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ORIGINAL ARTICLE

Overexpression of an alfalfa (*Medicago sativa*) gene, *MsDUF*, negatively impacted seed germination and response to osmotic stress in transgenic tobacco

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Abstract

Many stress-responsive genes have been identified in alfalfa (*Medicago sativa* L.). The function of these genes, however, are mostly not understood. We reported previously a novel stress-responsive gene, *MsDUF*, from alfalfa that was up-regulated under drought stress. In the present study, we examined its function by overexpressing the gene in *Nicotiana tabacum*. We found that overexpression of *MsDUF* reduced seed vigor and germination percentage under normal conditions or osmotic stress. The reduced seed vigor and germination was associated with an increased ABA content in the overexpressor seeds. Further analysis revealed that overexpression of *MsDUF* resulted in up-regulation of transcript levels of ABA biosynthesis genes (*ZEP, NCED1* and *NCED6*) in the seeds. Compared with wild type, *MsDUF*-overexpression seedlings displayed significantly lower chlorophyll content and reduced soluble sugar content under normal conditions. MDA content was significantly higher in *MsDUF*-overexpressors compared to wild type under ABA treatment, while soluble sugar content and peroxidase activities were significantly lower in *MsDUF*-overexpressors. Our results suggest that *MsDUF* may act as a negative regulator in controlling seed vigor and responses to osmotic stress in plants.

Keywords *MsDUF* · Overexpression · ABA · Alfalfa · Osmotic stress · Germination

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Yafang Wang and Zhiqiang Zhang have contributed equally to this work.

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Abbreviations

ABA	Abscisic acid
GA	Gibberellic acid
GI	Germination index
GP	Germination percentage
SVI	Seed vigor index
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time PCR
DUF	Domains of unknown function
WT	Wild type
OS	Osmotic stress

Introduction

The domain of unknown function (DUF) families in the Pfam database (http://pfam.xfam.org/family) include many highly conserved DUFs presumably with important biological functions. There are about 3000 DUF families within the Pfam database, representing over 20% of known families (Lia et al. 2017). Recently, the functions of some DUF protein domains have been identified in different biological

processes in plants including growth and development (Ge et al. 2010; Sharma et al. 2012), defense response to diseases and insect pests (Chen et al. 2013; Kondou et al. 2013), and response to abiotic stresses (Gu and Cheng 2014; Luo et al. 2014).

Some DUF-containing proteins have been reported to enhance abiotic stress tolerance in plants. TaSRG is an unknown salt-induced gene containing a conserved DUF662 domain. The overexpression of TaSRG in *Arabidopsis* resulted in increased salt tolerance compared with wild-type plants (He et al. 2011). *RING-DUF 1117*, encoding E3 ubiquitin ligases, played important roles in plant tolerance to ABA-mediated drought stress (Kim et al. 2012). Overexpression of *OsDUF946.4* in *Escherichia coli* significantly improved the resistance to salt and drought stress (Li et al. 2017), and overexpression of *OsDUF866.1* could enhance cell viability and significantly improve the resistance to heat stress conditions (Lia et al. 2017). Another *DUF 1644* gene, *OsSIDP366*, functions as a regulator of the PBs/SGs and positively regulates salt and drought resistance in rice (Guo et al. 2016). However, some *DUF* genes negatively regulated plant response to abiotic stress. It was reported that overexpression of a stress-repressive gene *OsDSR2* encoding a protein with a *DUF966* domain increases the sensitivity to salt and simulated drought stress and reduces ABA sensitivity in rice (Luo et al. 2014).

As sessile organisms facing various environmental challenges, higher plants have adaptive robustness at molecular, cellular and physiological levels to survive environmental stress. Abscisic acid (ABA), a key plant stress-signaling hormone, is accumulated under many abiotic stress conditions (Yoshida et al. 2014), and regulates many key processes in growth and development (Hoffmann-Benning and Kende 1992), seed dormancy and germination (Siriwardana et al. 2014), and responses to environmental stresses involving loss of water (Cuevas et al. 2008; Huang et al. 2012). A number of ABA-responsive genes are normally expressed during late embryogenesis when seed tissues desiccate and the embryos become dormant (Finkelstein et al. 1985). It has been suggested that ABA inhibits water uptake by preventing cell wall loosening of the embryo during seed germination, thus reducing embryo growth potential (Xi et al. 2010). The action of ABA can target specifically guard cells for induction of stomatal closure but may also signal systemically for adjustment towards severe water shortage (Tuteja 2007). In maize leaves, water stress-induced ABA accumulation triggers the increased generation of ROS, which, in turn, leads to the up-regulation of the antioxidant defense system (Jiang and Zhang 2002).

Alfalfa (*Medicago sativa* L.), an important perennial forage crop with high nutritional content, is widely distributed in various environments worldwide. It fixes nitrogen through a symbiotic relationship with an N_2-fixing bacterium (*Sinorhizobium meliloti* L.), providing an extra source of nitrogen for plants and soils. Rotation of alfalfa with other crops in the field improves soil structure and increases soil organic matter (Bourgeois et al. 1990). Alfalfa has evolved to maintain a relatively strong stress-tolerant capacity. It can survive long term drought stress without any damage to its regrowth process (Hamidi and Safarnejad 2010) and endures 50 mM NaCl salt stress without yield loss (Castroluna et al. 2014). Despite its relatively strong stress tolerance, biomass production in alfalfa is frequently reduced by environmental stresses. Therefore, improving tolerance to adverse environment in alfalfa is critical to minimizing the reduction due to abiotic stress as well as improving soil fertility and enhancing its production on margin land.

In a previous study, we isolated a novel stress-responsive gene, *MsDUF* (GenBank accession No. JX183734) from alfalfa (cv Baoding). The gene was up-regulated under drought, salinity (NaCl), ABA, and GA_3 treatments (Han et al. 2013). The protein was localized in the cytoplasm based on subcellular localization study. To understand the function of *MsDUF* in osmotic stress response, we constructed a transgenic tobacco overexpressing *MsDUF* and examined the transgenic plants for seed germination. We found that *MsDUF*-overexpression in tobacco negatively impacted seed germination and response to osmotic stress in seedlings.

Materials and methods

Plant materials

Transgenic tobacco overexpressing MsDUF was generated, selected and confirmed by Han et al. (2013). Genomic DNA was isolated from T1 plants (selected and regenerated plants after transformation) and wild type (WT). We conducted polymerase chain reaction (PCR) with hygromycin (Hyg) primers (see Table S1 in Supplementary Material) and identified seven transgenic lines (including Lines 8 and 9) (see Fig. S1 in Supplementary material). RNA was isolated from young seedlings and reverse transcribed into cDNA. Synthesized cDNA samples were used to perform PCR with primeSTAR® HS DNA polymerase and DUF gene primers (see Table S1 in Supplementary Material). Lines 8 and 9 were the only two lines producing enough seeds for physiological characterization. RT-PCR confirmed the genes were expressed in the transgenic plants (714 bp, see Fig. S2 in Supplementary material). T1 plants were self-pollinated to produce T2 seeds. T2 seeds and seedlings were used for this study.

Sequence analysis of *MsDUF*

The deduced amino acid sequence of the alfalfa gene was used to blast search the NCBI sequence database (http://www.ncbi.nlm.nih.gov/BLASTP). The sequences with highest similarity (cutoff value of $e = 2 \times e^{-62}$) were retrieved and aligned with the alfalfa sequence. A phylogenetic tree was constructed using the neighbor-joining method (DNAMAN version 8.0, Lynnon Biosoft, Vaudreuil, QC, Canada).

Germination assays

Seeds of WT and transgenic lines were harvested at the same time from tobacco, and dried for 1 week before used for the germination assay. Seeds were surface sterilized with 70% ethanol, rinsed with distilled water, and air-dried in a sterile hood. Seeds were germinated in 1/2 MS medium (control) (Murashige and Skoog 1962) or 1/2 MS medium supplemented with 150 mM D-mannitol or 10 μM ABA in Petri dishes, at 24 °C under a 12-h photoperiod. Germination was scored daily with visible radicle protrusion (> 1 mm) as the defining criterion. Twelve days later, the root length of each plant was measured. Seed germination characteristics such as germination percentage (GP), germination index (GI) and seed vigor index (SVI) were determined. Three independent experiments were performed and each experiment is represented by 50 seeds.

$$\text{Germination percentage (GP)} = \frac{\text{Number of germinated seeds}}{\text{Number of tested seeds}} \times 100\%$$

$$\text{Germination index (GI)} = \sum \frac{G_i}{D_i}, \text{where Gi is the number}$$

of seeds germinated at the *i* day, Di is the corresponding day of germination.

Seed vigor index (SVI) = GI × R, where R is root length (cm).

Quantification of ABA and gibberellin (GA_3)

Extraction and purification of ABA, and GA_3 were carried out according to Park et al. (2008) and Zhang et al. (2016) with some modifications. In brief, about 50 mg tobacco seeds were frozen in liquid nitrogen and grounded to a powder with a Tissue Lyser (Qiagen). The powder was mixed with 2 mL 80% (v/v) methanol and incubated in dark at 4 °C overnight. The extract was centrifuged at 4000×*g* for 10 min at 4 °C, and the resulting pellet was re-extracted with another 1 mL 80% (v/v) methanol, as described above. The supernatants were combined and dried under a stream of nitrogen. The dried extract was re-dissolved in 0.8 mL methanol, then filtered through a 0.45 μm syringe filter. Quantification of hormones by LC–MS/MS was performed as described by Sasaki et al. (2015). Raw values for ABA and GA_3 levels were normalized by plant mass and extraction volume.

Quantitative RT-PCR (qRT-PCR) analysis of genes related to hormone synthesis

Total RNA was extracted from tobacco seeds and the first strand cDNA was synthesized with a PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instructions. qRT-PCR was performed with Roche FastStart Universal SYBR Green Master on the Roche 480 II Real-Time PCR Detection System (Roche Diagnostics), using tobacco *actin* gene (*NtActin*) as a reference gene. Three independent biological replicates and three technical replicates for each sample were used for the qRT-PCR. A melt curve was performed at the end of each reaction to verify PCR product specificity. The qRT-PCR gene expression was quantified from three technical replicates using the $2^{-\Delta\Delta CT}$ comparative methods and calibrated by amplification efficiency, where $\Delta\Delta CT = (CT, Target - CT, NtActin)_{transgenic\ line} - (CT, Target - CT, NtActin)_{WT}$. The primers used are listed in Table S1.

Physiological and biochemical analysis of *MsDUF*-overexpression tobacco in response to osmotic and ABA treatment

Twelve-day-old seedlings were used for chlorophyll content, lipid peroxidation and peroxidase (POD, EC, 1.11.1.7) activity measurement. For chlorophyll content determination, 0.2 g leaves were frozen in liquid nitrogen, ground with a mortar and pestle, and placed into 1.5 mL conical tubes. The sample was suspended in 90% acetone, vortexed for 10 s, incubated at 4 °C in the dark for 24 h, and centrifuged at 2700×*g* at room temperature for 5 min to collect the supernatant. Chlorophyll absorbance was measured at 645 and 665 nm using 90% acetone as a blank. Chlorophyll concentration was calculated using the equations described by Villicaña et al. (2016) and expressed on a fresh weight basis (mg g^{-1} FW).

Lipid peroxidation was determined using the thiobarbituric acid (TBA) reaction as described by Puckette et al. (2007). The concentration of malondialdehyde (MDA) was calculated as a measure of lipid peroxidation and expressed as nanomole per gram fresh weight. The content of soluble sugar was determined following the method of Dreywood (1946). POD activity was measured using guaiacol (1-Hydroxy-2-methoxybenzene) as a substrate. The level of enzyme activity was expressed as the amount of guaiacol oxidized by POD per minute.

Statistical analyses

All data are presented as means ± standard errors (SE) from three biological replicates of each experiment. Statistical significance was calculated by analysis of T-test. The significant differences are represented by $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$. Analyses were performed with IBM SPSS Statistics 16.0 software. Figures were created using SigmaPlot 12.5 (Systat Software, Inc., Germany).

Results

The stress-responsive gene in alfalfa encodes a DUF containing protein

Sequence alignment analysis revealed that the stress-responsive gene in alfalfa is identical to an mRNA encoding a plastid movement impaired protein in *Medicago truncatula* (XM_013601405.1) and the corresponding polypeptide sequence (XP_013456859.1) in *M. truncatula*. The deduced amino acid sequence from alfalfa showed 86, 73, 73, 67, and 53% identities to the sequences in *Trifolium subterraneum* (GAU29092.1), *Glycine max* (XP_003555905.1), *Cicer arietinum* (XP_004505259.1), *Cajanus cajan* (KYP42580.1), and *Arabidopsis thaliana* (NP_178243.2) (Fig. 1a). These protein sequences are common, sharing a DUF 4228 domain (DUF4228) shown in Fig. 1a. For this reason, the stress-responsive gene was annotated as *MsDUF* gene. Phylogenetic analysis showed that MsDUF is clustered with the protein sequences of other legume plants, such as *M. truncatula, T. subterraneum, C. arietinum, G. max, Phaseolus vulgaris* and *Vigna angularis* while three non-legume species formed a separate cluster (Fig. 1b).

Overexpression of *MsDUF* in tobacco negatively impacted seed germination

To understand the function of *MsDUF*, we overexpressed the gene in tobacco plants (Han et al. 2013). Seed germination test revealed that overexpression of *MsDUF* significantly reduced seed GP (Figs. 2, 3) under normal conditions and osmotic stress. Most seeds germinated between day 3 and day 4, reaching the maximum germination on day 6 for both WT and overexpressor seeds under normal condition. WT seeds showed a 94.5% germination rate, while overexpressors had an 87.6% germination rate (Figs. 3a, 4a). Osmotic stress treatment delayed seed germination, with a major germination occurring at day 6, reaching 86 and 56% for WT and overexpressors, respectively. WT seeds continued to germinate, reaching a similar GP to that at normal conditions. Overexpressor seeds also continued to germinate with a lower rate to reach a maximum rate at day 12 (Fig. 3b). Treatment of 10 µM ABA also delayed germination of both WT and overexpressor seeds. All the seeds germinated at day 5, reaching 83.7 and 77.3% for WT and overespressors respectively and showing no further germination afterward (Fig. 3c).

A greater reduction of seed germination in *MsDUF*-overexpression lines also reflected in GI when compared with WT seeds under normal, or osmotic stress conditions. ABA treatment reduced GI more in WT seeds than the overexpressors, reaching the same GI for WT and overexpressors (Fig. 4b). SVI were also significantly reduced in *MsDUF*-overexpressor lines under normal and osmotic stress conditions, when compared to WT seeds. Osmotic stress and ABA caused 59.5 and 89.7% reduction, respectively, in SVI in WT. Both ABA-treated WT and overexpressor seeds showed a drastic reduction in SVI and radical length and showed no significant difference among genotypes after ABA treatment (Fig. 4c, d).

Overexpression of *MsDUF* increased ABA content and transcript level of ABA synthesis genes in transgenic tobacco seeds

To determine whether reduced seed germination in *MsDUF*-overexpressors is associated with change in phytohormone content, ABA and GA_3 levels were determined in WT and line 8 of *MsDUF*-overexpressor. Our results showed that the level of ABA in *MsDUF*-overexpressing seeds was about 1.7-fold higher than those in WT (Fig. 5). GA_3 was undetectable in both *MsDUF*-overexpressor and WT seeds (data not shown). In addition, the transcript levels of three ABA biosynthetic genes, *ZEP, NCED6*, and *NCED1* were about 3.4-fold, 4.0-fold and 42.5-fold higher in *MsDUF*-overexpressing seeds than those in WT (Fig. 5). *MsDUF*-overexpressor line showed 2.9-fold increase in transcript level of *SOM*, a gene involved in regulating ABA content in *Arabidopsis* seeds (Kim et al. 2008), when compared with WT seeds (Fig. 5).

MsDUF-overexpression compromised health and response to mannitol and ABA treatment in transgenic tobacco plants

MsDUF-overexpression lines exhibited lower levels of chlorophyll a, chlorophyll b and thus total chlorophyll content than WT plants (Fig. 6). However, the ratio of chlorophyll a/b in *MsDUF* overexpression lines were not significantly different from that in WT.

MAD content was increased in both WT plants and overexpressors, but the content was comparable under mannitol treatment. While MAD content showed little change under ABA treatment in WT plants compared to normal condition, ABA greatly increased MDA content in overexpressors,

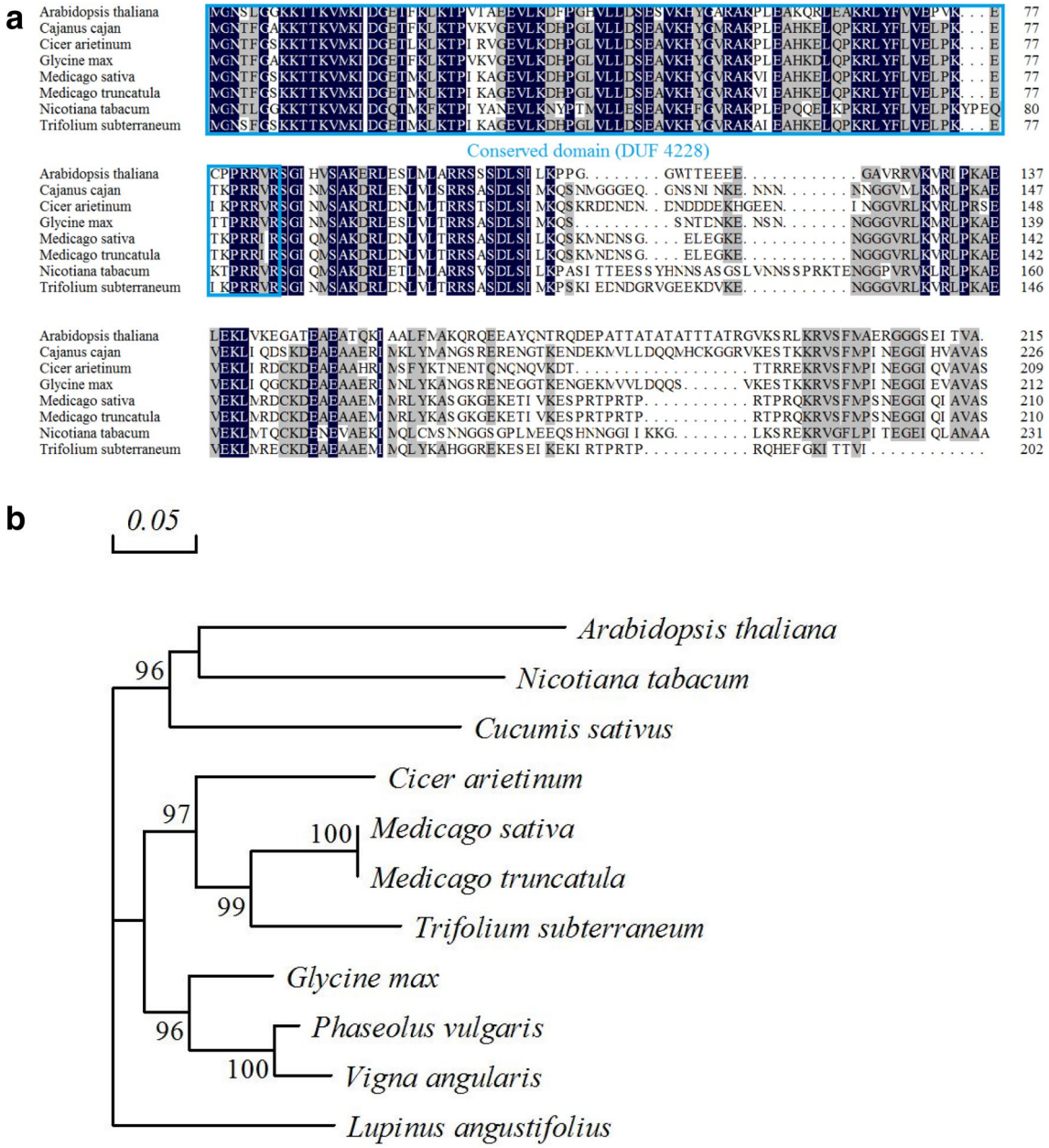

Fig. 1 Amino acid sequence alignment (**a**) and phylogenetic tree (**b**) of MsDUF with other selected plant species. Identical residues are shaded in dark blue. Gray shading indicates similar residues in more than 75% of all the sequences. The predicted DUF conserved domain is highlighted with blue boxes. Phylogenetic tree was constructed using neighbor-joining method with 1000 bootstrap. The species and corresponding GenBank accession number are as follows: *A. thaliana* (NP_178243.2), *C. cajan* (KYP42580.1), *C. arietinum* (XP_004505259.1), *Cucumis sativus* (XP_004140437.1), *G. max* (XP_003555905.1), *Lupinus angustifolius* (OIV93290.1), *M. sativa* (AFP87383.1), *M. truncatula* (XP_013456859.1), *T. subterraneum* (GAU29092.1), *Nicotiana tabacum* (XP_016496165.1), *P. vulgaris* (XP_007157721.1), and *V. angularis* (XP_017435869.1)

resulting in significant difference between WT and overexpressor plants (Fig. 7a). Soluble sugar content was lower in *MsDUF* overexpressor plants under normal and ABA treatment conditions but was comparable under mannitol treatment due to an increase in soluble sugar content in overexpressors under stress condition (Fig. 7b). POD activity was comparable between WT and overexpressors under normal growth. ABA treatment, however, resulted in nearly

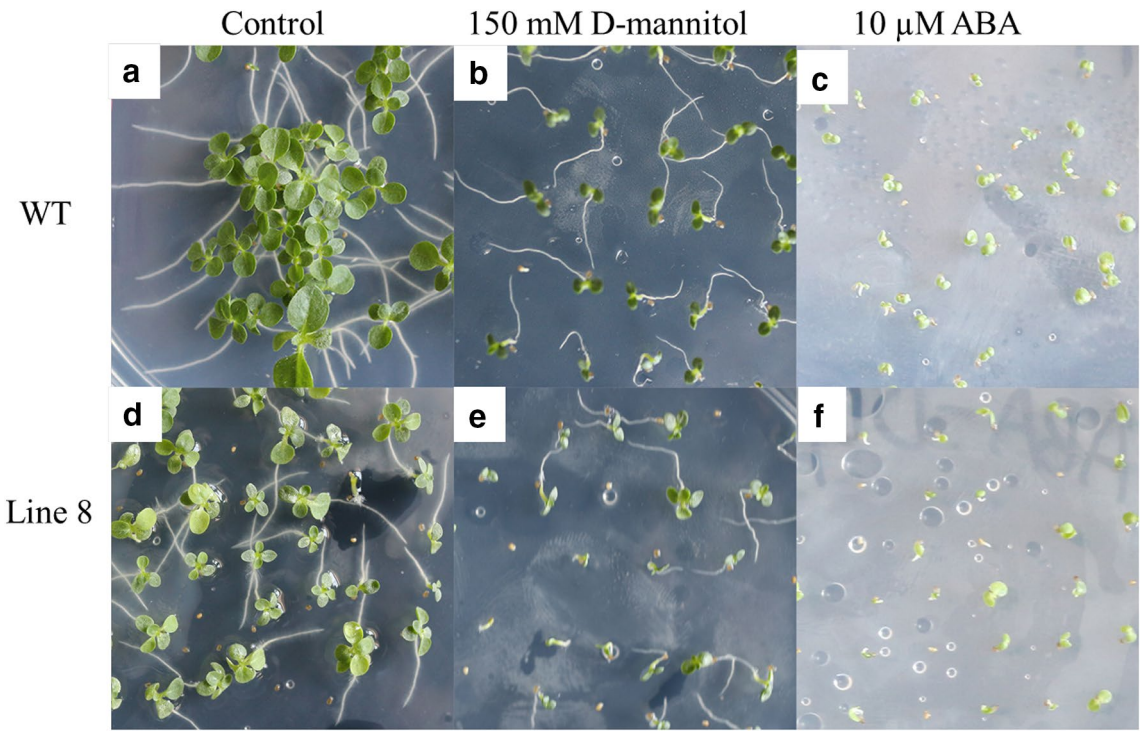

Fig. 2 Effect of *MsDUF* overexpression on tobacco seed germination and seedling phenotype. Wild type and *MsDUF*-overexpression (line 8, T_2) tobacco seeds were germinated in 1/2 MS medium (**a** and **d**), 1/2 MS medium supplemented with 150 mM D-mannitol (**b** and **e**), and 1/2 MS medium supplemented with 10 μM ABA (**c** and **f**), respectively. Pictures were taken 12 days after germination

doubling of POD activity in WT but little change in overexpressors when compared to that at normal growth conditions. Consequently, overexpressors showed significantly lower POD activity than WT plants (Fig. 7c). Mannitol treatment greatly increased POD activity in both WT plants and transgenic plants, but the difference between them was not significant.

Discussion

A large number of stress responsive genes have been identified in plants including alfalfa (Jin et al. 2010; Luo et al. 2009; Mohapatra et al. 1989). However, our understanding of the function of stress responsive gene is very limited. The main objective of this study was to examine the function of a stress responsive gene, *MsDUF*, in the process of seed germination and seedling growth. Ectopic expression of the alfalfa gene in tobacco supports its potential function in regulating seed germination and plants' health and stress response.

Our study supports a model that *MsDUF* is involved in seed germination through regulating ABA synthesis. First, the reduction in seed germination due to *MsDUF* overexpression is associated with a greater accumulation of ABA in the seeds of overexpressors. Second, overexpressor seeds showed a greater expression of several key genes in ABA synthesis. Third, overexpressors showed a higher level of *SOM* transcripts compared to WT. *SOM* had been shown to promote ABA level in *Arabidopsis* (Park et al. 2011). These results suggested that *MsDUF* may regulate ABA content in seeds through up-regulating the transcript levels of ABA synthesis genes.

ABA is a sesquiterpene hormone that is well known for its physiological role in the processes of seed development, such as seed maturation and dehydration (King 1982; Nakashima et al. 2009) and in germination (El-maroufbouteau et al. 2015; Fujii et al. 2007) as well as in plant adaptation to different types of environmental stresses (Kang et al. 2002; Pastori and Foyer 2002). It has been suggested that ABA inhibits water uptake by preventing cell wall loosening of the embryo during seed germination, implying that ABA is able to reduce embryo growth potential (Schopfer and Plachy 1985). This could explain why *MsDUF* overexpressors showed reduced germination rate, vigor and index and showed compromised germination

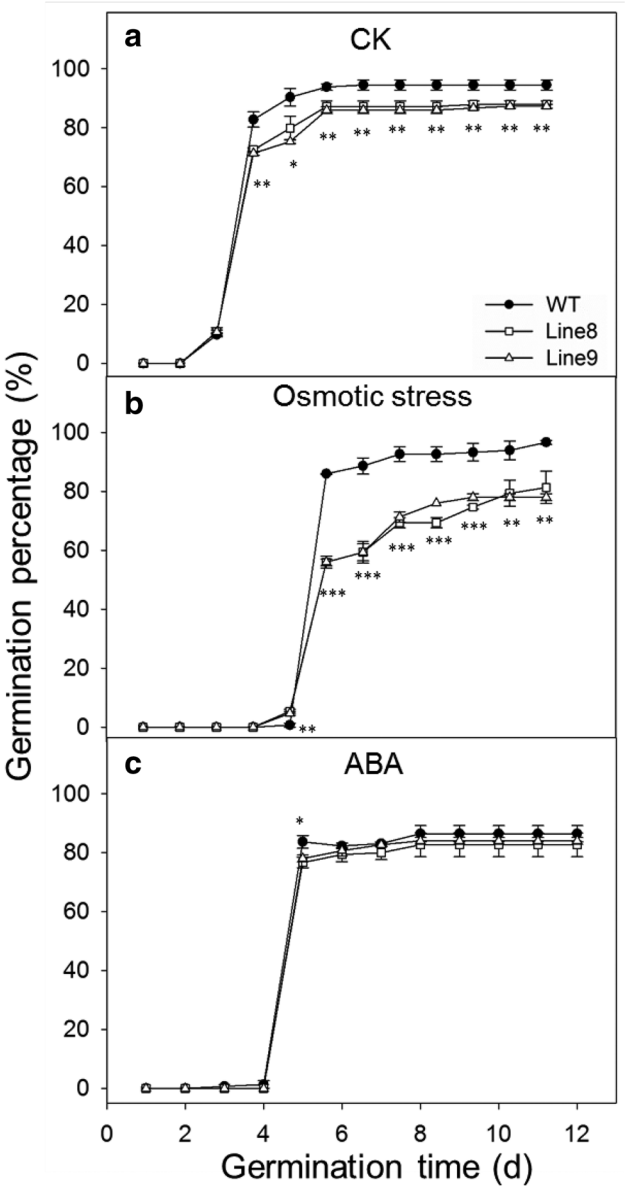

Fig. 3 Effect of *MsDUF* overexpression on tobacco seed germination rate. The seeds were germinated in 1/2 MS medium (CK), 1/2 MS medium supplemented with 150 mM D-mannitol (osmotic stress), and 1/2 MS medium supplemented with 10 μM ABA (ABA). WT, wild type; line 8 and line 9, two T_2 *MsDUF*-overexpression lines. *, ** and *** indicate significant difference between overexpressors and WT at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Three independent experiments were performed and each experiment is represented by 50 seeds

rate under osmotic stress Osmotic stress is known to link to ABA accumulation (Hoad 1975; Lehmann et al. 1995), thus further accumulation of ABA in osmotic stressed seeds may lead to more inhibition in seed germination. Interestingly, the ABA treated WT and overexpressor seeds showed similar lower germination rates. It is possible that the high exogenous ABA application has masked the smaller difference in endogenous ABA content in WT and overexpressor seeds.

GA also plays an essential role in promoting seed germination (Peng and Harberd 2002). In fact, seeds germination in many species depend on a balance of GA and ABA (Razem et al. 2006). Quantification of GA in WT and *MsDUF*-overexpressor seeds was not successful, due to a trace amount of GA_3 in mature tobacco seeds. Thus, whether overexpression of *MsDUF* has any effect on GA content or signaling is inconclusive. However, the fact that *SOM* expression was enhanced in *MsDUF*-overexpressors may suggest a role of *MsDUF* in regulating GA level, since overexpression of *SOM* inhibits seed germination partly by activating the expression of ABA synthesis genes and by inhibiting the expression of GA synthesis genes in *Arabidopsis* (Kim et al. 2008). The *som* mutant of Arabidopsis contained a lower level of ABA and an elevated level of GA (Kim et al. 2008).

Overexpression of *MsDUF* also compromised plant health and stress response. Transgenic tobacco plants appeared light green in leaf color compared to dark green in WT plants, which was consistent with the findings that *MsDUF*-overexpressors contained less chlorophyll a and b. Overexpressors showed significantly higher level of MDA which is associated with a significant decrease in POD activity after exogenous ABA treatment, suggesting that *MsDUF* may suppress oxidative stress response under severe stress conditions or when ABA is accumulated to high levels in plants. Various abiotic stresses lead to an overproduction of reactive oxygen species (ROS) in plants which are toxic and damage proteins, lipids, carbohydrates and DNA (Gill and Tuteja 2010). Thus controlling oxidative stress is one of the key processes in stress response. Our results thus suggest that MsDUF may act as a negative regulator of stress response. The notion is further supported by the fact that the overexpressors accumulated less soluble sugar under normal conditions and after ABA treatment, which may compromise its osmotic adjustment capacity during stress response. Since overexpression of *MsDUF* resulted in an accumulation of ABA in the seeds, one may assume the effect of *MsDUF* overexpression on stress response in seedlings may be associated with ABA also. ABA has been demonstrated important in stress response including enhancing osmotic adjustment and alleviating oxidative stress (Yoshida et al. 2014). The results from our *MsDUF* overexpression showed the opposite of the ABA effect. Thus, the negative impact of *MsDUF* overexpression on plant stress response may not be directly associated with ABA content in the plants.

In summary, our results support a potential role of *MsDUF* in regulating seed germination through enhancing

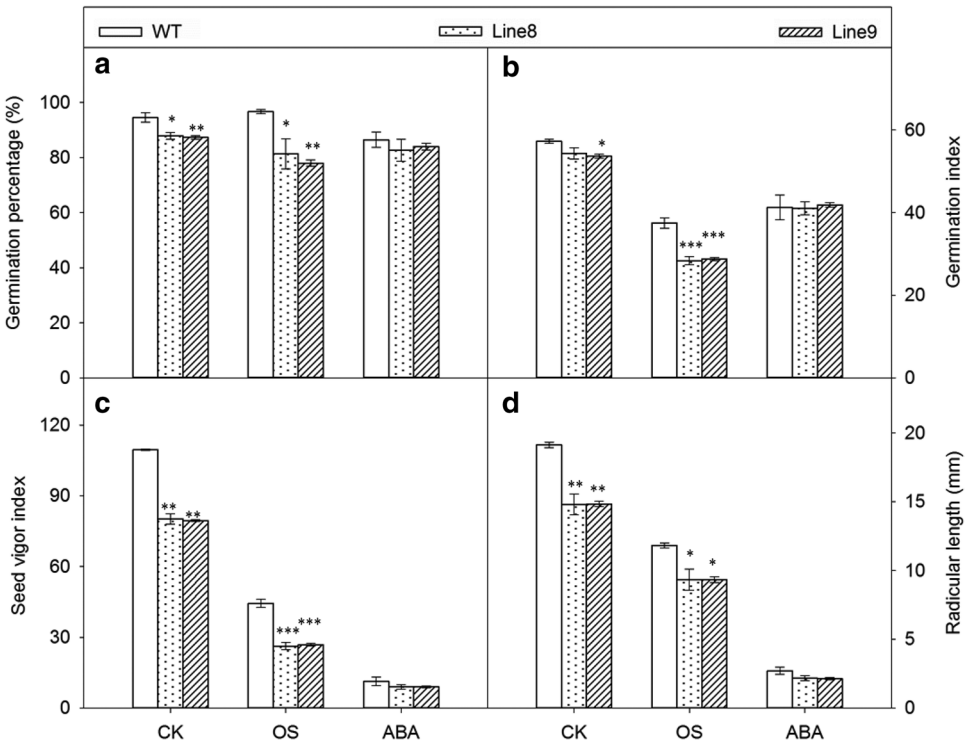

Fig. 4 Effect of *MsDUF* overexpression on seed GP (**a**), GI (**b**), SVI (**c**) and radicular length (**d**). The seeds were germinated in 1/2 MS medium, 1/2 MS medium supplemented with 150 mM D-mannitol (OS), and 1/2 MS medium supplemented with 10 μM ABA (ABA). WT wild type; line 8 and line 9, two T_2 *MsDUF*-overexpression lines; OS osmotic stress; Bars represent SE (n = 3). *, ** and *** indicate significant difference when compared to WT at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Three independent experiments were performed and each experiment is represented by 50 seeds

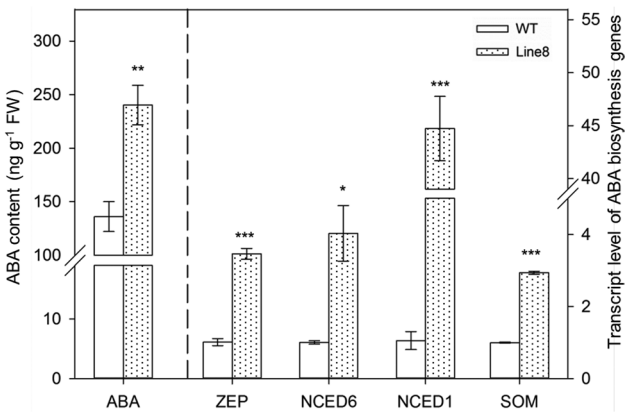

Fig. 5 Effect of *MsDUF* overexpression on ABA content and transcript level of ABA biosynthesis genes in tobacco seeds. WT wild type; line 8 T_2 *MsDUF*-overexpression line; ABA abscisic acid; FW fresh weight

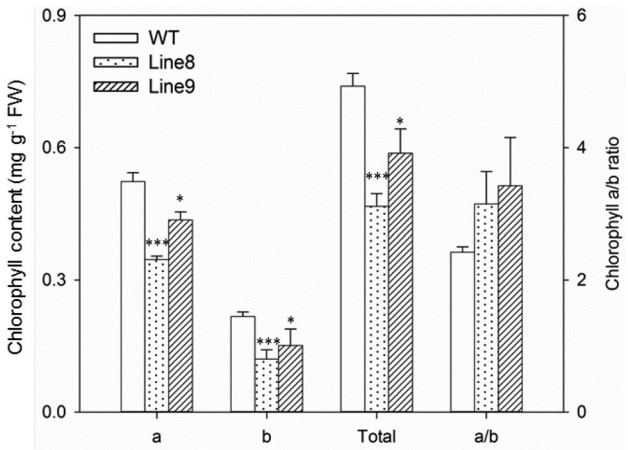

Fig. 6 Effect of *MsDUF* overexpression on chlorophyll content and chlorophyll a/b ratio in tobacco seedlings. a, Chlorophyll a content; b, chlorophyll b content; Total, total chlorophyll content; a/b, chlorophyll a/b ratio. WT, wild type; line 8 and line 9, two T_2 *MsDUF*-overexpression lines

ABA synthesis in seeds. Overexpression of *MsDUF* also compromised plant performance and response to stress, suggesting a role of *MsDUF* as negative regulator in plant growth. However, additional studies are needed to determine the specific working mechanisms of *MsDUF* in plants and its role in stress response. While caution is needed to extrapolate the gene function directly into alfalfa, the high similarity of DUF protein in both alfalfa and tobacco suggests that findings from this study can be meaningful for both alfalfa and tobacco plants as well as other plants possessing this *DUF* gene.

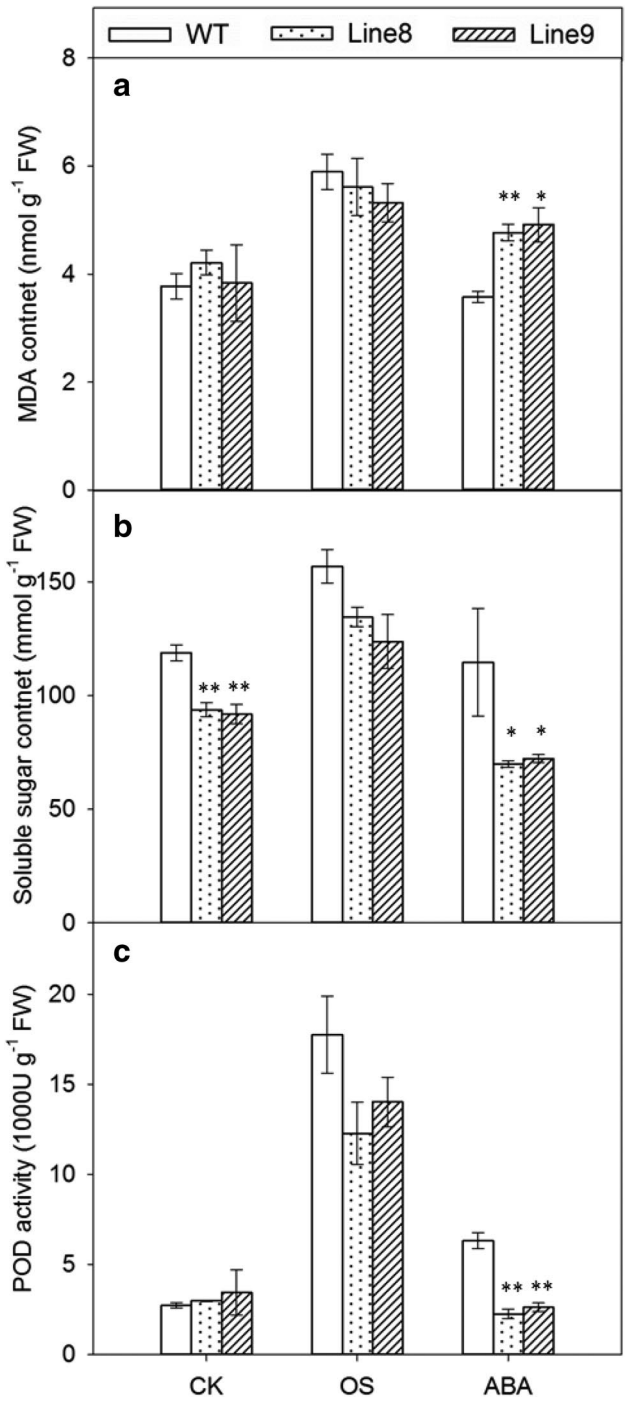

Fig. 7 Effect of *MsDUF* overexpression on MDA content (**a**), soluble sugar content (**b**), and POD activity (**c**) in tobacco seedlings. Measurements were done on 12-day-old plants that were grown in 1/2 MS medium (CK), 1/2 MS medium supplemented with 150 mM D-mannitol (OS), and 1/2 MS medium supplemented with 10 µM ABA (ABA). *WT* wild type; line 8 and line 9, two T_2 *MsDUF*-overexpression lines; *OS* osmotic stress; Bars represent SE (n = 3). * and ** indicate significant difference when compared to WT at $p \leq 0.05$ and $p \leq 0.01$, respectively

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Author contributions YW and ZZ performed the whole experiment. YW and YW analyzed the data and wrote the manuscript. BH provided the transgenic tobacco seeds. HL, YA and LC participated in the gene expression, antioxidant enzyme and soluble sugar measurement. PY and TH proposed the ideas, designed the experiment, and edited the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

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

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