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Overexpression of *ZmDUF1644* from *Zoysia matrella* enhances salt tolerance in *Arabidopsis thaliana*

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Abstract:

The DUF1644 family is a highly conserved and functionally unknown protein family and has been demonstrated significantly improved the salt tolerance of rice (non-halophytes). However, little is understood about the function of the *DUF1644* gene in halophytes. Further studies are required to determine whether it has a similar function in halophyte species. In our study, the functions of *ZmDUF1644* from halophyte *Zoysia matrella* Z123 species were investigated under salt stress. Phylogenetic analysis clustered *ZmDUF1644* and *OsSDIP361* in the same group, which function as a salt-tolerant genes in rice. Our experimental data suggested that the *ZmDUF1644* gene significantly alleviated the growth inhibition of transgenic *Arabidopsis thaliana* seedlings caused by salt stress. The maximum root length, fresh weight, and photosynthetic pigment content of transgenic seedlings were obviously better than that of the wild type. Moreover, a relatively lower relative electrolyte leakage rate, toxic ion (Na^+) content, and Na^+/K^+ ratios were also found in the transgenic line when compared with the wild type under salt stress. In conclusion, this study revealed that the *ZmDUF1644* gene was overexpressed in *Arabidopsis thaliana* significantly enhancing the salt tolerance of seedlings. It is the first report on the functions of *ZmDUF1644* from halophyte *Zoysia matrella* which has a similar function in halophyte species.

Keywords: *ZmDUF1644* gene, salt tolerance, transgenic line, halophyte, *Zoysia matrella*.

1. Introduction

Salt stress is one of the most important abiotic stresses for plant farming and is intimately linked with the two other significant abiotic stresses, drought and cold (Sharifi Alishah et al. 2022). Aside from the practical purpose of genetically enhancing salt tolerance of plants, salt tolerance study is an important aspect of basic plant biology,

adding to our understanding of issues that include transcriptional regulation, signal transduction, oxidative damage, ion homeostasis, and nutritional imbalance (Sharifi Alishah et al. 2022; Wu et al. 2021). The most typical salt in the environment is sodium chloride (Hao et al. 2021). Plants suffering from salt stress will exhibit osmotic stress and ionic stress in the beginning phase due to the altered water potential (Hossain et al. 2021). Then Na^+ and Cl^- are over-accumulated inside the plants which would affect their growth and development (Gao et al. 2022). Plants usually alleviate the effects of salt stress through the excretion of excess ions outside the body or compartmentalization of ions into the vacuoles under the regulation of salt stress-responsive genes, especially for the salt tolerance gene (Adillah et al. 2022; Theerawitaya et al. 2019).

The domain of the unknown function (DUF) is proteins with specific conserved structural domains, and their functions are still uncharacterized (Smith et al. 2020). The DUF protein family is ubiquitous in all organisms, with approximately 2700 DUF proteins distributed in bacteria and over 1500 DUF proteins distributed in eukaryotes (Goodacre et al. 2013). Despite the functions are not annotated, DUF proteins play vital roles in plant biological processes, including growth and development and response to stress (biotic and abiotic), etc (Grützner et al. 2021; Yang et al. 2022; Ying et al. 2021). Recently, research on DUF proteins in various plants has increased dramatically. For example, in the pattern plant *Arabidopsis thaliana*, the gene family of *DUF581* is induced to be expressed by environmental factors and hormones and interacts with SnRK1 to regulate the relocalization of kinases to specific regions in the nucleus (Nietzsche et al. 2016). Through gene knockout experiments, Kim demonstrated that *AtRDUFs* could regulate drought stress mediated by ABA (Kim et al. 2012). Gu and Zhou reported a *DUF1517* gene from *Ammopiptanthus mongolicus*, which has a significant response to cold stress and has been confirmed to reduce the sensitivity to cold stress in *Arabidopsis* mutant seedlings through transgenic technologies, which were the deletion of the *AtDUF1517* gene (Gu and Cheng 2014; Zhou et al. 2020). In addition, Gholizadeh also found that DUF538 proteins from *Celosia cristata* plant had strong resistance to oxidation, and the gene expression was in response to drought stress (Gholizadeh 2015; Gholizadeh and Baghban Kohnehrouz 2010). The latest research even discovered that DUF1644 protein interacted with WD40 protein to synergistically regulate the number of secondary branches in maize and the number of grains in rice, which could enhance grain yield in both (Chen et al. 2022).

With the continuous excavation of the *DUF* gene family in plants, numerous DUF proteins have been found to be intimately linked to adversity stresses, such as DUF221 (Cao et al. 2020), DUF810 (Li et al. 2018), DUF1645 (Liu et al. 2022) and DUF1644 (Zhang et al. 2021). Especially for the DUF1644 protein family, which is a highly conserved and functionally unknown protein family belonging to a plant-specific DUF protein family (Zhang et al. 2021). Distinct from other families of plant DUF, the *DUF1644* family genes were mainly involved in response to salt stress in rice (Li et al. 2016). Luo et al. (2016) reported that the gene *OsSIDP301* was negatively regulated in response to salt stress and enhanced the sensitivity of rice to high salt stress which belongs to the *DUF1644* gene family (Luo et al. 2016). In parallel, *OsSIDP361*, another gene of *DUF1644* family was identified to be induced by 200 mM/L NaCl and significantly enhanced the salt tolerance of rice at the seedling and heading stage but the gene *OsSIDP361* positively regulated the expression of stress-responsive genes and was highly sensitive to exogenous ABA (Li et al. 2016). Nevertheless, Guo et al. (2016) found that the expression of gene *OsSIDP366*, a member of the *DUF1644* gene family was not affected by ABA and could also significantly improve the salt tolerance of rice (Guo et al. 2016). Previous studies have shown that the *DUF1644* gene family can regulate both ABA-dependent and non-ABA-dependent signaling pathways to improve salt tolerance in rice and which is a non-halophytic herb, and its response to salt stress is different from halophytic herbs. However, the function of the *DUF1644* gene in halophytes is poorly understood. Whether it has a similar function in halophyte species remains to be further demonstrated.

In our study, a salt-tolerant gene of the *DUF1644* family was screened from halophyte *Zoysia matrella* Z123 species. Which was one of the most salt-tolerant warm-season turf grasses (Zhang and Hu 2021). To investigate the functions of the gene *ZmDUF1644* in response to salt stress, we heterologously expressed the gene *ZmDUF1644* in wild-type *Arabidopsis thaliana* by using a transgenic method and discovered that the salt tolerance of the *ZmDUF1644* transgenic *Arabidopsis thaliana* seedlings was drastically increased via regulating osmotic potential and ion homeostasis.

2. Materials and Methods

2.1. Plant materials and treatment

The halophyte *Zoysia matrella* Z123 species and *Arabidopsis thaliana* (Columbia

ecotype, Col-0) were used in this study. Seedings treatment, germination, and culture conditions were performed as described in a previous study (Wei et al. 2016). After being sterilized and seeded into pots containing a sterilized peat moss and vermiculite combination, seeds of the *Arabidopsis thaliana* WT and *ZmDUF1644*-transgenic line (L1) were cultivated for 35 days before being treated with 150 mM NaCl. The physiological and biochemical indicators of salt stress were measured after 15 days NaCl stress.

2.2. Cloning and analysis of *ZmDUF644*

The *ZmDUF1644* gene was amplified from *Zoysia matrella* Z123 species by specific primers (Table S1). The amino acid sequence of ZmDUF1644 was aligned with 10 *Arabidopsis thaliana* DUF1644 proteins and 9 rice DUF1644 proteins (Table S3) using clustalx. Phylogenetic trees using the neighbor-joining method of MEGA 7.0

2.3. *ZmDUF1644* gene overexpression in *Arabidopsis thaliana*

The flowering dip strategy was used to convert the p1300-*ZmDUF1644* plasmid into *Arabidopsis thaliana* (Clough and Bent 1998). The transgenic plants were identified, and the seeds from the T2 generation were utilized in further investigations as previously reported (Wei et al. 2016).

2.4. Measurement of maximum root length and fresh weight

To measure the maximum root length and fresh weight of *Arabidopsis thaliana* under salt stress, seedlings of WT and *ZmDUF1644*-transgenic lines were treated with 150 mM NaCl at 35 days. After 15 days treatment, remove the seedlings from the pot using the flowing water, and the maximum root length and fresh weight of both were measured.

2.5. Determination of photosynthetic pigment content

Refer to the determination method described by He and Chen (2015) (He and Chen 2015). Each test sample was taken 0.1 g of leaves in a tube, and the leaves were extracted in the dark for 48 h with the mixture which contained anhydrous ethanol and acetone in a volume ratio of 1:1. Then the absorption of the chlorophyll and carotenoids extracts was measured at OD644 nm, OD663nm and OD470 nm using a UV-Spectrophotometer. The contents of various photosynthetic pigments in the leaves were calculated according to the formulas in Table S2.

2.6. Determination of relative electrolyte leakage and ion content

The WT and *ZmDUF1644*-transgenic *Arabidopsis thaliana* seedlings were

divided into shoots and roots, weighed separately for fresh weight, then air-dried and weighed separately for dry weight. The relative electrolyte leakage of the WT and *ZmDUF1644*-transgenic seedlings in shoots and roots was assayed using the method described by Cao et al. (2016) (Cao et al. 2016). And the Na^+ and K^+ contents in shoots and roots of both was measured and analyzed as described in a previous study (Wei et al. 2015)

3. Results

3.1. The simple bioinformatics analysis of ZmDUF644

We obtained the complete *ZmDUF1644* CDS via PCR amplification from the halophyte *Zoysia matrella* Z123 which contains 1026 nucleotides (**Supplementary Figure S1**). The phylogenetic analysis with DUF1644 family proteins of model plant rice and *Arabidopsis thaliana* shown that ZmDUF1644 protein clustered together with protein OsSDIP361 in the Group II of DUF1644 protein family (**Figure 1A**). To further investigate the potential function of protein ZmDUF1644, we performed a simple bioinformatics analysis of protein ZmDUF1644 by using the protein structure domain online analysis tool (https://swissmodel.expasy.org/interactive/) and the protein modular architecture research tool (http://smart.embl-heidelberg.de/). It was found that the protein contains a typical DUF1644 domain and that the domain contains a C2H2-type zinc finger structural region (**Figure 1B**) which was consistent with the prediction of protein tertiary structure (**Figure 1C**).

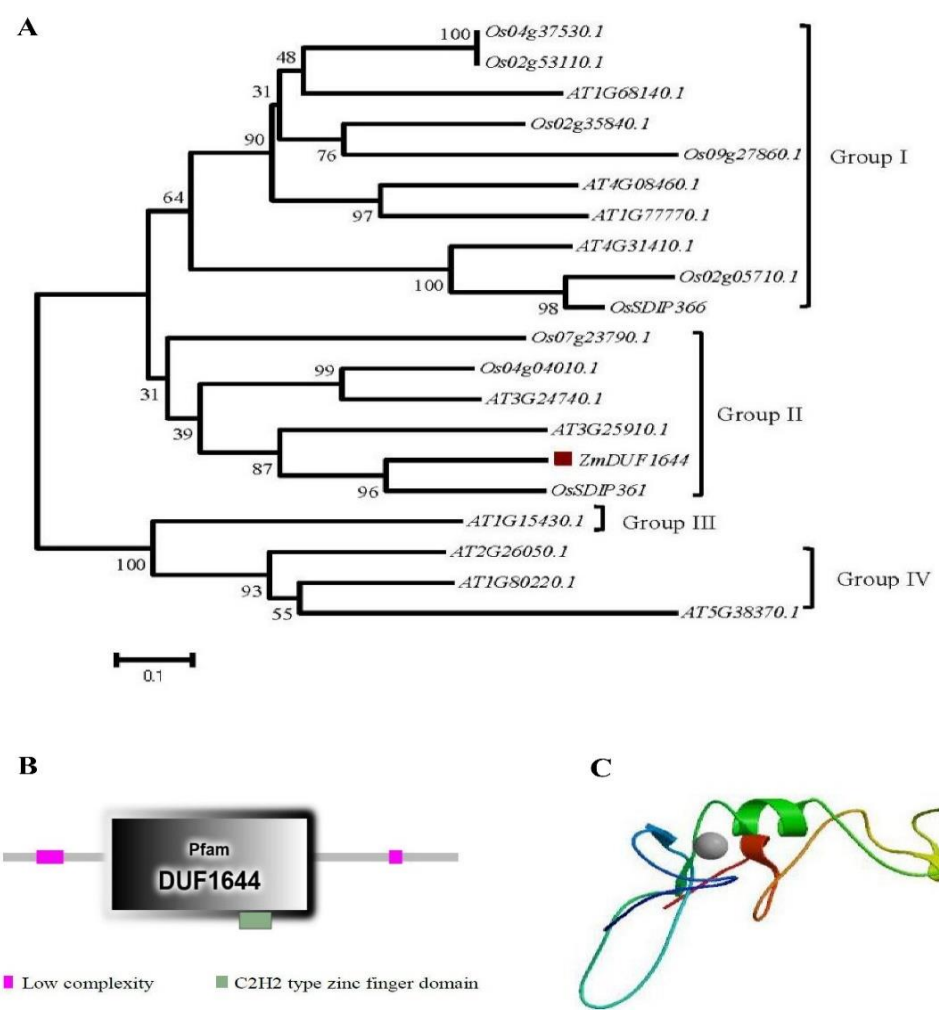

Figure 1. The phylogenetic and structure domain analysis of ZmDUF1644 protein: (A) The ZmDUF644 phylogenetic analysis with DUF1644 family proteins of model plant rice and *Arabidopsis thaliana*. MEGA-X was used to create unrooted NJ trees. At each branch, Bootstrap values from 1000 replicates are displayed; (B) The conserved structural domain analysis of ZmDUF644 protein; (C) The Tertiary structure prediction of protein ZmDUF644;

3.2. The variation of fresh weight and maximum root length

In the *Arabidopsis thaliana* transgenic experiment, four individual transgenic lines which were overexpressed gene *ZmDUF1644* have been created. Since salt tolerance was presented in all transgenic lines (**Supplementary Figure S2**), we chose one representative line (L_1) for in-depth study. Under normal conditions, both WT and *ZmDUF1644* transgenic seedlings were grown well and no significant differences in growth phenotypes. In contrast, the growth of both was inhibited under salt stress, but the *ZmDUF1644* transgenic *Arabidopsis thaliana* seedlings displayed a better growth

trend compared with the WT (**Figure 2A**). Under salt stress, the leaves of *ZmDUF1644* transgenic seedlings appeared to be larger than WT which were already presented wilting. Here we determined the root length and fresh weight of both. Under salt stress, the results showed that the *ZmDUF1644* transgenic seedlings increased 27.40% (**Figure 2B**) and 76.44% (**Figure 2C**) over wild-type seedlings, respectively.

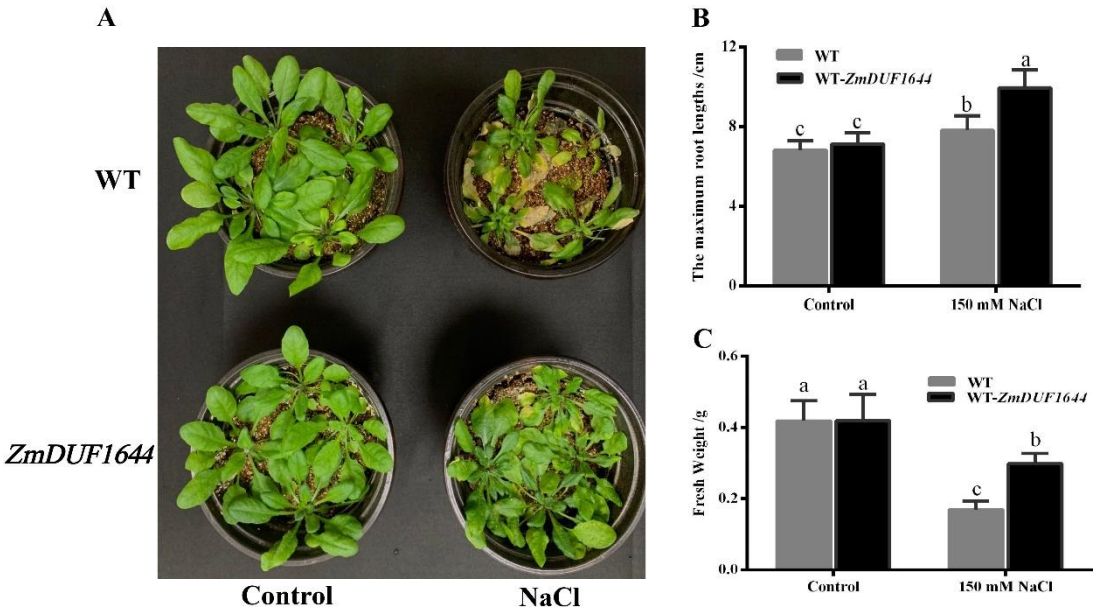

Figure 2. The growth (A), maximum root length (B), and fresh weight (C) of WT and *ZmDUF1644* transgenic *Arabidopsis thaliana* seedlings. Each column represents the mean value (n = 10) ±SD. The different letters that indicate a significant difference at $p <$ 0.05using Duncan's test after one-way ANOVA demonstrate statistical significance.

3.3. The variation of photosynthetic pigment content

To establish the photosynthetic pigment disruption caused by salt stress, we determine the contents of chlorophyll and carotenoid to compare the difference between WT and *ZmDUF1644* transgenic *Arabidopsis thaliana* seedlings. Except for the contents of carotenoid, no significant differences were found between WT and *ZmDUF1644* transgenic *Arabidopsis thaliana* under normal conditions. After 10 days of 150 mM NaCl stress, the contents of chlorophyll b and carotenoid in both were decreased dramatically compared with the non-salt treated control. However, the photosynthetic pigment damage is more severe in wild type than in transgenic line. The contents of chlorophyll a, chlorophyll b, chlorophyll (a+b), and carotenoids were 100.00% (**Figure 3A**), 83.17% (**Figure 3B**), 95.87% (**Figure 3C**), and 66.10% (**Figure**

3D) higher than those of wild type, respectively. In particular, the chlorophyll a and chlorophyll (a+b) content of *ZmDUF1644* transgenic seedlings, although still slightly lower than the control group, it was already without significant difference between the two groups.

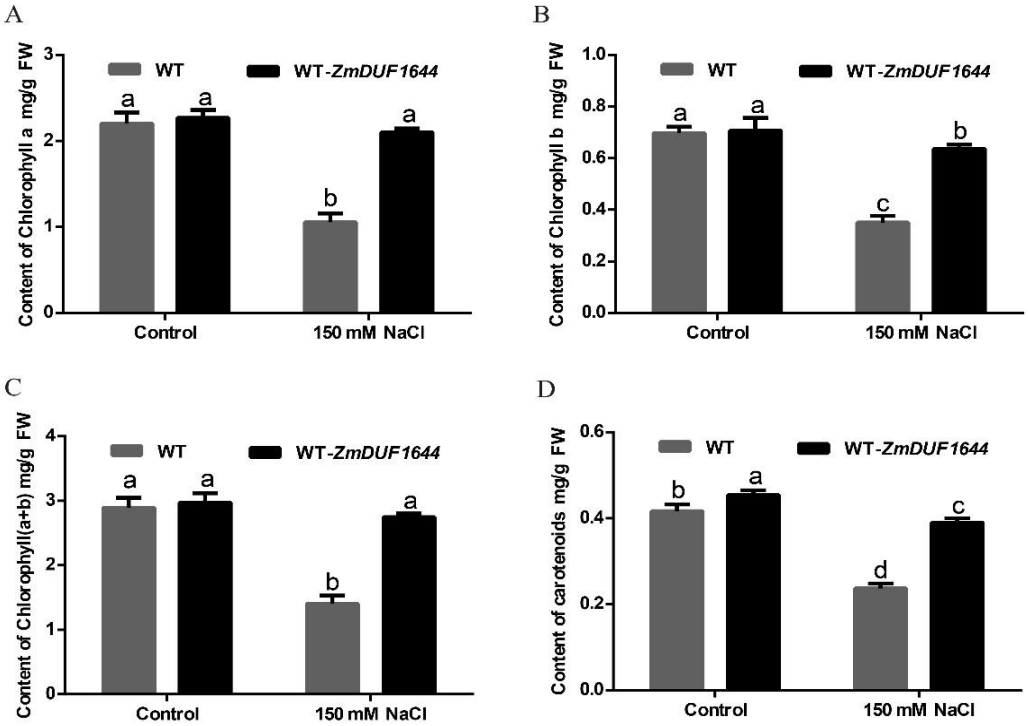

Figure 3. The content's variation of photosynthetic pigment content in WT and *ZmDUF1644* transgenic *Arabidopsis thaliana* seedlings. The chlorophyll a (A), chlorophyll b (B), chlorophyll (a+b) (C) and carotenoids (D) contents of WT and *ZmDUF1644* transgenic seedlings under control and salt stress. Each column represents the mean value (n = 3) ±SD. The different letters that indicate a significant difference at $p < 0.05$using Duncan's test after one-way ANOVA demonstrate statistical significance.

3.4. The alteration of the relative electrolyte leakage

To compare the differences in plant cell permeability between WT and *ZmDUF1644* transgenic *Arabidopsis thaliana* under salt stress, the relative electrolyte leakage (REL) of roots and shoots was also measured. Compared with the control, the REL of *Arabidopsis thaliana* seedlings of both WT and transgenic line were displayed significantly increased under salt stress. The REL value of WT seedlings in leaves and roots increased by 45% and 50% respectively, while the REL of *ZmDUF1644* transgenic seedlings in leaves and roots increased by only15% and 30% respectively. Obviously, the increase of REL of transgenic seedlings was significantly lower ($p <$

0.05) than the WT both in roots and shoots (**Figure 4**).

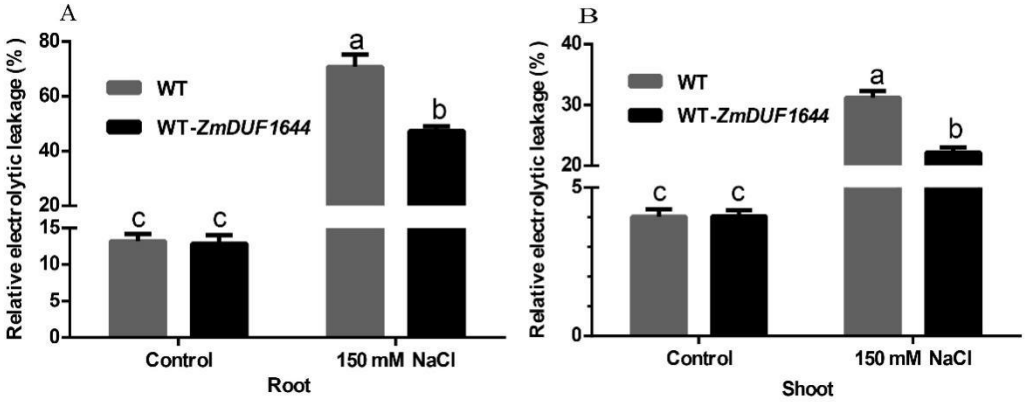

Figure 4. The REL value of WT and *ZmDUF1644* transgenic *Arabidopsis thaliana* seedlings in roots (A) and shoots (B) under control and salt stress. Each column represents the mean value (n = 3) ±SD. The different letters that indicate a significant difference at $p < 0.05$using Duncan's test after one-way ANOVA demonstrate statistical significance.

3.5. The alteration of Na^+ and K^+ content

To explore the role of the *ZmDUF1644* gene in alleviating the ion toxicity caused by salt stress (NaCl), we measured the Na^+ and K^+ content of both WT and *ZmDUF1644* transgenic *Arabidopsis thaliana* seedlings. Under 150 mM NaCl treatment, the Na^+ content was increased sharply and K^+ content was decreased significantly in both seedlings. The Na^+ content accumulated in the roots and shoots of the *ZmDUF1644* transgenic seedlings was all lower than that of the WT, which was 16.03% (**Figure 5B**) and 18.28% (**Figure 5A**) lower than the WT in the roots and shoots, respectively. In contrast, the variation of K^+ content was exactly the opposite of Na^+ content. Under salt stress, the K^+ content of the *ZmDUF1644* transgenic seedlings was much higher than that of the WT. And it was 176.53% (**Figure 5D**) and 40.00% (**Figure 5C**) higher than the WT in the roots and shoots, respectively. When the Na^+/K^+ ratio of both was further compared, the result showed that the trend of the ratio was similar to that of the Na^+ content, which was much lower than the WT in the roots (**Figure 5F**) and shoots (**Figure 5E**), respectively.

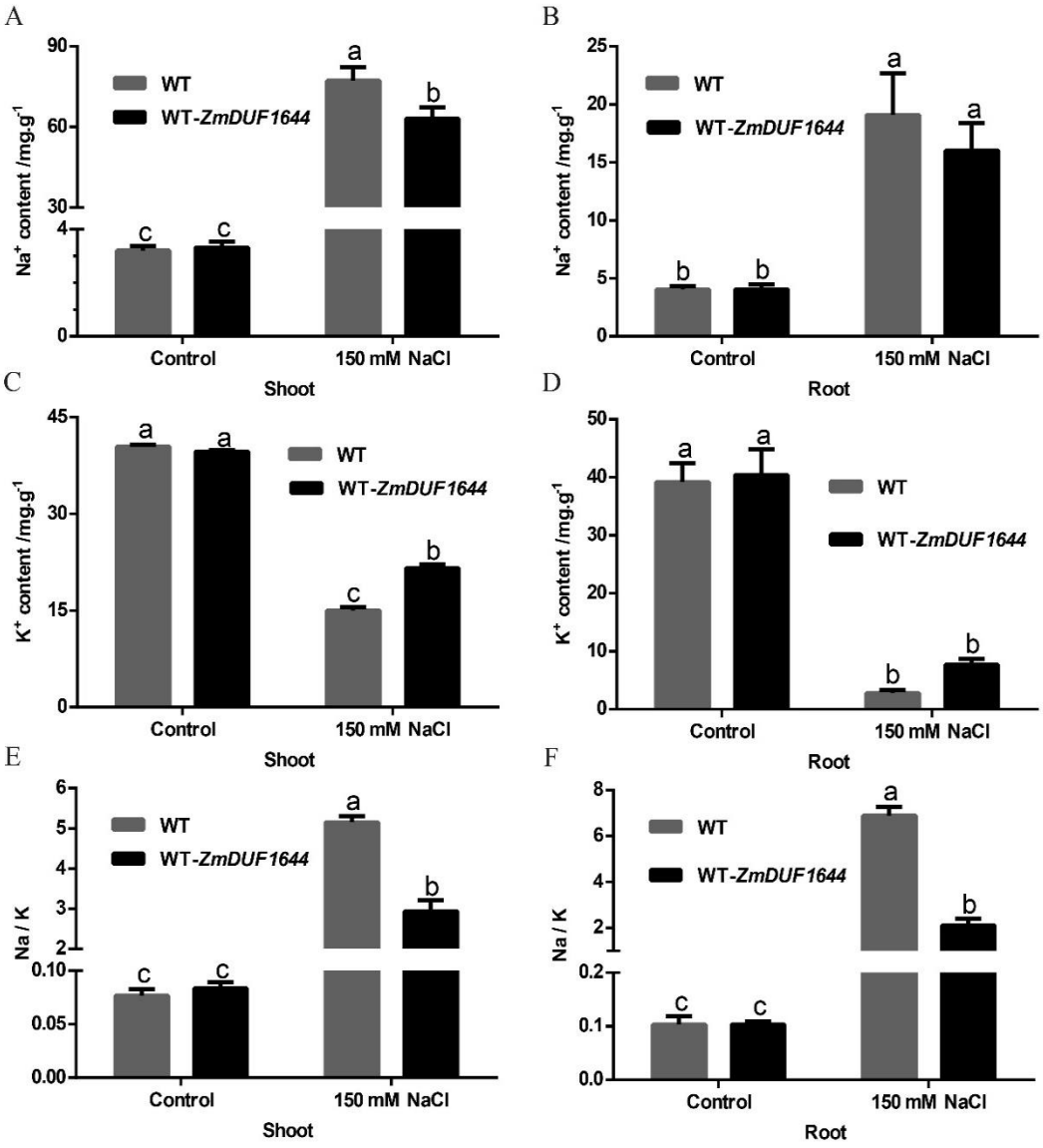

Figure 5. The Na^+(A, B), K^+(C, D) content and Na^+/K^+ ratio (E, F) of WT and *ZmDUF1644* transgenic *Arabidopsis thaliana* seedlings in roots and shoots under control and salt stress. Each column represents the mean value (n = 3) ±SD. The different letters that indicate a significant difference at $p < 0.05$using Duncan's test after one-way ANOVA demonstrate statistical significance.

4. Discussion

The DUF1644 family is a highly conserved and functionally unknown protein family belonging to a plant-specific DUF protein family which is mainly involved in response to salt stress in a non-halophytic herb, rice (Li et al. 2016; Zhang et al. 2021). However, the role of the *DUF1644* family gene in the halophytic herb is still not clear. In our study, to explore the functions performed by *DUF1644* family gene in the halophytic herb, we have screened a *DUF1644* gene from halophyte *Zoysia matrella*

Z123 species, overexpressed it in *Arabidopsis thaliana*，and measured the physiological and biochemical indicators.

Gene *ZmDUF1644* belongs to Group II of the *DUF1644* gene family and was shown to be clustered together with the gene *OsSDIP361* (**Figure 1A**) which could increase salt tolerance in rice positively (Li et al. 2016). It implies that the gene *ZmDUF1644* probably performs a similar function to the *OsSDIP361* gene. Under salt stress, overexpression of the *ZmDUF1644* gene significantly improved the maximum root length (**Figure 1B**) and fresh weight (**Figure 1C**) of *Arabidopsis thaliana.* This finding was similarly to that of Li who found that the gene *OsSIDP361* improved salt tolerance and increased shoot length and fresh weight in transgenic rice (Li et al. 2016). It is common knowledge that photosynthesis plays a vital role in plant growth (Yan et al. 2022) (Zhang et al. 2022) (Bullaín Galardis et al. 2022). Under salt stress, plant chlorophyll synthesis is inhibited and photosynthesis decreased (Wahid et al. 2022) (Naseer et al. 2022). Hence, chlorophyll content was often used as an indicator to evaluate plant salt tolerance in plant salt stress research (Wang et al. 2019). Our study found that overexpression of the *ZmDUF1644* gene alleviated the damage to photosynthetic pigments in leaves of *Arabidopsis thaliana* seedlings under salt stress (**Figure 3**). And, the contents of chlorophyll a (**Figure 3A**), chlorophyll b (**Figure 3B**), chlorophyll (a+b) (**Figure 3C**) and carotenoids (**Figure 3D**) were significantly higher in *ZmDUF1644* transgenic seedlings than in WT. This also suggested that overexpression of the *ZmDUF1644* gene in *Arabidopsis thaliana* could improve the salt tolerance of seedlings.

When plants suffered damage from stress, cell membranes were disrupted to different degrees, membrane permeability increased, selective permeability was lost, and some intracellular electrolytes leaked out (Feng et al. 2021; Maiti and Daschakraborty 2021; Sun et al. 2020). Therefore, the change in electrolyte leakage rate was closely related to the degree of plant injury under stress and was an important parameter to examine the cell membrane permeability of plants after suffering stress (Hu et al. 2021; Taha and El-Samad 2022). Furthermore, the increase in electrolyte leakage rate was frequently lower in plants with stronger stress tolerance and higher in species with weaker stress tolerance (Salehi et al. 2018; Sun et al. 2020b). To evaluate

the impact of the *ZmDUF1644* gene on plant salt tolerance, we determined the relative electrolyte leakage of WT and *ZmDUF1644* transgenic seedlings under salt stress (**Figure 4**). Our results clearly showed that overexpression of the *ZmDUF1644* gene could dramatically decrease the relative electrolyte leakage both in the roots (**Figure 4A**) and shoots (**Figure 4B**) of *Arabidopsis thaliana* seedlings under salt stress. Meanwhile, it also suggested that *ZmDUF1644* gene performs similar functions in halophytic and non-halophytic.

Salt stress disturbed the intracellular ion homeostasis and decreased the metabolic activity of plants caused by ionic stress (Zhang et al. 2021a; Zhang et al. 2021b). In addition, excessive Na^+/K^+ also caused plant cellular dehydration, membrane dysfunction, and ion toxicity (Gupta et al. 2021; Zhang et al. 2021c). To alleviate ion stress, plants mainly reduce salt ion concentration in the cytoplasm through reduced uptake (Sofy et al. 2020), increased exclusion (Wei et al. 2022), or compartmentalized salt ions in the vesicles (Lang et al. 2017). Consequently, the salt tolerance of plants is associated with the ability to exclude sodium ions from excess uptake and maintain low sodium to potassium ratio (Xie et al. 2021). Under salt stress, excess toxic ions disrupted the ionic balance in *Arabidopsis thaliana seedlings* which resulted in Na^+ accumulating excessively in the seedlings and K^+ decreasing significantly, eventually leading to a significant increased Na^+/K^+. When the *ZmDUF1644* gene was introduced into the *Arabidopsis thaliana* seedlings, the excess Na^+ accumulated inside was reduced (**Figure 5A, B**), while K^+ was relatively increased (**Figure 5C, D**), which allowed the *ZmDUF1644* transgenic seedlings to maintain a relatively lower Na^+/K^+ than the WT seedlings (**Figure E, F**). Our research further revealed that the *ZmDUF1644* gene alleviated salt injury through regulating ion homeostasis, which enabled enhanced salt tolerance of the transgenic seedlings.

In conclusion, we firstly reported a DUF6144 family gene from halophyte *Zoysia matrella* Z123 species, and found that it could significantly improve the salt tolerance of the *ZmDUF1644* transgenic *Arabidopsis thaliana*, which is rather similarly to the function performed by the *DUF1644* gene of non-halophyte plants. These initial results further demonstrated that the *DUF1644* gene family played an important role in response to plant salt stress. However, the molecular mechanism of salt tolerance in halophyte plant species still needs further intensive study, although a similar function was exhibited with the *DUF1644* family genes in non- halophyte plants.

Statistical analysis

SPSS 22.0 was used to analyze the data, which were represented as mean±SD for each treatment (n=3 or 10). The different letters that indicate a significant difference at $p < 0.05$ using Duncan's test after one-way ANOVA demonstrate statistical significance. Data from various transgenic lines were compared under control and NaCl treatment conditions.

Conflicts of Interes

The authors declare no conflicts of interest.

Authors' Contributions

Peipei Wei and Guosi Li: Writing- Original draft preparation and Conceptualization. Qihui Yin: Methodology. Yuting Chen: Software. Xiaoxue Li and Yuting Chen: Data curation. Xuelei Chen:Investigation. Fucheng Zhu and Hui Deng: Writing- Reviewing and Editing.

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Supplementary materials

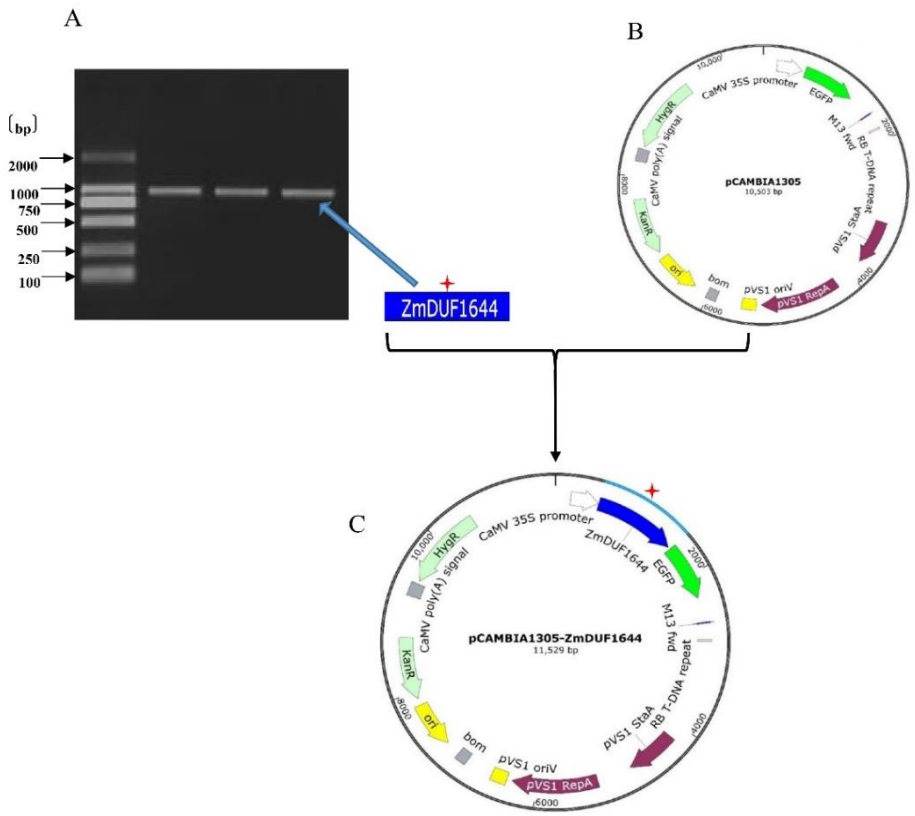

Figure S1. Construction of plant overexpression vectors

(A)PCR-cloned *ZmDUF1644* gene from the halophyte *Zoysia matrella* Z123 species; (B)The empty vector of pCAMBIA1305; (C) Recombinant vector of pCAMBIA1305-*ZmDUF1644*

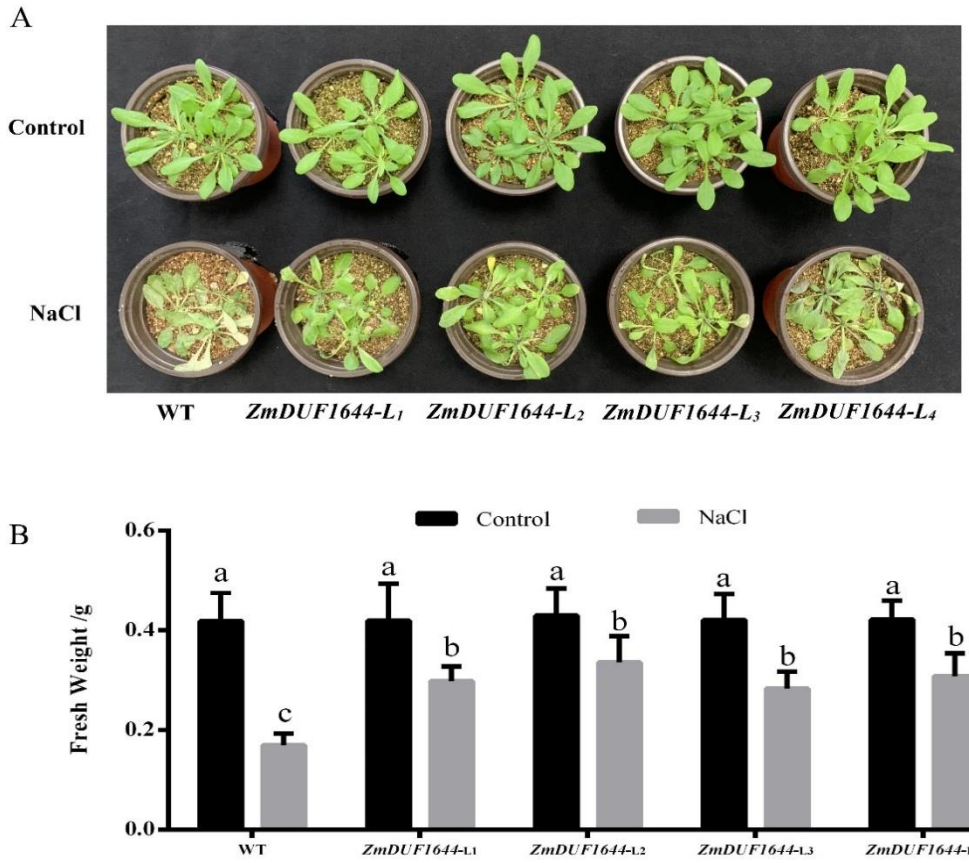

Figure S2. The growth (A)and fresh weight (B) of WT and ZmDUF1644 transgenic lines under control and salt stress. Each column represents the mean value (n = 10) ±SD. Statistical significance is shown in the different letters that indicate a significant difference at $p < 0.05$ using Duncan's test after one-way ANOVA.

Table S1 Primers used in the study

Program	Forward	Reverse
ZmDUF1644 for gene cloning	5'-ATGCCAAAGGACAGGAGCTC-3'	5'-TCATTGTGCAGGGTCGCC-3'
ZmDUF1644 for overexpression	5'AGGACAGGGTACCCGGGGATCCA TGCCAAAGGACAGGAGCTC-3'	5'TGGTACTAGTGTCGACTCTAGA TTGTGCAGGGTCGCCGTC-3'

Table S2 Formulas for calculating photosynthetic pigment content

Pigment content (mg/g-1 FW)	Calculation formula
Chlorophyll a	(12.71×A663–2.59×A644) V/ l000W
Chlorophyll b	(22.58×A644–4.67×A663) V/ l000W
Chlorophyll (a+b)	(5.03×A663 +20.29×A644) V/ l000W
Carotenoids	(4.37×A470+l.94×A663-10.35×A644) V/ l000W

A663, A644 and A470 represented the absorbance values at 663, 644 and 470 nm, respectively.

V and W represent the volume of the extraction solution and the mass of the test sample.

Table S3. DUF1644 protein sequences used in the study

>ZmDUF1644

MPKDRSSHGASSESRWSRGSPYIVSRGGSSTSRRSKESSAATATANLSPNRRSRGSHRSEESSAAAAVAAVK
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SECQQPVNLSCPLCRGPVSHWIKDYNARRHMDCKVRSCTMESCEFRGVYSVLRKHAKEEHPLVKPMEV
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