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Molecular Cloning and Functional Analysis of *MsDUF* from Alfalfa (*Medicago sativa* L.)

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ABSTRACT

Background: As a high-quality legume forage, *Medicago sativa* is restricted by various abiotic stresses during its growth and development. Proteins with domains of unknown function (DUF) are reportedly involved in abiotic stress tolerance in plants. However, the biological functions of DUF-containing proteins in alfalfa (*Medicago sativa* L.) related to environmental stress remain poorly understood.

Methods: We reported previously a novel stress-responsive gene, *MsDUF*, from alfalfa that was up-regulated under drought stress. In this study, MsDUF, a 210 amino acid protein containing a DUF4228 domain, was identified in alfalfa. Furthermore, *MsDUF*-overexpressing tobacco plants were constructed to explore the functions of MsDUF in plant resistance to abiotic stress.

Result: Compared with wild-type tobacco, relative water content, osmoregulator (proline and soluble sugars) content and superoxide dismutase activity were elevated and malondialdehyde content was decreased in leaves of *MsDUF*-overexpressing plants under drought and salt stress (p<0.05). The results indicate that MsDUF plays a vital role in responses to drought and salt stress in tobacco. Thus, this protein could be used to improve drought and salt resistance of tobacco and other crops.

Key words: Drought stress, DUF protein, MsDUF, Salt stress, Stress tolerance, Tobacco.

INTRODUCTION

Domains of unknown function (DUF) protein families in the Pfam database (Jaina *et al*., 2020) (http://pfam.xfam.org/family) mostly comprise highly conserved DUFs that likely perform key biological functions. The functions of DUF proteins have been revealed in many plant biological processes, including plant responses to abiotic stress (Gu and Cheng 2014; Luo *et al*., 2014) . Some DUF-containing proteins reportedly enhance abiotic stress tolerance in plants. In particular, genes encoding DUF domains play an essential role in plant resistance to drought and salt stress (Zhou *et al*., 2020).

Expression levels of *OsDUF829.2* and *OsDUF829.4* genes in rice (*Oryza sativa*) are markedly increased under salt stress, while salt stress resistance in *Escherichia coli* overexpressing the two DUF-encoding genes is markedly improved (Li *et al*., 2018). In addition, expression of *OsDUF810.7* in rice is upregulated under stress conditions, such as drought, salt, cold, heat and abscisic acid (ABA) treatments. The activities of catalase and peroxidase are enhanced in *OsDUF810.7*-overexpressing *E. coli* cells, which in turn increases resistance to drought and salt stress (Li *et al*., 2018). Furthermore, overexpression of *TaSRHP*, a gene encoding the DUF581 domain in wheat (*Triticum aestivum* L.), improves the resistance of transgenic *Arabidopsis* plants to drought and salt stress(Hou *et al*., 2013). The transcription factor LcFIN1 in Chinese wild rye (*Leymus chinensis*), which contains a DUF761 domain, has a positive regulatory effect on plant cold stress, thereby increasing the survival rate and fresh weight of *LcFIN1*-overexpressing (OE) plants (Gao *et al*., 2016).

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DUF genes also play a crucial regulatory role in plant growth and development, including cell wall development, plant growth, flowering and fruiting. A DUF640 protein affects the grain shape, size and quality of rice by altering the expression of *GW2*, a gene related to cell division and grain width (Yan *et al*., 2013). Moreover, overexpression of *AtDUF4* in *Arabidopsis* and rape (*Brassica napus* L.) affects the size of corresponding vegetative organs and seeds, possibly through its effect on the expression of cell wall and auxin transport genes that regulates cell size (Chen *et al*., 2018). In addition, DUF genes act in plant defences against pests and diseases. For example, the *Arabidopsis IRN1* gene, which has the DUF581 domain, may be related to resistance to aphids. Overexpression or deletion of this gene in *Arabidopsis* enhances or decreases plant resistance to aphids, respectively (Chen *et al*., 2013).

Alfalfa (*Medicago sativa* L.) is a widely-grown forage legume crop that has evolved a high stress tolerance capacity. It can survive long-term drought stress without any damage to its regrowth process and endures 50 mM NaCl salt stress without yield loss (Hamidi and Safarnejad, 2010). However, its stress tolerance, biomass production in alfalfa is frequently reduced by environmental stresses. Therefore, improving tolerance to adverse environments in alfalfa is critical to minimise yield reduction caused by abiotic stress. Previously, we identified a *DUF* gene in alfalfa *(MsDUF)*, whose expression is upregulated under abiotic stress (Han *et al*., 2013). Overexpression of *MsDUF*in transgenic tobacco (*Nicotiana tabacum* L.) reduces seed vigor and germination percentage under normal conditions or osmotic stress (Wang *et al*., 2018). However, there is still no report on the molecular mechanisms involving DUF genes that operate in alfalfa in response to drought stress, although the physiological mechanisms contributing to drought tolerance in alfalfa have been reported (Castroluna *et al*., 2014).

Therefore, this study explored the possible role of alfalfa *MsDUF* gene in plants under drought and salt stress. The results could broaden our understanding of the functions of DUF-containing proteins in *M. sativa* and provide a framework for further application of these DUF proteins in improving abiotic stress resistance in other crops.

MATERIALS AND METHODS

Plants and growth conditions

Baoding alfalfa (*Medicago sativa* L. cv. Baoding) seeds were purchased from the Chinese Academy of Agricultural Sciences (Beijing, China). Tobacco (*Nicotiana tabacum* L. cv. 89) seeds were purchased from the Tobacco Research Institute of Chinese Academy Agricultural Sciences (Qingdao, Shandong Province, China). All plants were cultured on MS basal medium (Murashige and Skoog, 1962) for 3 weeks at 25±2°C.

Bioinformatics analysis

A homolog analysis of DUFs was conducted by comparing the amino acid sequences of homologs from *M. sativa* (AFP87383.1), *Medicago truncatula* (XP_013456859.1), *Trifolium pratense* (PNX71568.1), *Glycine max* (XP_003555905.1), *Nicotiana tabacum* (XP_016496165.1), *Arachis ipaensis* (XP_016196584.1), *Juglans regia* (XP_018843726.1), *Cucurbita maxima* (XP_022986741.1), *Ipomoea nil* (XP_019160474.1) and *Ziziphus jujuba* (XP_015882404.1).

All sequences were obtained from the National Centre for Biotechnology Information database (NCBI; http://www.ncbi.nlm.nih.gov) and aligned with DNAMAN version 8.0 (Lynnon Biosoft, Vaudreuil, QC, Canada). The domains of the 10 putative proteins were analysed using SMART software version V (http://smart.emblheidelberg.de/smart/set_mode.cgi? NORMAL=1). The phylogenetic relationship of DUF homologs from these species was analyzed using Molecular Evolutionary Genetic Analysis (MEGA) v7.0.26 (http://www.megasoftware.net/).

Preparation of MsDUF-overexpressing tobacco plants

To engineer *MsDUF*-overexpressing tobacco plants, the overexpression vector *MsDUF*-OE was constructed and transferred into tobacco by *Agrobacterium tumefaciens*-mediated transformation (Krenek *et al*., 2015). Specifically, recombinant plasmid PMD-MsDUF and expression vector pCAMBIA1301 were digested with *Nhe*I and *Bgl*II (Takara). The digested products were recovered, ligated, then transformed into competent *A. tumefaciens* cells. Next, *MsDUF*-containing cells were transformed into tobacco and the resultant transformants were selected on MS medium containing 50 mg/L kanamycin and 200 mg/L cefalotin over ~20 days. DNA and RNA from transformants were respectively extracted using cetyltrimethylammonium bromide (Springer, 2010) and TRIzol (Invitrogen) reagents, then used to verify the correctness of transformants by PCR and RT-PCR. Primers used for PCR amplification of *Hyg* (hygromycin resistance gene) and RT-PCR amplification of *MsDUF* are listed in Table 1.

Physicochemical measurements

Tobacco leaves of wild-type (WT) plants and two transgenic lines (*MsDUF*-OE#2 and *MsDUF*-OE#8) were collected, weighed (fresh weight, W_f, g) and divided into two equal parts. One half of leaf samples was placed into a 105°C oven to dry then weighed (dry weight, W_d, g). The other half was placed in a vacuum dryer for 1 h to absorb surface moisture and immediately weighed (W_t). The relative water content (RWC) was calculated as follows:

$$RWC\ (\%) = \frac{(W_f - W_d)}{(W_t - W_d)} \times 100$$

Table 1: Primers used for PCR in this study.

Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (5'–3')
MsDUF (*Medicago sativa* DUF gene)	F1: CTAATGGGGAACACTTTTGG F2: GCGTTTGTATAAGGCCAGTGGCAA	AAATCTCCTCAGTTTGGTGAG TTCTGTCGTGGTGTTCTCGGAGTT
GFP (green fluorescent protein gene)	GGCTCTAGACTAATGGGGAACACTTTTG	GTTCGCGGGTACCTGAAGCCACAGCTATTTGAATCC
Hyg (hygromycin resistance gene)	CGATTCCGGAAGTGCTTGAC	CGTCTGCTGCTCCATACAAG
actin (reference gene)	TTTGAGACTTTCAATGTGCCCGCC	TAGCATGTGGGAGTGCATAACCCT

The proline content in leaf samples was determined using the method of Batesÿ Bates, 1973 ÿ and calculated as follows: Proline content

$$\text{mass fraction} = \frac{(c \times V_t)}{(W \times V_s \times 10^6)}$$

Where:

c = Mass of proline (µg) calculated from the standard curve.

V_t = Total volume of the sample extracted (mL).

W = Dry weight of the sample (g).

V_s = Volume of crude enzyme solution taken during the assay (mL).

Superoxide dismutase (SOD) activity was determined using the nitroblue tetrazolium method (Giannopolitis and Ries 1997; Wang *et al*., 2009) based on the following formula:

$$\text{SOD activity } (U \cdot g^{-1}FW \cdot h^{-1}) = \frac{[(A_0 - A_s) \times V_t \times 60]}{(A_0 \times 0.5 \times FW \times V_s \times t)}$$

Where:

A_0 = Absorbance at 560 nm of the control tube under light.

A_s =Absorbance at 560 nm of the sample tube.

V_t = Total volume of the sample extracted (mL).

V_s = Volume of crude enzyme solution taken during the assay (mL).

t = Light duration of the colour reaction (min).

FW= Sample fresh weight (g).

Malondialdehyde (MDA) content as determined using the thiobarbituric acid method (Puckette *et al*., 2007) and calculated as follows:

$$C\ (\mu M) = 6.45 \times OD_{532} - 0.56 \times OD_{450}$$

Where:

OD_{532} and OD_{450} = Optical densities of samples at 532 and 450 nm, respectively.

Further, soluble sugar content was obtained as follows:

$$C\ (mM) = 11.71\ OD_{450}$$

Stress assay

To explore the role of the *MsDUF* gene in response to drought and salt stress in tobacco, WT, OE#2 and OE#8 plants were cultivated in seedling bowls(plastic pots: 7 by 31 cm). Plants that grew consistently for 30 days were subjected to drought (air drought) and salt stress (200 mM NaCl added to daily water). Samples were collected on days 1, 4, 7, 14, 20 and 23 of treatments to measure RWC, osmotic-pressure-regulating substances (OPRS; proline and soluble sugar), SOD activity and MDA content as described above.

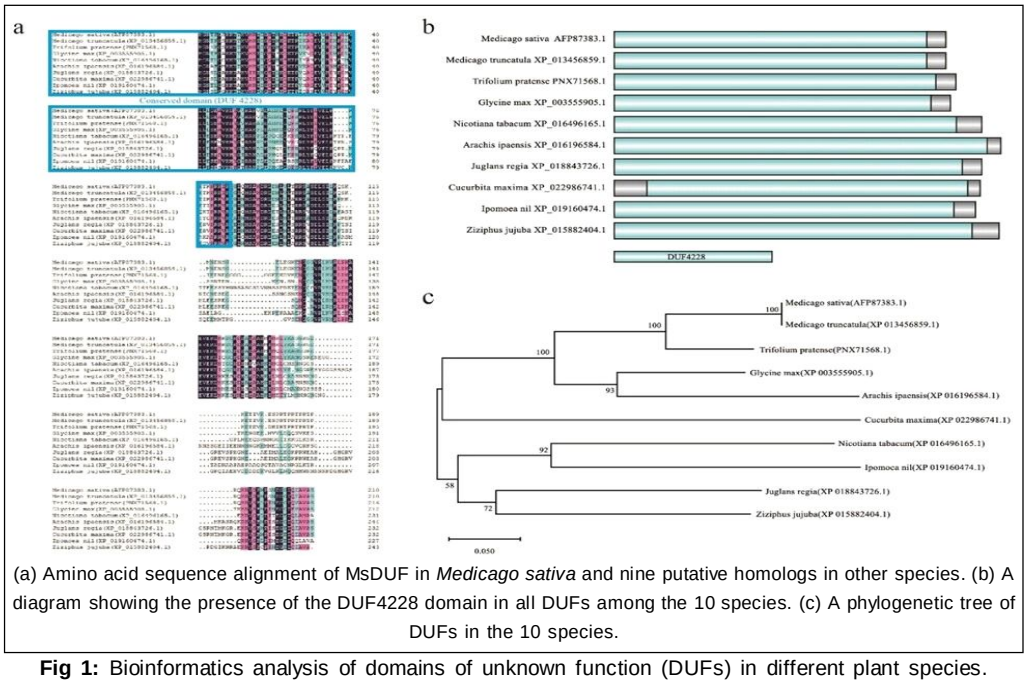
Statistical analysis

Results are presented as means ± standard deviation. Significant differences were determined by one-way analysis of variance using GraphPad Prism v5 (Graph Pad Software, Inc., San Diego, CA, USA) and statistical significance was assumed at $P<0.05$. All experiments in this study were repeated at least three times.

RESULTS AND DISCUSSION

Sequence characteristics of MsDUF inalfalf

MsDUF was predicted to be a DUF protein with 210 amino acids. Sequences of *MsDUF* and its homologs in another nine species (*M. truncatula, T. pratense, G. max, N. tabacum, A. ipaensis, J. regia, C. maxima, I. nil* and *Z. jujuba)* were aligned and DUFs in *M. sativa* and *M. truncatula* share the



(a) Amino acid sequence alignment of MsDUF in *Medicago sativa* and nine putative homologs in other species. (b) A diagram showing the presence of the DUF4228 domain in all DUFs among the 10 species. (c) A phylogenetic tree of DUFs in the 10 species.

Fig 1: Bioinformatics analysis of domains of unknown function (DUFs) in different plant species.

highest similarity (100%), while the lowest similarity (53%) was observed between DUFs in *M. sativa* and *Z. jujuba* (Fig 1a). The domains in DUFs were then analysed and the highly conserved DUF4228 domain was found in all 10 species (Fig 1b). A phylogenetic tree was constructed based on comparing the MsDUF sequence with amino acid sequences of homologs in other species and MsDUF is most closely related (98%) to the DUF from *M. truncatula* (Fig 1c).

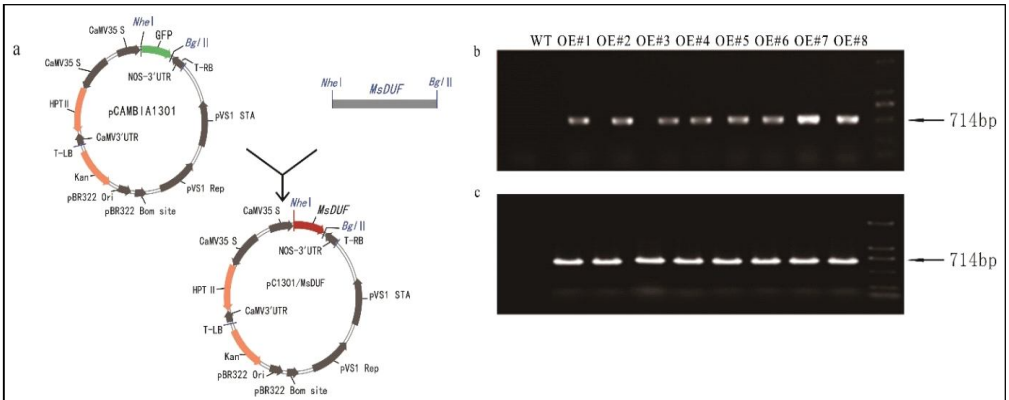
DUF proteins span many families containing DUFs with conserved amino acid sequences and unknown functions (Bateman *et al*., 2010). These proteins have been linked to stress tolerance in plants (Zhou *et al*., 2020). Herein, we characterized a *DUF4228* gene in *M. sativa*, designated *MsDUF* (JX183734). Recently, a DUF protein encoded by the *ATDUF4228* gene, which contains a DUF4228 domain, was found to play a role in response to abiotic stress (osmotic, cold and salt) in *Arabidopsis* (Yang *et al*., 2020). In the present study, we explored the classification, functions and evolution of *MsDUF*, which broadens our knowledge of DUF genes in plants.

Construction and verification of tobacco transformants overexpressing the MsDUF gene

The overexpression vector MsDUF-OE was constructed (Fig 2a) and transferred into tobacco. Candidate OE transformants were tested by PCR and RT-PCR(reverse-transcription PCR) (Fig 2b). Diagnostic PCR revealed that a 521 bp fragment of the pCAMBIA1301-MsDUF vector was amplified from OE plants only. After cDNA was prepared from total RNA, a 714 bp MsDUF gene was detected by RT-PCR analysis in OE plants, but not in WT plants (Fig 2c). These results confirmed that OE tobacco plants were constructed successfully.

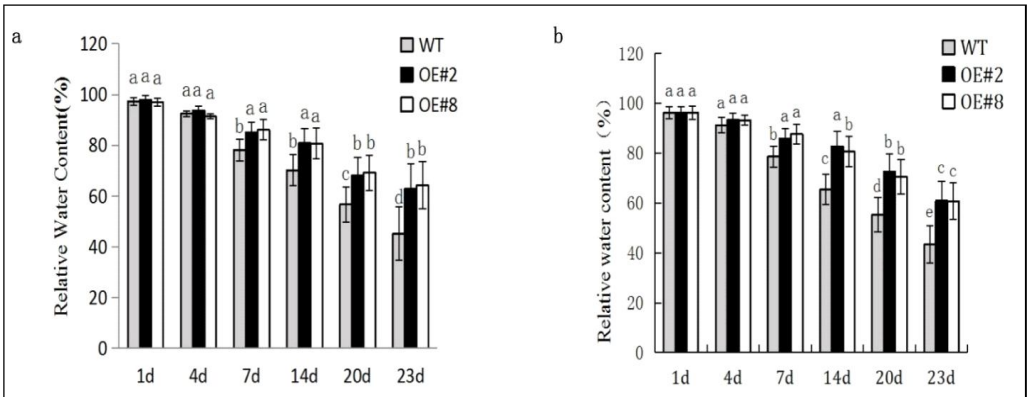
MsDUF plays an essential role in maintaining leaf water content

After drought stress (air drought) and salt stress (hydroponics in 200 mM NaCl), tobacco leaves (WT and OE) were collected on days 1, 4, 7, 14, 20 and 23 of the experiment to determine RWC. With the extension of drought and salt stress duration, RWCs of all plants gradually decreased (Fig 3). Moreover, RWCs of OE plants were



(a) The con-struction strategy. (b) PCR verification. (c) Reverse-transcription PCR verification.

Fig 2: Construction of MsDUF overexpression vector (MsDUF-OE) in tobacco.



(a) Relative water content of leaves treated with drought stress (air drought). (b) Relative water content of leaves treated with salt stress (200 mM NaCl). WT, wild-type plants; OE, MsDUF-overexpressing plants. Values are means ± standard deviation (n = 6). Different lowercase letters above the error bars represent significant differences between the treatment groups at $P<0.05$.Same as below.

Fig 3: The role of MsDUF in maintaining relative water content in tobacco.

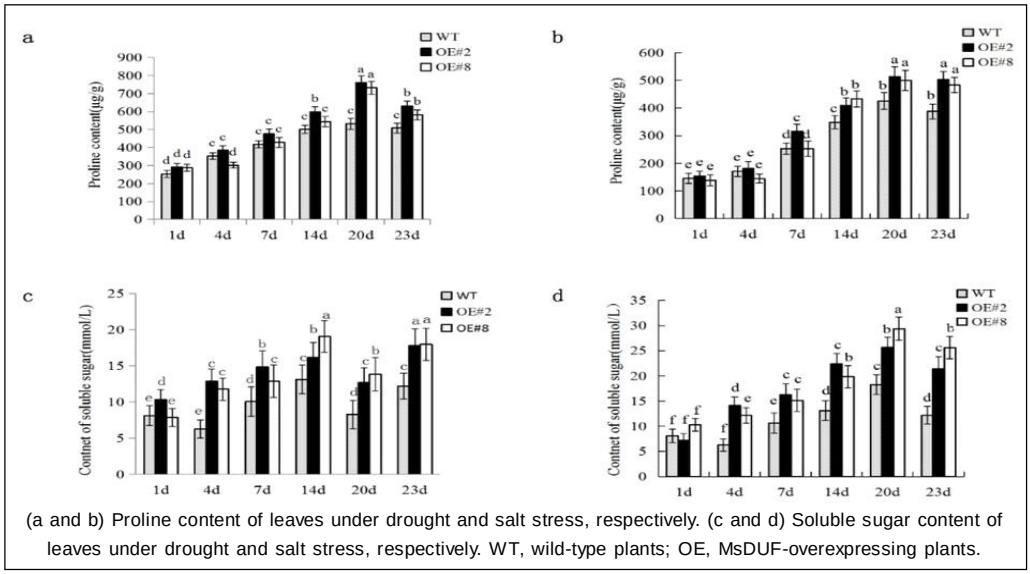
significantly higher than those of WT plants at different timepoints, except day 1 and 4 ($p<0.05$). For example, under drought stress, RWCs of OE#2 and OE#8 lines were 1.3- and 1.2-fold higher than those of WT plants on day 14, respectively. On day 23, RWCs of OE#2 and OE#8 were 2.4- and 2-fold those of WT plants.

RWC directly indicates the water retention capacity and hence drought resistance of plants. Plant leaves with a higher RWC have superior osmotic regulation and stronger drought resistance (Zegaoui *et al*., 2017). This indicates that MsDUF plays a positive role in slowing down the rate of water loss in tobacco leaves under drought and salt stress.

MsDUF is involved in adjusting osmoregulator levels under environmental stress

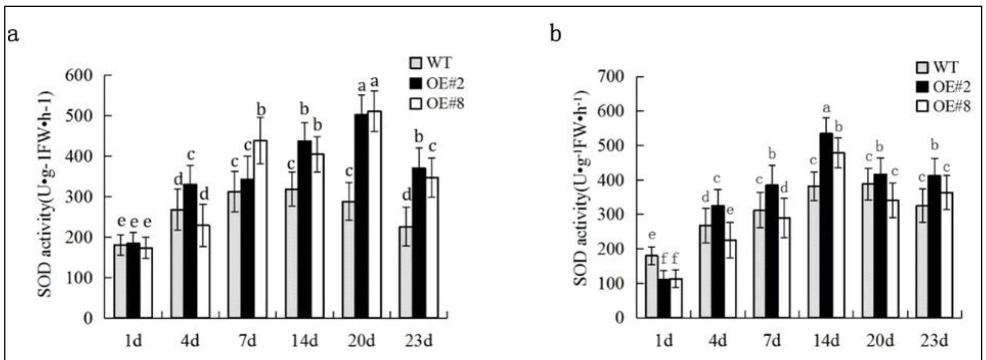
Levels of proline and soluble sugars in tobacco leaves (WT and OE) increased with the extension of drought stress duration and reached a maximum on days 20 and 14, respectively. In particular, proline and soluble sugar levels of OE plants were 1.4 and 1.6-fold higher than those of WT plants ($P<0.05$) on day 20(Figure 4a and c). In addition, the contents of both osmotic pressure substances increased with the extension of salt stress duration and reached a maximum on day 20. Proline and soluble sugar levels in OE plants were significantly higher than those of WT plants on days 20 and 23($P<0.05$). For example, the soluble sugar contents of OE plants were 1.6- and 1.9-fold higher than those of WT plants on days 7 and 23, respectively (Figure 4b and d).

Osmotic regulation is the most effective protective strategy for plants when facing drought and salt stress, which can increase cytosol concentration, reduce osmotic potential, maintain turgor and ease dehydration stress, all of which are beneficial to maintaining the water content and physiological processes of cells (Zegaoui *et al*., 2017; Hongyu Xu *et al*., 2022). Proline and soluble sugars are important OPRS components that accumulate in large amount in plants upon exposure to drought, salt and other adverse conditions (Yooyongwech *et al*., 2017). Proline helps to maintain water



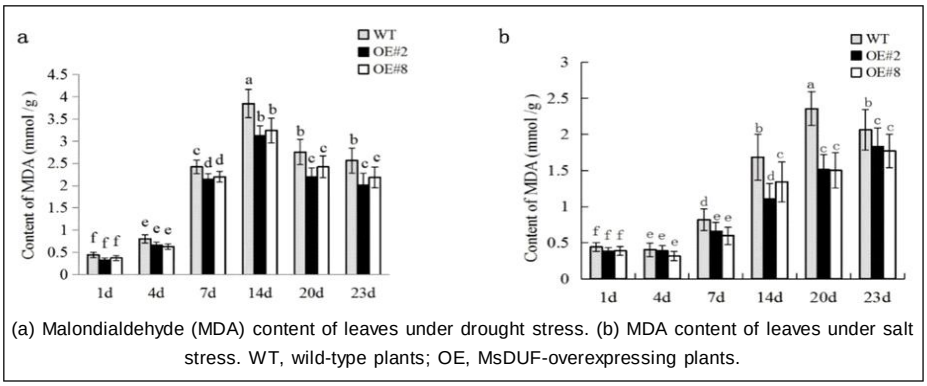
(a and b) Proline content of leaves under drought and salt stress, respectively. (c and d) Soluble sugar content of leaves under drought and salt stress, respectively. WT, wild-type plants; OE, MsDUF-overexpressing plants.

Fig 4: The involvement of MsDUF in regulating the content of osmotic-pressure-regulating substances in tobacco.



(a) SOD activity of leaves under drought stress. (b) SOD activity of leaves under salt stress. WT, wild-type plants; OE, MsDUF-overexpressing plants.

Fig 5: The contribution of MsDUF to enhancement of superoxide dismutase (SOD) activity in tobacco.



(a) Malondialdehyde (MDA) content of leaves under drought stress. (b) MDA content of leaves under salt stress. WT, wild-type plants; OE, MsDUF-overexpressing plants.

Fig 6: The role of MsDUF in protecting cell membrane stability in tobacco.

content in cells or tissues and it can also serve as a source of carbohydrates, enzymes and cell structure protection agents when plants are exposed to environmental stress (*e.g.*, drought and high salinity) (Hayat *et al*., 2012). In addition, soluble sugars can effectively reduce the osmotic potential of plants, maintain turgor pressure and create conditions for plants to maintain normal life activities under drought conditions (Yang *et al*., 2008; Jing Li *et al*.,2017). Herein, we found that both proline and soluble sugar levels in tobacco leaves increased first with the extension of drought and salt stress duration, then decreased moderately thereafter. OPRS levels were much higher in *MsDUF*-OE plants than in WT tobacco, suggesting that MsDUF improves the stress resistance of tobacco plants by elevating OPRS levels.

MsDUF contributes to enhancement of antioxidant enzyme activity

On the first day of stress treatment, SOD activity in tobacco leaves of WT and OE plants was similar (Fig 5). However, as drought and salt treatment durations were prolonged, SOD activity of plants tended to increase first then decrease and SOD activity of OE plants on day 23 was still significantly higher than the original level. In addition, SOD activity of OE plants was significantly higher than that of WT plants ($P<0.05$). For example, under drought stress, SOD activity of OE#2 and OE#8 plants was 1.7- and 1.8-fold higher than that of WT plants on day 20, respectively.

SOD is the main enzyme in the antioxidative defence system that protects against membrane lipid peroxidation. When external stress causes the production of reactive oxygen species (ROS) in plants, SOD can effectively remove free radicals (Sharma *et al*., 2012). High SOD activity is the physiological basis by which plants resist adversity and stress (Tian *et al*., 2023). Our results showed that the SOD activity of *MsDUF*-OE plants was markedly higher than that of WR tobacco. This suggests that MsDUF has a strong ability to remove ROS and thereby improve the antioxidative capacity of plants.

MsDUF enhances cell membrane stability by lowing malondialdehyde level

The change in MDA content is shown in Fig 6. With prolonged stress duration, the content of MDA tended to increase, but levels began to decrease slowly on day 20. The MDA content of WT plants was significantly higher than that of OE plants ($P<0.05$).

CONCLUSION

The study reports for the first time the functions of a DUF gene in M. sativa. MsDUF encodes a protein localized in the cytoplasm. MsDUF overexpression enhances plant resistance to drought and salt stress in tobacco by increasing RWC and OPRS (proline and soluble sugars) levels and SOD activity and decreasing MDA content. The findings provide a reference for genetic improvement of tobacco and other crops against environmental stress.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessairily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handing techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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