Molecular cloning and characterization of a novel gene *MsKMS1* in *Medicago sativa*

B. HAN[1,2], P. ZHANG[3], Z.-Q. ZHANG[4], Y.-F. WANG[2], T.-M. HU[2,*], and P.-Z. YANG[2,*]

[1] *Faculty of Animal Science and Technology, Yunnan Agriculture University, Kunming 650201, Yunnan, P.R. China*
[2] *College of Grassland Agriculture, Northwest A&F University, Yangling 712100, Shaanxi, P.R. China*
[3] *Department of Grassland Science, College of Animal Science and Technology, Northeast Agricultural University, Harbin 150030, Heilongjiang, P.R. China*
[4] *Key Laboratory of Grassland Resources, Ministry of Education P.R. of China, College of Grassland Resources and Environment, Inner Mongolia Agricultural, University, Hohhot 010011, Inner Mongolia, P.R. China*

[*]Corresponding author: Fax: +86 29 87092164, E-mail: hutianming@126.com, yangpeizhi@126.com

Abstract

Vacuole membrane proteins play a critical role in the regulation of plant physiological processes including normal growth and development, and responses to stresses. The *killing me slowly 1* (*KMS1*) gene that encodes a soluble N-ethylmaleimide-sensitive fusion attachment receptor (SNARE) domain-containing vacuole membrane protein was first reported in *Arabidopsis*. Currently, the function of *KMS1* in other plants under stress is poorly understood. In this study, we report cloning, expression, and characterization of a novel *KMS1* gene in alfalfa (*Medicago sativa* L.), designated *MsKMS1* (GenBank accession No. JX467688). The full-length cDNA of *MsKMS1* was 1 396 bp and contained a complete open reading frame of 1 257 bp, which encoded a putative protein of 418 amino acids. The *BLASTp* analysis showed that *MsKMS1* shared high amino acid sequence similarities with *KMS1* from other plants such as *Medicago truncatula* (99 %), *Cicer arietinum* (89 %), *Glycine max* (77 %), *Prunus mume* (76 %), *Ricinus communis* (72 %), *Populus euphratica* (72 %), *Theobroma cacao* (72 %), and *Arabidopsis thaliana* (67 %). Transient transformation of onion (*Allium cepa*) bulb scale epidermal cells by biolistic bombardment showed that *MsKMS1* was localized to the plasma membrane. Quantitative real-time PCR revealed that *MsKMS1* expression was upregulated under different abiotic stresses (200 mM NaCl, 20 % (m/v) polyethylene glycol 6000] and 10 mg dm^{-3} abscisic acid. Transgenic tobacco plants were obtained *via Agrobacterium*-mediated transformation and treated with 200 mM NaCl. Reverse-transcription PCR data showed that *MsKMS1* was successfully transcribed and expressed in the leaves of transgenic plants. The *MsKMS1*-overexpressors showed a lower malondialdehyde content and maintained a higher relative water content and proline content compared with non-transgenic controls under salt stress. These results indicate that the introduction of the *MsKMS1* gene could improve salt stress resistance in tobacco plants. This study reveals the role of *MsKMS1* in the regulation of plant responses to abiotic stress and provides evidence for further functional studies of the *KMS1* family in alfalfa.

Keywords: abscisic acid, functional analysis, NaCl, onion, PEG 6000, SNARE domain, transgenic tobacco plants.

Introduction

Drought, salinity, and extreme temperatures are common environmental stresses that adversely affect plant growth and development. Plants respond to these abiotic stresses through various pathways, and a growing number of stress resistance genes have been identified. For example, transgenic plants overexpressing aquaporin (*AQP*) genes such as *Oryza sativa plasma membrane intrinsic protein 1*, *Solanum tuberosum tonoplast intrinsic proteins 2*, *Sesuvium portulacastrum aquaporin protein* (*AQP*) *1*, *Triticum turgidum plasma membrane intrinsic protein 1*, *Triticum aestivum nodulin26-like intrinsic protein*, and *Triticum aestivum AQP 8* have been shown to present a salt-tolerant phenotype (Gao *et al.* 2010, Hu *et al.* 2012, Chang *et al.* 2016, Kapilan *et al.* 2018). Several other *AQP* genes are also involved in plant tolerance to multiple stresses. The *TaAQP7* from *Triticum aestivum* has been shown to confer tolerance to drought, osmotic, and cold stresses, whereas *Panax ginseng tonoplast intrinsic proteins 1* enhances drought and salt tolerance (Peng *et al.* 2007, Zhou *et al.* 2012, Huang *et al.* 2014).

Recently, it has been found that the introduction of the CCCH-tandem zinc finger protein gene *Oryza sativa tandem zinc finger protein 5* reduces rice yield loss under drought conditions (Selvaraj *et al.* 2020). Overexpression of *Thellungiella halophila homeobox protein 1* and the *Thellungiella halophila stress related NaCl*, the homeodomain transcription factor gene, improves the heat stress resistance of *Thellungiella halophila* (Liu *et al.* 2019). Moreover, many *myeloblastosis* family genes participate in plant responses to one or more stresses including low temperature, high salinity, and water stress (Seo *et al.* 2009, Chen *et al.* 2013, Kim *et al.* 2013, Song *et al.* 2019). Therefore, transforming such genes into crops has the potential to generate genetically modified plants with improved tolerance to abiotic stresses.

Soluble N-ethylmaleimide-sensitive fusion attachment protein receptors (SNAREs) are also known to participate in plant defense responses against abiotic stresses (Bao *et al.* 2005). Zhu *et al.* (2002) have identified an *Arabidopsis thaliana* mutant that is sensitive to salt and osmotic stresses, *osm1*, in which the *Qc-SNARE* gene of the trans-Golgi network, *Arabidopsis thaliana syntaxin of plants 61*, is missing. Additionally, Deng *et al.* (2007) have reported that the SNARE-related gene *R295* in the *Hevea brasiliensis* responds to the external stress caused by rubber tapping and thus may be involved in stress signal transduction. The *killing me slowly 1* (*KMS1*) gene that encodes a SNARE domain-containing protein was first reported in *Arabidopsis thaliana* and knock-out mutation showed a slow lethal effect (Wang *et al.* 2011). So far, there have been no reports on the role of *KMS1* in the abiotic stress resistance of plants.

Alfalfa (*Medicago sativa* L.) is a high-quality forage legume that is planted worldwide (Geng 1995). However, alfalfa production is often severely limited by abiotic stresses, such as salinization, drought, and cold damage (Boyer 1982, Long *et al.* 2006). Breeding of salt- and drought-resistant cultivars of alfalfa is therefore of practical significance. In this study, we cloned a KMS1-encoding gene from *M. sativa*, designated *MsKMS1*. We analyzed the amino acid sequence characteristics of *MsKMS1* and its expression patterns in different tissues of alfalfa plants subjected to various abiotic stresses. We further explored the role of *MsKMS1* in the salt tolerance of transgenic tobacco seedlings. The results could be useful for the breeding of high-quality stress-resistant alfalfa cultivars.

Materials and methods

Plants and experimental design: Alfalfa seedlings (*Medicago sativa* L. cv. Zhongmu No. 1) were cultured on a Murashige and Skoog basal medium for 3 weeks and divided into 2 groups, with a minimum of 100 plants per group. One group was directly used for gene cloning and tissue-specific expression analysis. The other groups were treated with 200 mM NaCl, 20 % (m/v) polyethylene glycol (PEG) 6000, or 10 mg dm^{-3} abscisic acid (ABA). Root, stem, and leaf samples were taken at 0, 2, 4, 8, 12, and 24 h of treatment for quantitative real-time PCR analysis.

Cloning of *MsKMS1* cDNA: Total RNA was extracted from the leaves of untreated alfalfa seedlings using a *Trizol* reagent (*Invitrogen*, Carlsbad, CA, USA). The extracted RNA was reverse-transcribed into cDNA using a cDNA synthesis kit (*Takara*, Otsu, Shiga, Japan) following the manufacturer's instructions.

In accordance with the methods used by Chen *et al.* (2008), the sequence of the drought-resistant upregulated gene *TC83565* was retrieved from the expressed sequence tag database of the model legume *Medicago truncatula* (http://www.tigr.org/). Three' rapid amplification of cDNA ends (RACE)- and 5' RACE-specific primers were designed using *Primer Premier v. 5.0* (*Premier Biosoft International*, Palo Alto, CA, USA): P1 (5'-ACTTTTGTTCTATACCTGGG-3') and P2 (5'-ACCCAGGTATAGAACAAAAG-3'). The 3' and 5' ends of *TC83565* were amplified using P1/P2 and an abridged universal amplification primer (5'-GGCCACGCGTCGACTAGTAC-3') using a *SMART*^{TM} *RACE* cDNA amplification kit (*Clontech Laboratories*, Mountain View, CA, USA) following the manufacturer's instructions.

After sequencing (Beijing Genomics Institute, Beijing, China), the reads were assembled in *DNAMAN v. 6.0* (*Lynnon Biosoft*, Quebec, QC, Canada) to obtain the full-length open reading frame (ORF). Specific primers were subsequently designed for the full-length cDNA of *TC83565*: P3 (5'-CCCATTTTTGACTCACTTGC-3') and P4 (5'-CTAATCAGTTGAGGTAGAAATG-3'). The full-length cDNA of *TC83565* was subjected to PCR amplification using the primer set P3/P4 and high-fidelity polymerase. PCR amplicons were ligated to the PMD18-T vector and transformed into *Escherichia coli* DH5α™ competent cells (Beijing Genomics Institute). Positive clones were screened and sent to Beijing Genomics Institute for sequencing.

Multiple sequence alignments of deduced amino acid sequences were performed using an internet *BLAST* search (http://www.ncbi.nlm.nih.gov/BLAST/) with *DNAMAN v. 6.0*. A phylogenetic tree was constructed using the neighbor-joining method.

Subcellular localization assay: *MsKMS1* was amplified using the newly designed specific primers P5 (5'-GGGTCTAGAATGGGTTCTGGAGAAATAGCTTCTTCATC-3', *Xba* I restriction site underlined) and P6 (5'-GGGCTCGAGCTAATCAGTTGAGGTAGAAATGTTTGATGC-3', *XhoI* restriction site underlined). The amplification conditions were as follows: pre-denaturation at 94 °C for 5 min, followed by 28 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.

The amplicons were double-digested with *XbaI* and *XhoI*, and then ligated to the PBI221-GFP (green fluorescent protein) vector. The ligated products were transformed into *E. coli* DH5α competent cells. The plasmids were extracted from positive clones using a commercial kit (*Promega*, Madison, WI, USA) and sent to Beijing Genomics Institute for sequencing. The positive recombinant plasmid 35S-MsKMS1-GFP was introduced into onion *(Allium cepa)* bulb scale epidermal cells *via* biolistic bombardment (Mare *et al.* 2004). Cells were incubated at room temperature in darkness for 24 h and then observed under a laser scanning confocal microscope (*LSM510META*, *META Zeiss*, Jena, Germany).

Real-time quantitative PCR (RT-PCR) assay: Total RNA was extracted from the roots, stems, and leaves of alfalfa seedlings treated with and without abiotic stress. In accordance with the full-length cDNA sequence of *MsKMS1*, specific primers P7 (5'-GGCCAAAGGTGGATTGCTTATGCT-3') and P8 (5'-TATGCAAACCAGACCCAAGACCGA-3') were designed for real-time quantitative PCR assays. The *β-actin* gene from *M. sativa* was used as the internal reference to design the primers P9 (5'-TTTGAGACTTTCAATGTGCCCGCC-3') and P10 (5'-TAGCATGTGGGAGTGCATAACCCT-3').

The PCR reaction was prepared using a *SYBR Premix Ex Taq*™ kit (*Takara*) following the manufacturer's instructions. PCR amplification was carried out in two steps on a *CFX96* real-time PCR system (*Bio-Rad*, Hercules, CA, USA) with denaturation at 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The cDNA samples were assayed in three biological replicates with three technical replicates each. The *MsKMS1* expressions in the roots, stems, and leaves of alfalfa seedlings were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Transformation of tobacco: The plant expression vector pCAMBIA1301 (preserved in our laboratory) and the plasmid MsKMS1 containing the target gene were double-digested with EcoR I and Hind III. The digested products were ligated with T4 DNA ligase overnight and the recombinant plasmid pCAMBIA1301-MsKMS1 was transformed into *E. coli* DH5α competent cells. The

plasmids were extracted from positive clones using a commercial kit (*Promega*) and sent to Beijing Genomics Institute for sequencing.

The expression vector pCAMBIA1301-MsKMS1 was introduced into *Agrobacterium tumefaciens* GV3101 using the freeze-thaw method (Qi and Zilong 2002). Positive clones were cultured in an *Agrobacterium rhizogenes* culture medium at 28 °C on a rotary shaker till the absorbance of the broth culture at 600 nm reached 0.6. Tobacco seedlings (*Nicotiana tabacum* L. cv. NC89) were obtained from the Tobacco Research Institute of Chinese Academy of Agricultural Science (Qingdao, China). Four-week-old seedlings were infected with the broth culture using the leaf disc method (Horsch *et al.* 1985). The calli were induced on different media containing 50 mg dm^{-3} hygromycin, and transgenic plants (T1) were obtained after differentiation and rooting. Positive transgenic plants screened by PCR were transplanted into soil and grown at a temperature of 25 °C, a 16-h photoperiod, an irradiance of 250 µmol m^{-2} s^{-1}, and a relative humidity of 70 %.

Identification of transgenic tobacco by PCR: After transformation, total genomic DNA was extracted from tobacco leaves using a DNA extraction kit (*BioTeke*, Beijing, China) following the manufacturer's instructions. The extracted DNA served as a template for the PCR amplification of a 521 bp gene fragment from the *hygromycin* (*HYG*) gene using the *HYG* universal primers: HygF (5'-CGATTCCGGAAGTGCTTGAC-3') and HygR (5'-CGTCTGCTGCTCCATACAAG-3'). The following PCR program was used: pre-denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 40 s, 57 °C for 40 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The negative control was prepared using the DNA of wild-type tobacco as the template, and the blank control was prepared with DNA-free water. The PCR results were used to examine whether the target gene was successfully transformed into tobacco. Reverse-transcription PCR (RT-PCR) was performed using the primers P3 and P4 to examine the expression of the target gene.

Stress resistance test of transgenic tobacco: The resistant tobacco seedlings were propagated by cuttings. Cutting seedlings with uniform growth were grown in a greenhouse for a month and then exposed to 200 mM NaCl. Leaf samples were taken at 1, 4, 7, 14, 20, and 23 d of treatment and immediately frozen at -80 °C until used. Relative water content (RWC), malondialdehyde (MDA) and proline content in the tobacco leaves were analyzed for the transgenic plants and non-transgenic controls as was described in Shekhawat *et al.* (2011).

Statistical analysis: Data are presented as means ± standard errors (SEs). The figures were drawn using *Excel 2010* (*Microsoft*, Redmond, WA, USA). One-way analysis of variance (*ANOVA*) was performed to analyze the significance of the differences between groups of data using *SPSS 16.0* statistics (*SPSS Inc.*, Chicago, IL, USA). The significance was defined at α = 0.05 according to the least significance difference (LSD) test.

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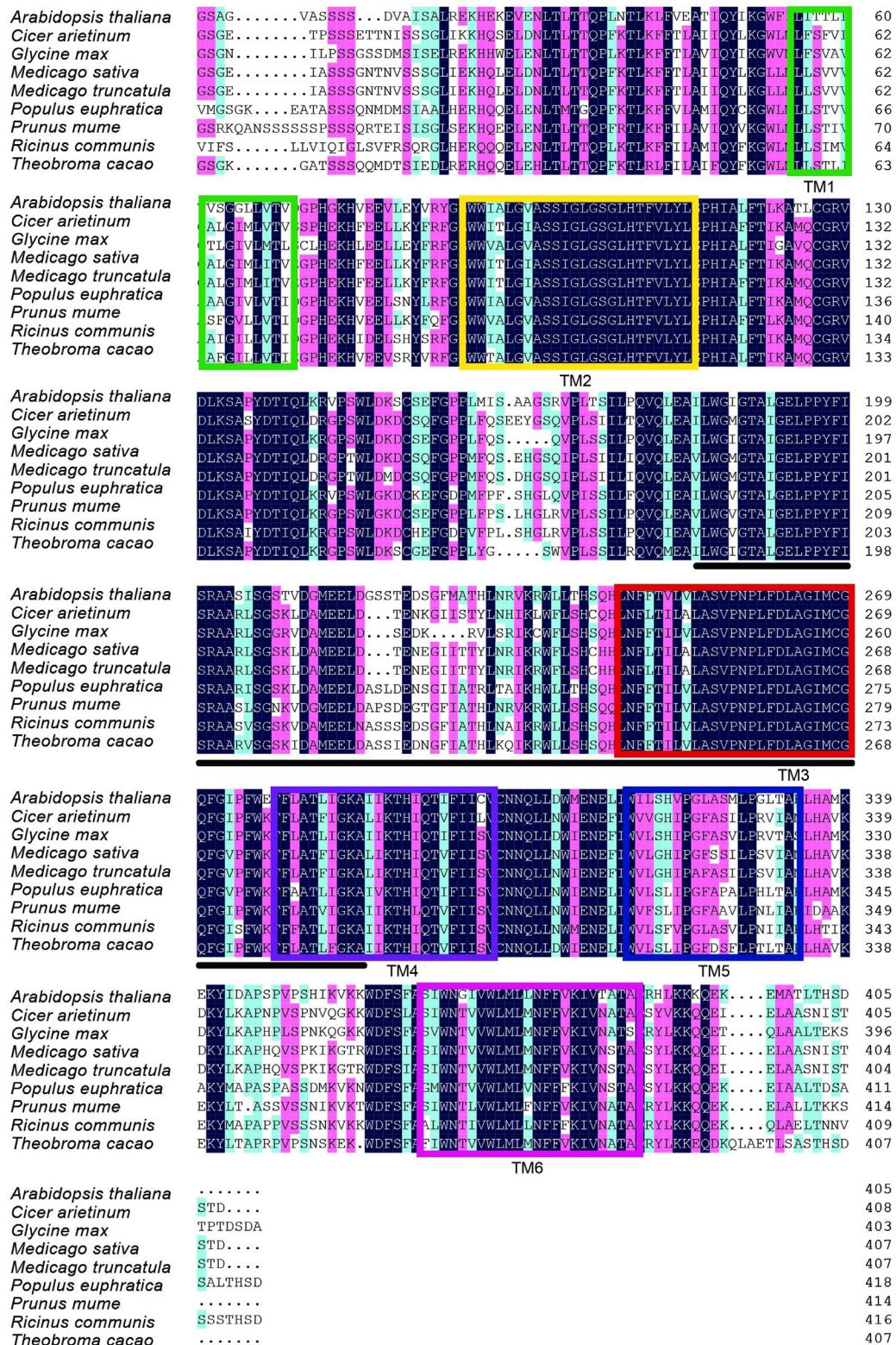
Fig. 1. The amino acid sequence alignment of Medicago sativa killing me slowly 1 (MsKMS1) with other selected plant species. Conserved residues are shaded *in black*. *Gray* shading indicates similar residues in more than 80 % of all the sequences. The predicted soluble N-ethylmaleimide-sensitive fusion attachment receptor (SNARE)-interacting conserved domain is shown with a *solid underline* and six transmembrane domains are highlighted with *colored boxes*. The species and corresponding *GenBan*k accession numbers are as follows: *Arabidopsis thaliana* (NP 567450), *Cicer arietinum* (XP 004496743), *Glycine max* (XP 003539287), *Medicago sativa* (AGE13753), *Medicago truncatula* (XP 003592411), *Populus euphratica* (XP 011021552), *Prunus mume* (XP 008224532), *Ricinus communis* (XP 002516770), and *Theobroma cacao* (XP 007034717).
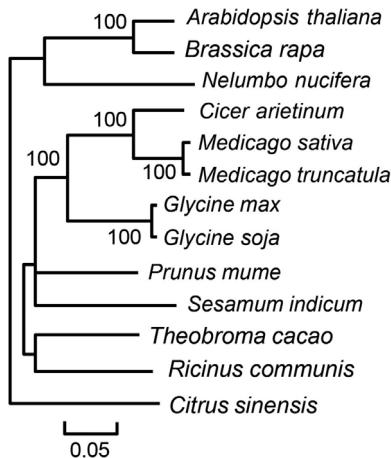
Fig. 2. A phylogenetic tree of killing me slowly 1 (KMS1) from alfalfa and other plants. The phylogenetic tree was constructed using a neighbor-joining method with 1 000 bootstrap replications. The *scale bar* indicates a 5 % sequence variance. *GenBank* accession numbers for amino acid sequences: *Arabidopsis thaliana* (NP 567450), *Brassica rapa* (XP 009107812), *Nelumbo nucifera* (XP 010256001), *Cicer arietinum* (XP 004496743), *Medicago sativa* (AGE13753), *Medicago truncatula* (XP 003592411), *Glycine max* (XP 003539287), *Glycine soja* (KHN35183), *Prunus mume* (XP 008224532), *Sesamum indicum* (XP 011071573), *Citrus sinensis* (XP 006489364), *Ricinus communis* (XP 002516770), and *Theobroma cacao* (XP 007034717).

Results

The 3'- and 5'-end gene fragments of *MsKMS1* were obtained *via* the rapid amplification of cDNA ends (RACE) method using the cDNA from the leaves of untreated alfalfa seedlings as the PCR template. Sequence analysis showed that the 3'- and 5'-end gene fragments were 1 036 and 600 bp in length, respectively. The full-length cDNA was 1 396 bp in length. Based on the nucleotide sequence of the full-length cDNA, specific primers (P3 and P4) were designed to amplify its ORF. Sequence analysis showed that the ORF, designated *MsKMS1* (GenBank accession No. JX467688), was 1 257 bp in length and encoded a putative protein of 418 amino acids.

Amino acid sequence alignment indicated that the deduced amino acid sequence of *MsKMS1* was highly homologous to that of *KMS1* from other plants. It was also found that MsKMS1 belonged to the SNARE-associated Golgi proteins. MsKMS1 contained one conserved SNARE domain and six transmembrane domains. According to the sequence alignment results (Fig. 1), *MsKMS1* shared high amino acid sequence similarities with the *KMS1* of *Medicago truncatula* (XP003592411; 99 %) and *Cicer arietinum* (XP004496743; 89 %). Relatively high sequence similarities were also observed with the *KMS1* of *Glycine max* (XP003539287; 77 %), *Prunus mume* (XP008224532; 76 %), *Ricinus communis* (XP002516770; 72 %), *Populus euphratica* (XP011021552; 72 %), *Theobroma cacao* (XP007034717; 70 %), and *A. thaliana* (NP 567450; 67 %). Phylogenetic analysis revealed that the amino acid sequence of MsKMS1 had the highest homology with other leguminous plants, such as *M. truncatula*, *C. arietinum*, *G. max*, and *G. soja* (Fig. 2).

Confocal microscopy analysis showed that the empty vector 35S-GFP was expressed both on the plasma membrane and in the nucleus of onion cells (Fig. 3A-C). By contrast, the green fluorescent signal of the 35S-MsKMS1-GFP plasmid was mainly distributed on the plasma membrane (Fig. 3D-F). These observations indicated that the protein encoded by *MsKMS1* was localized to the plasma membrane.

Using real-time quantitative PCR, it was found that *MsKMS1* was ubiquitously expressed in the roots, stems, and leaves of alfalfa. The highest expressions were observed in root samples, followed by leaf and stem samples (Fig. 4). The different expressions of *MsKMS1* in various plant tissues indicated that its expression pattern was tissue specific in alfalfa.

The quantitative PCR data showed that the *MsKMS1* expressions differed among alfalfa tissues under various abiotic stresses (Fig. 5). When the alfalfa plants were treated with PEG6000, the *MsKMS1* expressions displayed a varying pattern of increases and decreases over time in the roots, stems, and leaves. The highest expression of *MsKMS1* in the stems was observed at 2 h (1.7-fold in comparison with that at 0 h), in the leaves at 4 h (2.1-fold), and in the roots at 12 h (3.3-fold; Fig. 5A).

After subjecting alfalfa plants to NaCl treatment, the *MsKMS1* expressions peaked in the roots (1.7-fold), stems (1.4-fold), and leaves (2.6-fold) at 8 h. During the 24-h treatment period, the *MsKMS1* expressions in the roots
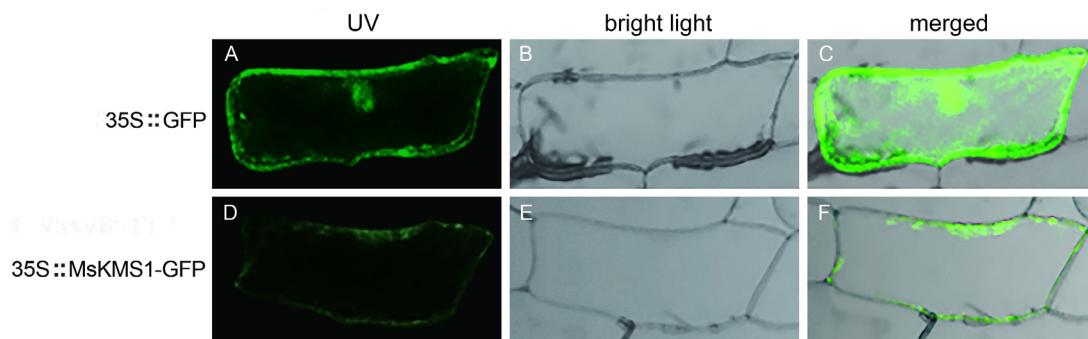

Fig. 3. The subcellular localization of Medicago sativa killing me slowly 1 (MsKMS1) in onion cells observed by laser scanning confocal microscopy; dark field (*A*, *D*), bright light field (*B*, *E*), and merged field (*C*, *F*).
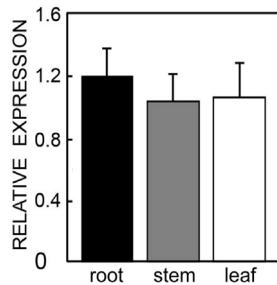
Fig. 4. Relative expression (fold-change) of *Medicago sativa killing me slowly 1* (*MsKMS1*) in various tissues. *M*eans ± SEs. No significant difference was found between groups.

and stems were not significantly different. All tissues had slightly higher *MsKMS1* expressions at 24 h than at 0 h (Fig. 5*B*).

After treating alfalfa plants with ABA, the *MsKMS1* expressions in the roots reached a maximum at 8 h (1.9-fold), and then declined, but remained slightly higher than at 0 h. In the stems, the *MsKMS1* expressions peaked at 12 h (1.8-fold) and then declined to a lower level at 24 h than at 0 h. In the leaves, the *MsKMS1* expression reached a maximum at 12 h (1.4-fold), and then declined but remained slightly higher than at 0 h (Fig. 5*C*).

Taken together, these results indicated that *MsKMS1* expression was upregulated in various alfalfa tissues after abiotic stress treatment.

The transgenic plants were verified with the primers of resistance gene, hygromycine. The target bands were amplified in the transgenic plants but not in the control, which indicated that the target gene has been successfully integrated into the tobacco genome (Fig. 6). Moreover, an RT-PCR assay using the leaves of positive transgenic seedlings confirmed that *MsKMS1* was successfully integrated into the tobacco genome and transcribed into mRNA.

The salt tolerance of transgenic tobacco plants was tested in terms of leaf RWC and MDA and proline content. The RWC in all tobacco plants gradually decreased with time under salt stress and the lowest values occurred at 23 d. Transgenic plants had significantly higher RWC values than non-transgenic controls at 20 d ($P < 0.05$). Although no significant differences were found at other time points, the RWC in the transgenic plants was slightly higher than in the non-transgenic controls throughout the experiment (Fig. 7*A*). This indicated that the transgenic plants expressing *MsKMS1* lost less water under salt stress and hence had a higher salt tolerance than the non-transgenic plants.
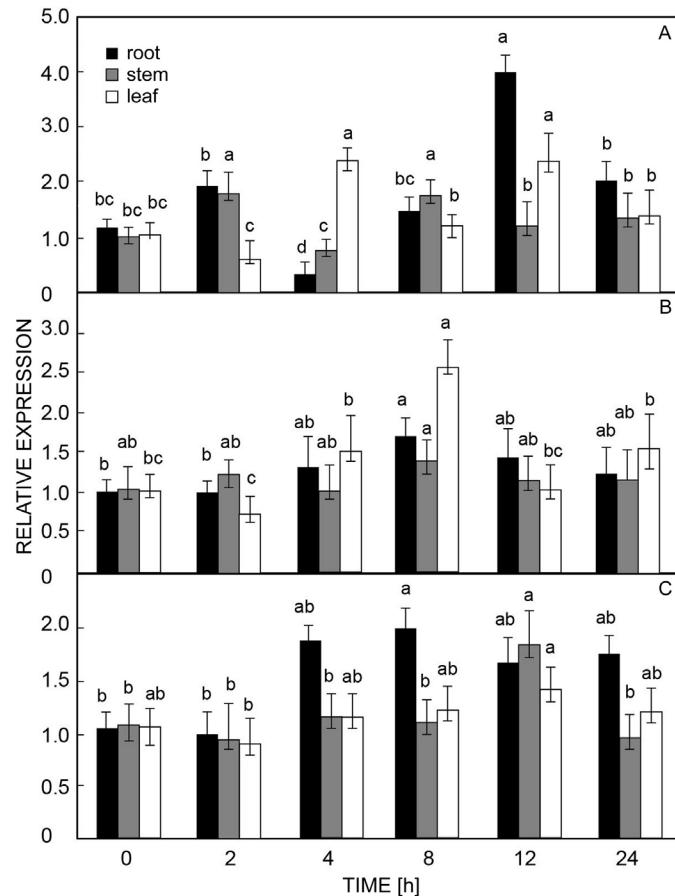

Fig. 5. Relative expression of *Medicago sativa killing me slowly 1* (*MsKMS1*) induced by abiotic stresses in different tissues. *A* - 20% (m/v) polyethylene glycol 6000, *B* - 200 mM NaCl, and *C* - 10 mg dm^{-3} abscisic acid. Means ± SEs. Different letters above the columns indicate significant differences at $P < 0.05$.
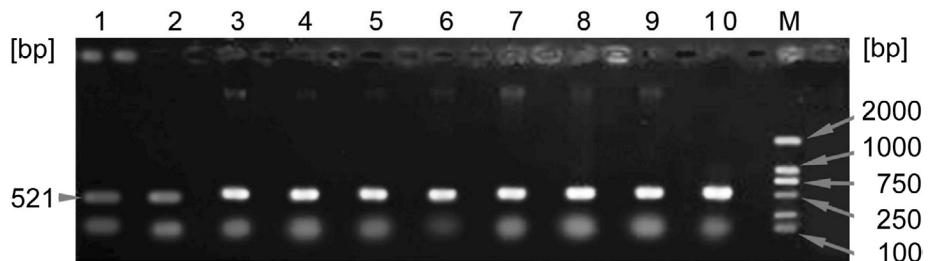
Fig. 6. Verification of the hygromycin resistance gene in transgenic tobacco seedlings by PCR amplification. Lane 1 - wild plant, lane 2 - blank control, lanes 3 to 9 - transgenic tobacco plants, lane 10 - positive control, and lane M - marker DL2000.
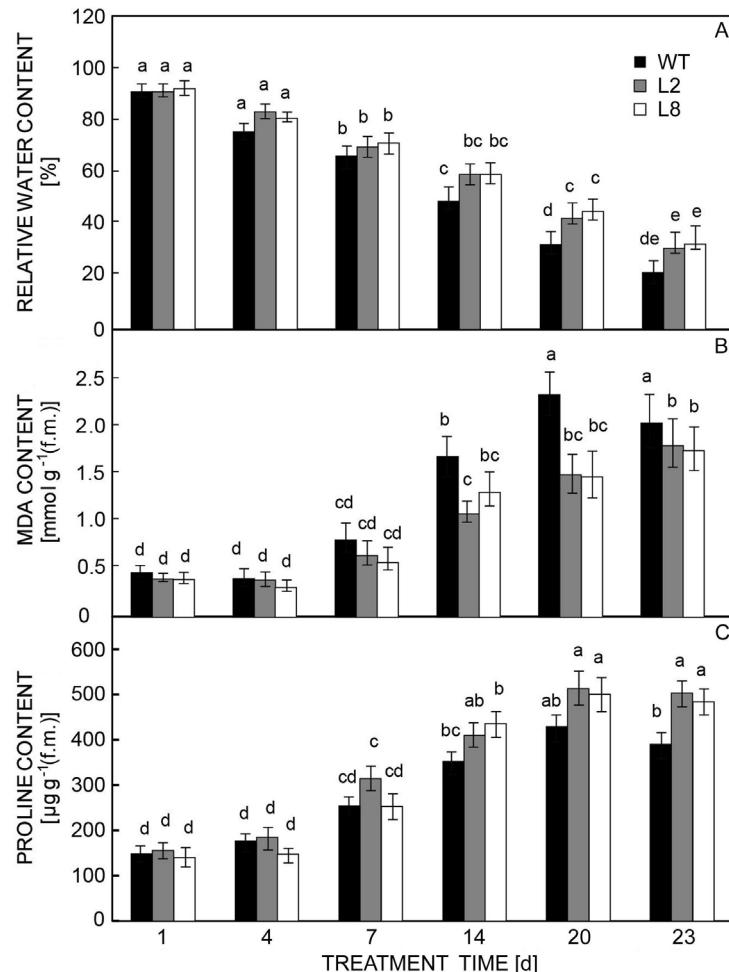
Fig. 7. Changes in relative water content (RWC, *A*), malondialdehyde (MDA) content (*B*), and proline content (*C*) in leaves of tobacco plants subjected to salt stress. WT - wild type, L2 - transgenic line 2, and L8 - transgenic line 8. Means ± SEs. Different letters above the columns indicate significant difference at *P* < 0.05.

The MDA content gradually increased with time in all plants under salt stress. The MDA content in transgenic plants was significantly lower than in the non-transgenic controls at 14 d and 20 d. A similar trend was observed at other time points, although the difference in MDA content was not significant between groups (Fig. 7*B*). This indicated that the expression of *MsKMS1* generally improved cell membrane stability and mitigated salt stress damage in transgenic plants, thereby contributing to higher salt tolerance.

The proline content rapidly increased with time in all tobacco plants under salt stress. Generally, transgenic plants had higher proline content than non-transgenic plants, and the difference between groups was significant at 23 d (Fig. 7*C*). This might also indicate that the transgenic plants expressing *MsKMS1* had a higher salt tolerance than the non-transgenic plants.

Discussion

In this study, specific primers were designed in accordance with an expressed sequence tag retrieved from the *M. truncatula* database. The designed primers were used in combination with the RACE technique to clone a novel KMS1-encoding gene, *MsKMS1,* from alfalfa. The *BLASTp* analysis showed that MsKMS1 shared high amino acid sequence similarities with the KMS1 of other plants, especially *M. truncatula* and *C. arietinum*. Further analysis indicated that *MsKMS1* encoded a vacuole membrane protein containing a conserved SNARE domain and six transmembrane domains.

The SNARE proteins are known to participate in plant defense responses against abiotic stresses (Bao *et al.* 2008a,b). The mechanisms underlying the role of SNARE proteins in defense responses have been investigated in many studies. Bao *et al.* (2008a,b) found that the expression of the *SNARE11* gene, *Oryza sativa Novel Plant SNARE 11 (NPSN11)*, in *Oryza sativa* was elevated by H_2O_2 but reduced by NaCl or PEG 6000 treatment. The transformation of *Oryza sativa NPSN11* into yeast and tobacco cells improved their resistance to H_2O_2 and increased their sensitivity to NaCl and mannitol. In *Arabidopsis*, the deletion of the SNARE gene *Arabidopsis syntaxins vesicle-associated membrane protein 7I* was found to reduce stomatal closure, increase the rate of water loss in leaves, and decrease plant tolerance to drought stress. In addition, *Arabidopsis syntaxins vesicle-associated membrane protein 7I* controlled the transport of H_2O_2 in vesicles and mediated the intracellular accumulation and distribution of reactive oxygen species, thereby regulating plant tolerance to salt stress (Leshem *et al.* 2010)

The KMS1 was first reported in *A. thaliana* as an endoplasmic reticulum-related protein that is involved in the early secretory pathway (Wang *et al.* 2011). KMS1 was localized to the endoplasmic reticulum in *A. thaliana*, which was similar to the intracellular location of homologs in other plants (Bard *et al.* 2006, Calvo-Garrido *et al.* 2008). In contrast, Sauermann (2008) claimed that the mammalian homolog of KMS (vacuole membrane protein 1) was located on the plasma membrane. In the present study, it was found that MsKMS1 was localized to the plasma membrane, which differed from the results of previous studies on plants. Taken together, these findings suggest that the MsKMS1 homologs are all located on the membrane system and participate in the transportation or secretion pathways, thereby playing an important role in plant defense responses to abiotic stresses.

Using real-time quantitative PCR, the expression of the *MsKMS1* gene was quantified in different alfalfa tissues subjected to various abiotic stresses. *MsKMS1* expression showed tissue-specific patterns in the roots, stems, and leaves of alfalfa, with the highest expression being found in the roots. These distinct tissue-specific expression patterns of *MsKMS1* suggest that this gene may have versatile functions in alfalfa. Moreover, an upregulation of *MsKMS1* expression was observed in alfalfa plants under different abiotic stresses. This indicates that *MsKMS1* is involved in the responses of alfalfa to salt (NaCl), drought

(PEG6000), and ABA. In addition, the transgenic tobacco plants had higher leaf RWC and proline content, but lower MDA content, compared with the non-transgenic plants under salt stress. This confirms that *MsKMS1* plays a role in improving the salt tolerance of alfalfa. To our knowledge, this study provides the first evidence for the function of *KMS1* in abiotic stress resistance in plants.

Conclusions

In this study, a novel KMS1-encoding gene, *MsKMS1*, was cloned from alfalfa. The *MsKMS1* encoded a vacuole membrane protein containing a conserved SNARE domain and six transmembrane domains. The MsKMS1 was localized to the plasma membrane and the expression was detected in leaves, stems, and roots of alfalfa, with the highest expression observed in the roots. Transgenic tobacco plants obtained *via Agrobacterium*-mediated transformation showed higher salt tolerance than non-transgenic plants, indicating that *MsKMS1* expression improved the stress tolerance of plants. These findings demonstrate that MsKMS1 participates in the regulation of different processes in response to abiotic stresses.

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