 2011). Under salt stress, transgenic alfalfa overexpressing *MsDIUPI* accumulated much more soluble sugar and proline than the WT plants, whereas knock-down of *MsDIUPI* in transgenic alfalfa accumulated less soluble sugar and proline (Fig. 4h, i), indicating the positive role of *MsDIUPI* in modulating the accumulation of osmoprotectants to mitigate the osmotic

damage caused by salt in alfalfa. This notion is supported by the observation that many osmotic adjustment-related genes including two *DHNs*, two *BGLUs*, and one *DnaJ11*, were more induced, whereas one *BAM3* was reduced less in the alfalfa OE plants than in the alfalfa WT or RNAi plants (Fig. 7d). BAMs and BGLUs are critical enzymes responsible for the accumulation of maltose and glucose, which act as key osmoprotectants in response to abiotic stress (Ahmad et al. 2017; Gao et al. 2018; Sun et al. 2021). Stress protein accumulation has been shown to be a central tolerance mechanism, such as, for example, DHNs or DnaJs that provide osmotic protection during stress events (Wang et al. 2004; Kumar et al. 2014; Jespersen et al. 2017). Studies on transgenic plants have proved that the overexpression of genes encoding BAMs, BGLUs, DHNs, and DnaJs could trigger a higher content of osmoprotectants and increased tolerance to various abiotic stresses (Brini et al. 2007; Wang et al. 2014b; Baba et al. 2017; Sun et al. 2021). Thus, our molecular discoveries demonstrate that *MsDIUP1* could improve the osmotic adjustment ability of salt-treated alfalfa plants through the accumulation of osmoprotectants.

Salt usually decreases the photosynthetic activity in plants, because of its disruption of ion homeostasis and osmotic adjustment in cells (Pan et al. 2016). Photosynthetic pigments including chlorophyll *a* and *b* are important components of the light reactions that occur during photosynthesis, and largely determine photosynthetic capacity (Shaheen et al. 2013).

As shown in several studies, a positive association exists between photosynthetic pigments and the rate of photosynthesis in plants exposed to salt stress, such as wheat (*Triticum aestivum*) (Arfan et al. 2007) and safflower (*Carthamus tinctorius*) (Siddiqi et al. 2009). In this study, although both the chlorophyll content and net photosynthesis rate declined under salt stress (Fig. 6), there was no apparent linear correlation between these two indicators among the salt-treated alfalfa WT, OE, and RNAi plants (Fig. 6). This observation suggests that chlorophyll content might not be the dominant factor controlling *MsDIUP1*-regulated photosynthesis, which is possibly more affected by other factors. Indeed, our transcriptome data showed that the two chloroplastic genes *BAM3* and *DnaJ11* were differentially expressed among the alfalfa WT, OE, and RNAi plants during salt stress (Fig. 7d). Although these two genes were shown to participate in the osmotic adjustment described above, several studies have reported that they are also involved in photosynthesis. In *Arabidopsis*, *BMX8* RNAi plants showed a reduced soluble sugar content (glucose, fructose, and sucrose) and chlorophyll fluorescence (*Fv/fm* ratio) when exposed to freezing stress, suggesting that *BMX8* may contribute to the protection of the photosynthetic electron transport chain and stromal proteins inside the chloroplast (Kaplan and Guy 2005). Additionally, in *Arabidopsis*, knockout of *DnaJ* genes including *AtJ8*, *AtJ11*, and *AtJ20* resulted in a series of events including the destabilization of PSII

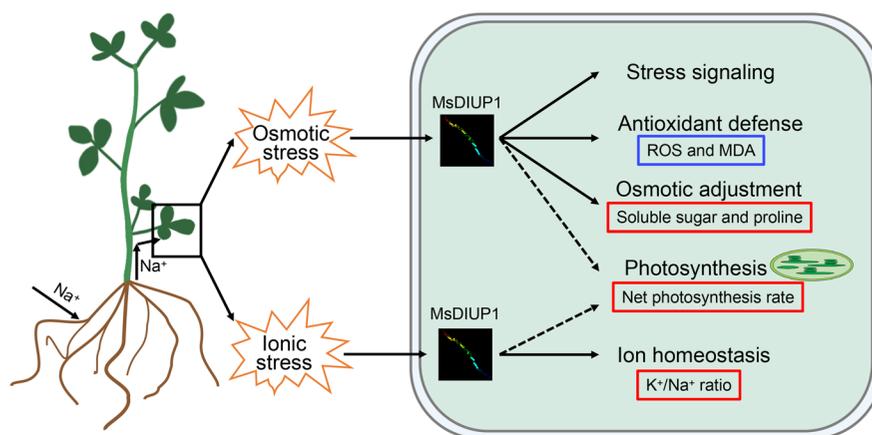


Fig. 8 Model description that *MsDIUP1* improves alfalfa salt tolerance, through modulating the processes of stress signaling, antioxidant defense, ion homeostasis, osmotic adjustment, and photosynthesis. Arrows represent the pathways with direct

relationships. The dotted arrows represent the pathways with uncertain direct relationships. Blue box represents decreased indicator. Red boxes represent increased indicators

complexes and a reduction in the photosynthetic efficiency in chloroplasts, suggesting that DnaJ proteins are involved in the optimization of various reactions of photosynthesis (Chen et al. 2010). Thus, it is reasonable to speculate that the improved photosynthesis conferred by *MsDIUPI* may be somewhat attributed to the enhanced osmoprotection capacity in the photosynthetic apparatus.

A key model proposed for LEA function is the molecular chaperone, which protects other proteins against aggregation under abiotic stresses (Lv et al. 2021a, b). Previous studies have demonstrated that some LEAs have a chaperone-like capacity to stabilize the structure and ensure the stability of antioxidant enzymes or protective proteins under stress conditions, such as through the modules of AtERD14/AtGSTF9 (Nguyen et al. 2020) and OpsDHN1/AtRAB18 (Hernández-Sánchez et al. 2017). In this study, *MsDIUPI* was found to be co-expressed with the antioxidant enzyme gene *GSTU4* and the protective protein gene *RAB18* (Fig. 7e), which was in accordance with our physiological findings that *MsDIUPI* improves the capacity of antioxidant defense and osmotic adjustment (Fig. 4f–i). This implies that *MsDIUPI* might act as a molecular chaperone and function to protect enzymes or proteins against salt stress-induced aggregation. However, this hypothesis must be confirmed by tests of chaperone function in vivo and in vitro in a future study.

Overall, our physiological and molecular discoveries revealed that *MsDIUPI* plays a positive role in regulating a broad range of processes including stress signaling, antioxidant defense, ion homeostasis, osmotic adjustment, and photosynthesis, ultimately, improving plant salt tolerance (Fig. 8). Since the upregulation of *MsDIUPI* in alfalfa plants has no apparent effect on their forage quality (Fig. S6), it is thus possible that alfalfa OE plants could be targeted to breed a salt-tolerant cultivar in future alfalfa breeding programs.

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Declarations

Competing interests The authors report no declarations of interest.

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