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ORIGINAL ARTICLE

Molecular cloning and functional analysis of the *phosphomannomutase* (*PMM*) gene from *Dendrobium officinale* and evidence for the involvement of an abiotic stress response during germination

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Abstract Phosphomannomutase (PMM, EC 5.4.2.8) catalyzes the interconversion of mannose-6-phosphate to mannose-1-phosphate, the precursor for the synthesis of GDP-mannose. In this study, the complementary DNA (cDNA) of the *Phosphomannomutase* (*PMM*) gene was initially cloned from *Dendrobium officinale* by RACE method. Transient transform result showed that the DoPMM protein was localized in the cytoplasm. The *DoPMM* gene was highly expressed in the stems of *D. officinale* both in vegetative and reproductive developmental stages. The putative promoter was cloned by TAIL-PCR and used for searched *cis*-elements. Stress-related *cis*-elements like ABRE, TCA-element, and MBS were found in the promoter regions. The *DoPMM* gene was up-regulated after treatment with abscisic acid, salicylic acid, cold, polyethylene glycol, and NaCl. The total ascorbic acid (AsA) and polysaccharide content in all of the *35S*::*DoPMM Arabidopsis thaliana* transgenic lines #1, #2, and #5 showed a 40, 39, and 31% increase in AsA and a 77, 22, and 39% increase in polysaccharides, respectively more than wild-type (WT) levels. All three *35S*::*DoPMM* transgenic lines exhibited a higher germination percentage than WT plants when seeded on half-strength MS medium supplemented with 150 mM NaCl or 300 mM mannitol. These results provide genetic evidence for the involvement of *PMM* genes in the biosynthesis of AsA and polysaccharides and the mediation of *PMM* genes in abiotic stress tolerance during seed germination in *A. thaliana*.

Keywords Phosphomannomutase · Subcellular localization · Ascorbic acid · Polysaccharides · Seed germination · Abiotic stress tolerance

Abbreviations

ABA	Abscisic acid
DW	Dry weight
FW	Fresh weight
GMP	GDP-mannose pyrophosphorylase
HAD	Haloalkanoic acid dehalogenase
PEG	Polyethylene glycol
PMM	Phosphomannomutase
ROS	Reactive oxygen species
SA	Salicylic acid

Introduction

Phosphomannomutase (PMM, E.C. 5.4.2.8), which belongs to the haloalkanoic acid dehalogenase (HAD) superfamily, catalyzes the interconversion of mannose-6-phosphate to mannose-1-phosphate, which is a substrate for the synthesis of GDP-mannose (Conklin et al. 1999). GDP-mannose is crucial for N-glycosylation in cellulose biosynthesis, L-ascorbic acid (AsA) production, and the composition of mannose-containing polysaccharides (Keller and Kossmann 1999; Lukowitz et al.

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2001). The enzymes in the HAD superfamily can be classified into three generic categories based on divergent cap domains and PMM is grouped into a subfamily with an αββ(α)αββ topology of the cap domain (Allen and Dunaway-Mariano 2004; Burroughs et al. 2006). The function of the PMM enzyme has been widely investigated in fungi and plants. For example, the PMM enzyme was purified from baker's yeast (*Saccharomyces cerevisiae*; Glaser et al. 1959), konjak (*Amorphophallus konjac* C. Koch; Murata 1976), *Cassia corymbosa* (Small and Matheson 1979), red alga (*Galdieria sulphuraria*; Oesterhelt et al. 1996), maize (*Zea mays* L.; Popova et al. 1998), and courgette (*Curbita pepo* L.; Hancock et al. 2003).

The gene encoding the PMM protein was isolated from *S. cerevisiae* and named *SEC53* (Kepes and Schekma 1988). *PMM* genes were also identified in fungi such as *Candida albicans* and *Kluyveromyces lactis* (Smith et al. 1992; Staneva et al. 2004), in mammals, including in humans and mice (Hansen et al. 1997; Heykants et al. 2001), and in several plants [Arabidopsis, *Arabidopsis thaliana* (L.) Heynh.; tobacco, *Nicotiana benthamiana* Domin; tomato, *Solanum lycopersicum* L.; wheat, *Triticum aestivum* L.; barley, *Hordeum vulgare* Betzes; soybean, *Glycine max* (L.) Merr.; stiff brome, *Brachypodium distachyon* (L.) P.Beauv.; acerola, *Malpighia glabra* L.] (Qian et al. 2007; Badejo et al. 2009; Yu et al. 2010). Two *PMM* genes, *PMM1* and *PMM2*, have been identified in both mice and humans (Hansen et al. 1997; Matthijs et al. 1997; Heykants et al. 2001). *PMM2* deficiency is the most frequent cause of congenital disorders of glycosylation-Ia (CDG-Ia) (Westpha et al. 2001; Grünewald 2009; Cline et al. 2012) and early embryonic lethality (Thiel et al. 2006), whereas *PMM1* is not associated with any disease (Cromphout et al. 2006). The yeast (*S. cerevisiae*) temperature-sensitive mutant *sec53-6* cannot produce GDP-mannose at 37 °C. *A. thaliana* temperature-sensitive *pmm-2* disrupts protein glycosylation and triggers cell death as these plants are deficient in the biosynthesis of GDP-mannose (Conzelmann et al. 1990; Hoeberichts et al. 2008). PMM is an upstream enzyme in GDP-mannose synthesis that has also been shown to be involved in AsA synthesis. For example, AsA content was increased when the *PMM* genes from higher plants (*A. thaliana* and *M. glabra*) were overexpressed in tobacco (Qian et al. 2007; Badejo et al. 2009). On the other hand, GDP-mannose, which is the downstream product of PMM, has been shown to act as a donor by providing mannose for the biosynthesis of mannose-containing polysaccharides (Elbein 1969; Dalessandro et al. 1986; Edwards et al. 1989). However, no evidence exists that the *PMM* gene is related to the synthesis of polysaccharides in plants.

Dendrobium officinale, which is a traditional Chinese medicinal plant with an abundance of polysaccharides, displays immunomodulatory and hepatoprotective activities (Ng et al. 2012). The stems of *D. officinale* are rich in bioactive polysaccharides composed predominantly of mannose residues (Meng et al. 2013; He et al. 2015). Based on an understanding of the importance of the *PMM* gene relative to the synthesis of GDP-mannose, which has been demonstrated to be involved in the synthesis of AsA and polysaccharides, in this study, *DoPMM* from *D. officinale* was cloned to investigate the relationship between *DoPMM* and AsA, polysaccharide biosynthesis, as well as abiotic stress tolerance during seed germination in *A. thaliana* transformed with this gene. Since abiotic stresses such as salinity and drought stress are major factors limiting crop productivity, this work is important for understanding the mechanisms underlying a plant's response to these stresses. Moreover, genetically modified crops would theoretically be able to add more AsA or bioactive polysaccharides to the human diet.

Materials and methods

Plant materials and growth condition

D. officinale seedlings were cultured on half-strength Murashige and Skoog (MS) (Murashige and Skoog 1962) medium containing 0.1% activated carbon, 2% sucrose, and 0.6% agar (pH 5.4) and were grown in a growth chamber at 26 ± 1 °C, 40 μmol m^{-2} s^{-1}, and a 12-h photoperiod. *D. officinale* plants used in gene cloning and expression analysis, and which sprouted in April, were grown and maintained in pots cultivated in a greenhouse (Guangzhou, China) under natural conditions. *A. thaliana* (ecotype Columbia) was used as the WT. WT and transgenic *A. thaliana* plants were grown in a mixture of topsoil and vermiculite (1:3, *v*/*v*) in a growth chamber under a 16-h photoperiod and at 22 °C. Liquid Hyponex fertilizer (N:P:K = 6-10-5, diluted 1000-fold; Hydroponic Chemicals Co., Ohio, USA) was used to water *A. thaliana* plants periodically.

Cloning of *PMM* gene from *D. officinale*

Stems from the adult stage of *D. officinale* (about 10 months after germination in April) were used to extract total RNA with Column Plant RNAout2.0 (Tiandz Inc., Beijing, China) according to the manufacturer's protocol. The first complementary DNA (cDNA) strands were synthesized by reverse transcription PCR (RT-PCR) using an M-MLV Reverse Transcriptase Kit (Promega, Madison, USA). The primers (forward: 5′-CAGGGCTAATTGTGCTGT-3′, reverse: 5′-TGCGTCCTATTGGAGAAA-3′) were designed for cDNA fragment amplification based on *Phalaenopsis PMM* sequences (Contig980, http://orchidbase.itps.ncku.edu.tw/est/home2012.aspx). A partially conserved 0.4-kb sequence was generated in a 20-μL PCR reaction mixture using LA *Taq* polymerase kit (Takara Bio Inc., Dalian, China) with the following amplification conditions: denaturation of cDNA at 94 °C for 2 min, 30 cycles of 94 °C for 30 s,

54 °C for 30 s, and 72 °C for 1 min. The PCR product fragment was purified with the Gel Extraction Kit (Dongsheng Biotech, Guangzhou, China), cloned into pMD18-T vector (Takara Bio Inc.) then sequenced at the Beijing Genomics Institute (BGI, Shenzhen, China).

The SMARTer RACE cDNA Amplification Kit (Clontech Laboratories) was used to generate both 5′ and 3′ RACE-Ready cDNA. From the 0.4 kb nucleotide sequence mentioned above, four other primers were designed for 5′ and 3′ RACE PCR, two GSPs antisense primers for 5′-RACE PCR [5′-GTTTGCCAATAAGTTCACCA-3′ (GSP1), 5′-CCAACTGTAACTACCTTTCG-3′ (GSP2)] and two sense primers for 3′-RACE PCR [5′-CGAAAGGTAGTTACAGTTGG-3′ (GSP3), 5′-GAACTTATTGGCAAACAGAA-3′ (GSP4)]. For 5′ RACE, 1 μL of 5′ RACE-Ready cDNA was used as the template with primer GSP1 and Universal Primer A Mix (UPM), which was provided in the kit. This served as the primary PCR and was run for 30 cycles. 0.5 μL was taken from the primary PCR product and used as the template for nested PCR with primer GSP2 and Nested Universal Primer A (NUP), which was also provided in the kit. For 3′-RACE, primers GSP3 and UMP were used for the primary PCR reaction and GSP4 and NUP for the nested reaction. The 5′ and 3′ RACE PCR products were purified using the Gel Extraction Kit (Dongsheng Biotech, Guangzhou, China), cloned into pMD18-T Vector and sequenced at BGI.

Subcellular localization of DoPMM

The full-length coding sequences (excluding the termination codon) of *DoPMM* were amplified and recombined into pSAT6-EYFP-N1 (Citovsky et al. 2006) at the *Noc*I site, downstream of the enhanced yellow fluorescence protein (YFP) sequence. The DoPMM-YFP fusion protein was driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter. The clone was verified by DNA sequencing at BGI. Mesophyll protoplasts were isolated from the fresh leaves of a 4–5-week-old WT *A. thaliana* plant and used to transfect an empty vector (35S::YFP) and construct 35S::DoPMM-YFP. The mesophyll protoplast isolation and transfection system was as described by Yoo et al. (2007). On the next day, YFP fluorescence was visualized by a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany). A primer pair was designed to construct the vector as follows: forward, 5′-CGAACGATAGCCATGGGAGACAGAATGACTGGGAGGATGC-3′; reverse, 5′-TGAGTCCGGACCATGGTGGCATCTCCAGCTTGCTTTGCT-3′.

Cloning and analysis of the putative promoter of *DoPMM*

The putative *DoPMM* promoter was cloned by using a thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) method described by Singer and Ellen Burke (2003). Three gene-specific primers (GSP1, GAAAAAGTGAGATTCAAATG; GSP2, TAGCATCTTCGGAGTTATAG; GSP3, TACATCAAACAGCACAATAA) were used together with arbitrary degenerate primers (AD primers, NGTCGASWGANAWGAA) for amplification. The tertiary TAIL-PCR product was purified, cloned into pMD18-T vector (Takara Bio Inc.), then sequenced at the BGI. An online database for prediction of plant *cis*-acting regulatory elements (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to search for *cis*-acting regulatory elements.

Plant hormone treatments and stress treatments

The plant hormones abscisic acid (ABA) and salicylic acid (SA) regulate a plant's adaptive response to various environmental stresses (Borsani et al. 2001; Ananieva et al. 2004; Lu et al. 2009). To explore the relationship between the expression of the *DoPMM* gene and ABA and SA, and stress resistance (cold, PEG and salinity), *D. officinale* seedlings were subject to stress treatments to monitor gene expression. In vitro-derived seedlings, 8–9 cm in height, were exposed to 100 μM ABA (Sigma-Aldrich, Shanghai, China), 0.5 mM SA (Sigma-Aldrich), 4 °C, 15% PEG (Sigma-Aldrich), and 250 mM NaCl (Guangzhou Chemical Reagent Factory, Guangzhou, China). After seedlings were treated for 6 h, roots were harvested, immediately frozen in liquid nitrogen and stored at −80 °C until RNA isolation. Control seedlings were treated with distilled water.

Generation of *DoPMM* overexpression lines

The full-length coding sequences of *DoPMM*, excluding the termination codon, was amplified with the PrimeSTAR HS DNA Polymerase kit (Takara Bio Inc., Dalian, China) with the following amplification conditions: denaturation of cDNA at 94 °C for 3 min, 30 cycles of 98 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min. The PCR product was purified with the Gel Extraction Kit (Dongsheng Biotech). *DoPMM* was then inserted into the pCAMBIA 1302 vector at the *Nco*I site using the In-Fusion® HD Cloning Kit (Takara Bio Inc.) according to the manufacturer's protocol. Correctness of the construct was confirmed by complete sequencing at BGI. Transformation by *A. thaliana* was performed using the floral dip method (Clough and Bent 1998) using *Agrobacterium tumefaciens* strain EHA105 harboring the *35S::DoPMM-mGFP* construct. Eight transgenic lines were identified and three homozygous lines (#1, #2, and #5) were used for further experiments. The primer pairs designed to construct an overexpression vector were as follows: forward, 5′-GGACTCTTGACCATGGGAGACAGAATGACTGGGAGGATGC-3′; reverse, 5′-GTCAGATCTACCATGGTGGCATCTCCAGCTTGCTTTGCT-3′.

Determination of AsA and polysaccharide content

Seven-day-old seedlings (50 mg) growing on half-strength MS (containing 1.5% sucrose and 0.8% agar, pH 5.7) were harvested to determine AsA content. Total AsA (reduced AsA and oxidized AsA) was determined by the ascorbate oxidase method described by Ueda et al. (2013). Each sample was assayed as three replicates. The aerial parts of 1-month-old *A. thaliana* plants in the vegetative stage were harvested, dried, and crushed into a powder by a DFT-50 pulverizer (Xinno Instrument Equipment Inc., Shanghai, China). Powdered samples were used to determine water-soluble polysaccharide content, as described by He et al. (2015) with a few modifications in the extraction step. Briefly, 0.5 g of sample powder was pre-extracted with 80% ethanol at 80 °C and then further extracted with 100 mL of distilled water for 2.5 h at 100 °C. The phenol-sulfuric acid method was used to determine water-soluble polysaccharides described by Dubois et al. (1956) and He et al. (2015).

Germination assay under salt and mannitol stress

Seeds of WT and *35S*::*DoPMM* transgenic lines were grown simultaneously, harvested, and stored under the same conditions. One hundred surface-sterilized seeds of each genotype were seeded on half-strength MS (containing 1.5% sucrose and 0.8% agar, pH 5.7) supplemented with 150 mM NaCl or 300 mM mannitol. Seeds of both genotypes sown on half-strength MS medium served as the control. After stratification for 2 days at 4 °C in the dark, plates were incubated in a 16-h photoperiod (100 μmol m^{-2} s^{-1}) at 22 °C and scored daily from the second day until the sixth day. Germination was defined as radicle emergence from the testa. Single experiments were performed in duplicate and every experiment was repeated in triplicate.

Quantitative real-time PCR analysis

Total RNA of *D. officinale* was extracted as indicated above. Two μg of total RNA isolated from samples were reverse transcribed for the first-strand cDNA using the M-MLV Reverse Transcriptase Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions and then used for quantitative real-time PCR (qRT-PCR). The *DoPMM* gene-specific primer pair (forward: 5′-GCTCCCAGAAAGGC TATAACTC-3′; reverse: 5′-CTCCAACGACACCA ACTGTAA-3′) was designed by online Primerquest software (http://www.idtdna.com/Primerquest/Home/Index). The *AtPMM* (chromosomal locus At2g45790) gene-specific primer pair (forward: 5′-ATTCCCTACCACCGGATTCA-3′; reverse: 5′-TTCTTGAGATGAGTAGTGTT-3′) was designed according to the 3′ and 5′ untranslated regions. qRT-PCR was performed using the SYBR Premix Ex Taq™ Kit (Takara Bio Inc.) according to the manufacturer's protocol in an ABI 7500 Real-time system (ABI, CA, USA). Amplification conditions were denaturation of cDNA at 95 °C for 2 min, followed by 40 cycles of amplification (95 °C for 15 s, 60 °C for 1 min) and plate reading after each cycle. The relative expression level was calculated by the threshold cycle (Ct) value and normalized using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The constitutively expressed gene, *D. officinale actin* (NCBI accession number: JX294908), was used as the internal control (forward: 5′-TCCCAAGGCAAACAGAGAAA-3′; reverse: 5′-GGCC ACTAGCATATAGGGAAAG-3′), based on the advice of He et al. (2015).

Semi-quantitative RT-PCR

Total RNA from 1-month-old *A. thaliana* WT and transgenic lines was extracted as indicated above. Two μg of each RNA sample was reverse transcribed for the first-strand cDNA with the M-MLV Reverse Transcriptase Kit (Promega). The DreamTaq™ Green PCR Master Mix kit (Takara Bio Inc.) was used for semi-quantitative RT-PCR analysis according to the manufacturer's instructions using the following thermocycling conditions: denaturation of cDNA at 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The pair primers for the *DoPMM* gene were forward, 5′-GAGGATGCCAGGGCTTATTGTACT-3′ and reverse, 5′-GGAAGTGGAGTTTACACTGCTCAG-3′. The pair primers for the *AtPMM* gene were the same pair primers used in qRT-PCR. The *A. thaliana* ubiquitin gene (*AtUBQ10*, TAIR accession number: AT4G05320.2) was used as the control (forward: 5′-GATCTTTGCCGGAAAACAATTGGAGG ATGGT-3′, reverse: 5′-CGACTTGTCATTAGAAAGAA AGAGATAACAGG-3′).

Statistical analyses

Data were analyzed using SigmaPlot12.3 software (Systat Software Inc., San Jose, California, USA). Analysis of *DoPMM* expression in different organs used one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). All other data were analysis by a *t* test.

Results

Cloning and analysis of the *DoPMM* gene

The complete cDNA of the *DoPMM* gene contains 1086 bp with an open reading frame of 759 bp encoding a protein of 252 amino acid residues with a calculated molecular mass of 28.618 kDa and an isoelectric point of 5.90. The cDNA of *DoPMM* has been submitted to GenBank with the following

accession number: KF195558. BLAST of the protein in NCBI (http://www.ncbi.nlm.nih.gov/BLAST) indicated that the DoPMM protein had high identity with PMM proteins from other plant species, sharing 86% similarity with *Elaeis guineensis* EgPMM (NCBI accession No.: XP_010934857), 86% similarity with *Vitis vinifera* VvPMM (NCBI accession No.: XP_00226767), and 86% similarity with *Populus euphratica* PePMM (NCBI accession No.: XP_011035818). The DoPMM protein shares four conserved motifs 1–4 with other reported PMM proteins that belong to the PMM family within the HAD superfamily (Fig. 1). Based on a previous PMM protein sequence analysis (Silvaggi et al. 2006), the secondary structure of DoPMM was predicted with online prediction software (http://distill.ucd.ie/porter/ and http://www.predictprotein.org/), revealing that DoPMM consists of 11 β-sheets and 9 α-helices (Supplementary Fig. 1). In order to better characterize the DoPMM protein, comparative modeling of its three-dimensional structure was performed using SWISS-MODEL (http://swissmodel.expasy.org/). The tertiary structure of the DoPMM protein has a cap domain and a core domain, the former in an open conformation, with the active site exposed to solvent (Supplementary Fig. 2).

DoPMM is localized in the cytoplasm

DoPMM prediction by TMHMM v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) topology prediction software showed that DoPMM had no transmembrane spanning domain (Supplementary Fig. 3). PMM from yeast (*S. cerevisiae*) and humans is localized in the cytoplasm (Eager et al. 1988; Hansen et al. 1997). To analyze the localization of DoPMM, a subcellular protein fractionation experiment was performed by using mesophyll protoplasts of *A. thaliana* seedlings expressing the DoPMM-YFP fusion protein. PEG-mediated transfection of the fusion construct in protoplasts followed by confocal laser scanning microscopy showed its localization in the cytoplasm (Fig. 2), consistent with the previously reported localization of PMM in yeast and humans (Eager et al. 1988; Hansen et al. 1997).

Expression pattern of the *DoPMM* gene in different organs

To investigate the expression pattern and level of *DoPMM* in the vegetative and reproductive stages, RNA was isolated from the organs (roots, stems, leaves, and flowers) of *D. officinale* in two stages of development (vegetative and reproductive, Supplementary Fig. 4), and monitored by qRT-PCR. The *DoPMM* gene displayed tissue-specific abundance patterns and showed high expression in the stems of both stages. Evidently, in the vegetative stage, *DoPMM* transcript was found in all the tested organs with the highest expression in the stems and lowest expression in the roots (Fig. 3a). In the reproductive stage, the *DoPMM* gene was expressed in all four organs (Fig. 3b). Similar to the vegetative stage, stems showed highest *DoPMM* expression—about threefold higher than roots and leaves—while flowers displayed second highest expression. The *D. officinale* stem is the principal vessel for the storage of polysaccharides (He et al. 2015). The highest *DoPMM* gene expression in the stem was consistent with a high polysaccharide content (He et al. 2015).

Analysis of stress-related *cis*-elements in the *DoPMM* promoter and response of the *DoPMM* gene to ABA, SA, and abiotic stress

A *cis*-regulatory element is a non-coding portion of DNA that is responsible for transcriptional regulation. To further understand the possible transcriptional regulatory mechanism of the *DoPMM* gene in abiotic or biotic stresses responses of *D. officinale*, we cloned and analyzed the *cis*-elements of the *DoPMM* promoter. About 1000 bp upstream of the translation start site was obtained by TAIL-PCR and was used to search for regulatory elements. A total of 21 TATA-box elements were found in the promoter regions and also several light-responsive elements, including the ATCT-motif, G-Box, I-box, and L-box. The ABRE *cis*-acting element, which is involved in responsiveness to abscisic acid (ABA), and a TCA *cis*-acting element involved in responsiveness to salicylic acid (SA) were found in the *DoPMM* promoter (Fig. 4). ABA and SA are plant hormones that play an important role in regulating plant adaptive responses to various environmental stresses (Aimar et al. 2011). Moreover, two MBS *cis*-elements, which had appropriate binding sites to MYB transcription factors that are involved in drought stress, were found in the *DoPMM* promoter. This indicates that the *DoPMM* gene may be related to stress response in *D. officinale*.

To explore the response of the *DoPMM* gene under stress hormones, namely ABA and SA, and adverse stress environments (cold, PEG and salt), *D. officinale* seedlings were subjected to these stresses. *DoPMM* gene expression was then determined by qRT-PCR. The *DoPMM* gene was induced by all five treatments, indicating that it may play an important role in stress tolerance in *D. officinale*. *DoPMM* was significantly up-regulated under ABA, SA, cold, PEG, and salinity treatments (Fig. 5).

AsA and polysaccharide content increased in *35S*::*DoPMM* transgenic lines

To generate *35S*::*DoPMM* transgenic lines, the *DoPMM* gene was cloned into the pCABIA 1302 vector at the *Nco*I site, driven by the CaMV 35S promoter (Fig. 6a), and transformed into *A. thaliana*. Expression of the *DoPMM* gene was detected in all three transgenic lines but not in the WT plant (Fig. 6b, c). The only gene (*AtPMM*) predicted to encode a putative PMM was also detected in all three transgenic lines and the WT

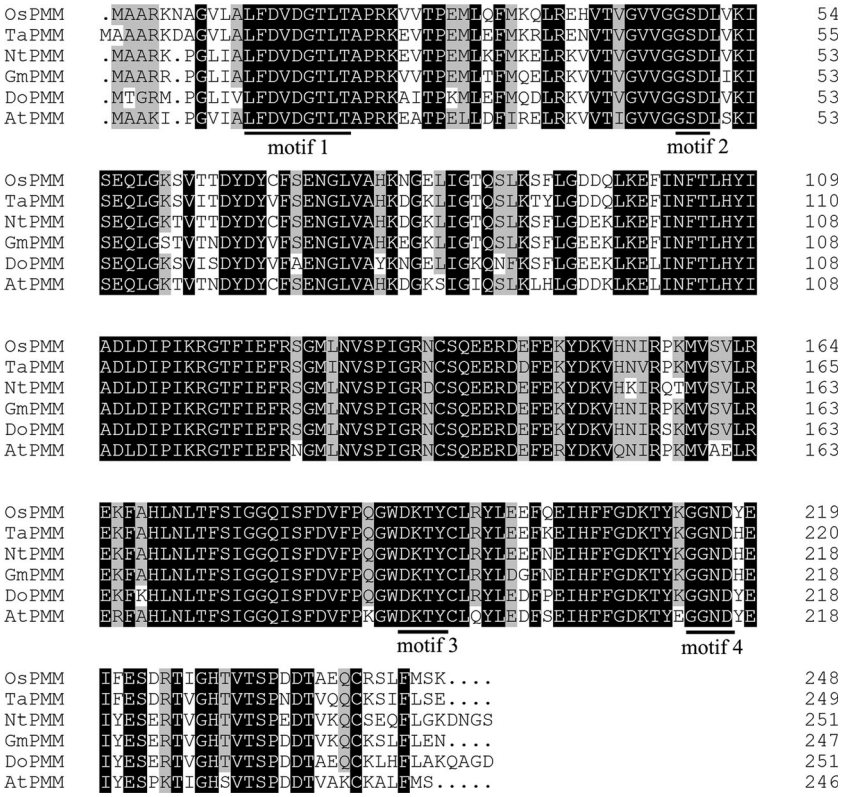


Fig. 1 Sequence alignment of various plant PMM proteins by ClustalX2. Identical residues in the same sequences are *shaded in black*, while similar residues are *shaded in gray*. Protein sequences used for alignment are as follows: NtPMM (*Nicotiana tabacum*), ABD97874; AtPMM (*Arabidopsis thaliana*), AEC10601; GmPMM (*Glycine max*), ABD97873; TaPMM (*Triticum aestivum*), DQ442996; OsPMM (*Oryza sativa* Japonica Group), NP_001054295

plant. The *AtPMM* gene showed no difference in expression level among the transgenic lines and the WT plant (Fig. 6c, d). This indicates that the *DoPMM* gene from *D. officinale* could be successfully transcribed in *A. thaliana* and enhanced the level of *PMM* gene expression. No phenotypic differences in plant size and fresh biomass were observed between the transgenic lines and the WT plant (Fig. 6e).

PMM catalyzes the formation of mannose-1-phosphate, which is the precursor of GDP-mannose. GDP-mannose is involved in the biosynthesis of AsA and polysaccharides (Conklin et al. 1999; Keller and Kossmann 1999). Thus, AsA and polysaccharide content were determined in the three transgenic lines and compared with the content of the WT plant. As expected, the AsA and polysaccharide contents increased significantly in the transgenic lines, about 4.7, 4.6, and 4.4 μmol g^{-1} FW of AsA, and about 104, 72, and 82 mg g^{-1} DW of water-soluble polysaccharides in line #1, #2, and #5, respectively (Fig. 7a, b). The WT plant only contained about 3.3 μmol g^{-1} FW of AsA and 59 mg g^{-1} DW of water-soluble polysaccharides (Fig. 7a, b). These results suggest that *DoPMM* is involved in the synthesis of AsA and polysaccharides in *A. thaliana*.

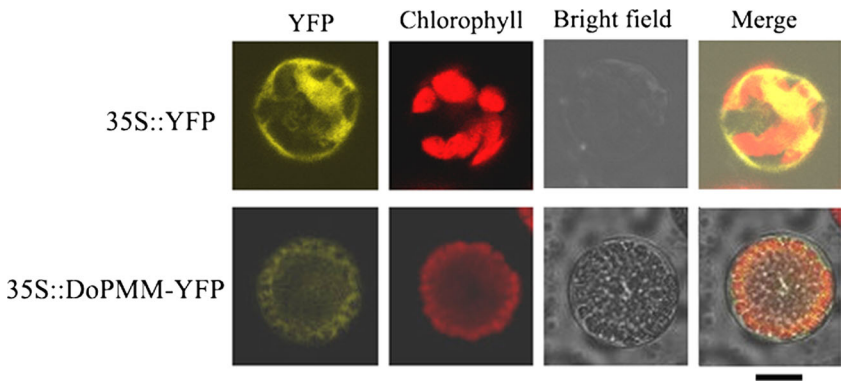


Fig. 2 Transient expression in protoplasts using the DoPMM-YFP fusion construct was used to determine the subcellular localization of DoPMM. An empty vector (35S::YFP) and 35S::DoPMM-YFP constructs were separately transferred into *Arabidopsis thaliana* mesophyll protoplasts and fluorescence was observed under a confocal laser scanning microscope. Images of the YFP signal (*yellow*), bright field, chloroplast fluorescence (*red*), and overlay (*yellow plus red*) from the same cell are shown. *Bars* 20 μm

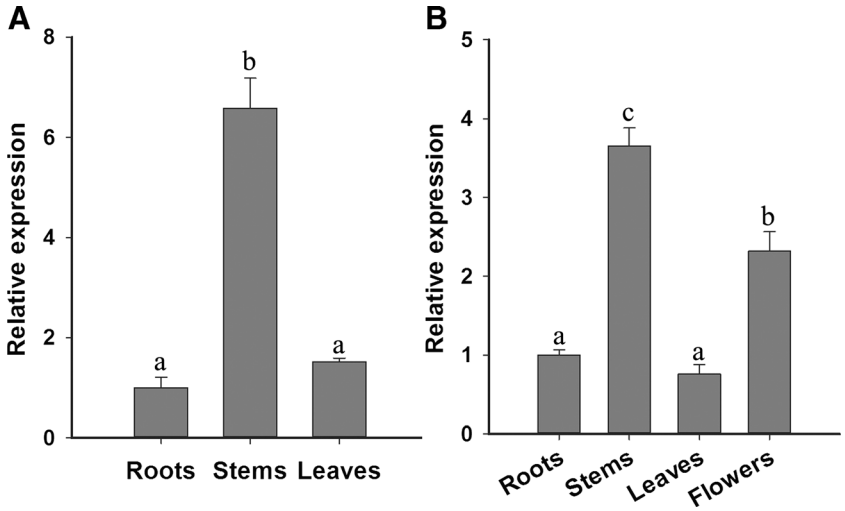
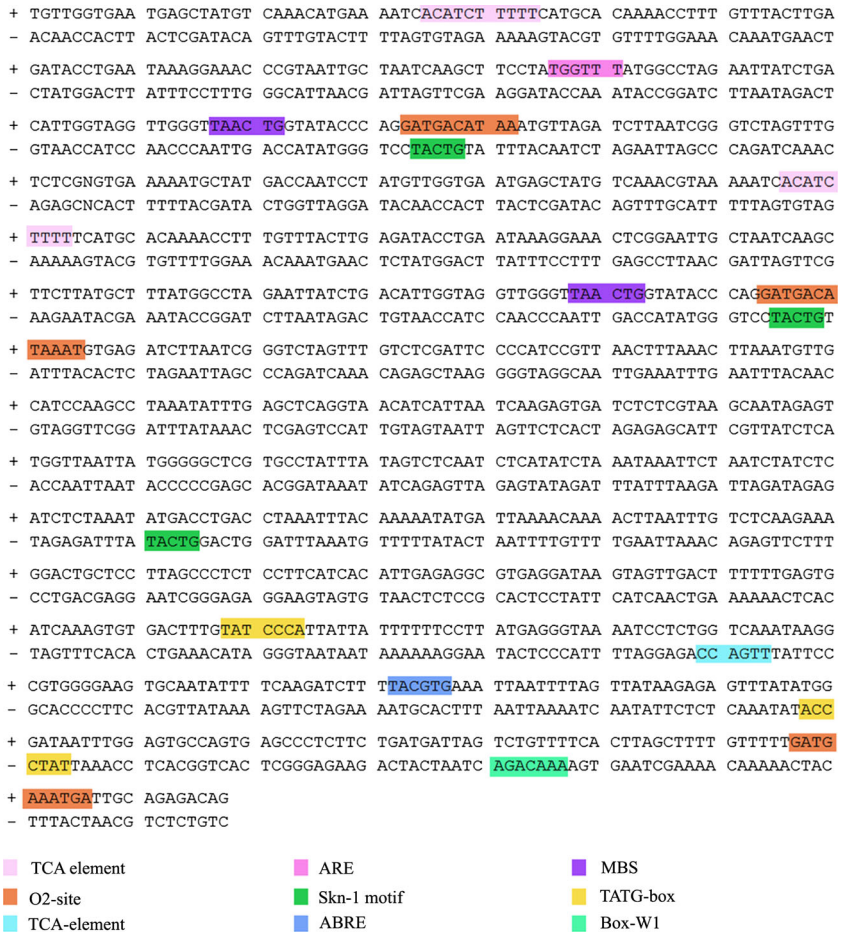


Fig. 3 qRT-PCR analysis of transcript levels for *DoPMM* in different organs. **a** The roots, stems, and leaves from *D. officinale* seedlings. The seedlings were cultured on half-strength MS medium about 10 months after germination. **b** The roots, stems, leaves, and flowers from the adult stage of *D. officinale*. Adult plants, about 13 months after germinating, were maintained in pots cultivated in a greenhouse. *Different letters* indicate significant differences between organs at $P < 0.05$ (DMRT). Each *data bar* represents the mean ± standard deviation (SD) ($n = 3$)

Fig. 4 Predicted *cis*-elements in the putative promoter regions of the *DoPMM* gene. The promoter sequence about 1000 bp upstream of the translation start site was analyzed. The various *colored sequences* represent different putative *cis*-elements identified in the PlantCARE database. *ABRE*, a *cis*-acting element involved in responsiveness to abscisic acid; *ARE*, *cis*-acting regulatory element essential for anaerobic induction; *Box-W1*, fungal elicitor-responsive element; *GARE-motif*, gibberellin-responsive element; *MBS*, MYB binding site involved in drought induction; *TATC-box*, *cis*-acting element involved in responsiveness to gibberellin; *TCA-element*, *cis*-acting element involved in responsiveness to salicylic acid; *Skn-1-motif*, *cis*-acting regulatory element required for endosperm expression; *O_2-site*, *cis*-acting regulatory element involved in the regulation of zein metabolism



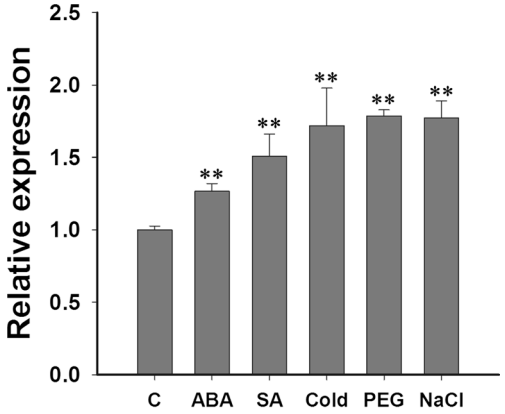


Fig. 5 qRT-PCR analysis of the transcript levels of *DoPMM* under ABA, SA, could, PEG, and salinity treatments in *D. officinale* seedlings. The *DoPMM* gene was induced by all treatments and was up-regulated under cold, PEG, and salt stresses. The seedlings were cultured on half-strength MS medium about 10 months after germination. $*P < 0.05$, $**P < 0.01$ between WT and transgenic lines by a *t* test. Each *data bar* represents the mean ± SD ($n = 3$)

Overexpressed *DoPMM* in *A. thaliana* increased germination percentage under abiotic stresses

Both salinity stress and water stress can induce osmotic stress and reduce the ability of a plant to take up water, and this is accompanied by a suite of metabolic changes (Munns 2002). Polysaccharides can serve as compatible solutes and contribute to the osmotic adjustment of a plant (Iraki et al. 1989). Based on this premise, the germination percentage under osmotic stress caused by salinity and mannitol was analyzed. WT and transgenic seeds germinated well in control medium and showed no obvious difference to WT but germinated and grew better than WT when supplemented with salt or mannitol (Fig. 8). On the third day after stratification, WT seed germination was just 14 and 31% under salt and mannitol stress, respectively (Fig. 8), while the germination of transgenic line #1, #2, and #5 seeds was 36, 24, and 22% under salt stress and 56, 50, and 46% under mannitol stress, respectively (Fig. 8). This result indicates that the *DoPMM* gene improved osmotic stress tolerance caused by salinity and mannitol during seed germination.

Discussion

PMM catalyzes the interconversion of mannose-6-phosphate to mannose-1-phosphate, which is the precursor for the biosynthesis of GDP-D-mannose (Hoeberichts et al. 2008). GDP-mannose participates in different types of glycosylation and in the biosynthesis of AsA and mannose-containing polysaccharides (Hinman and Villemez 1957; Lukowitz

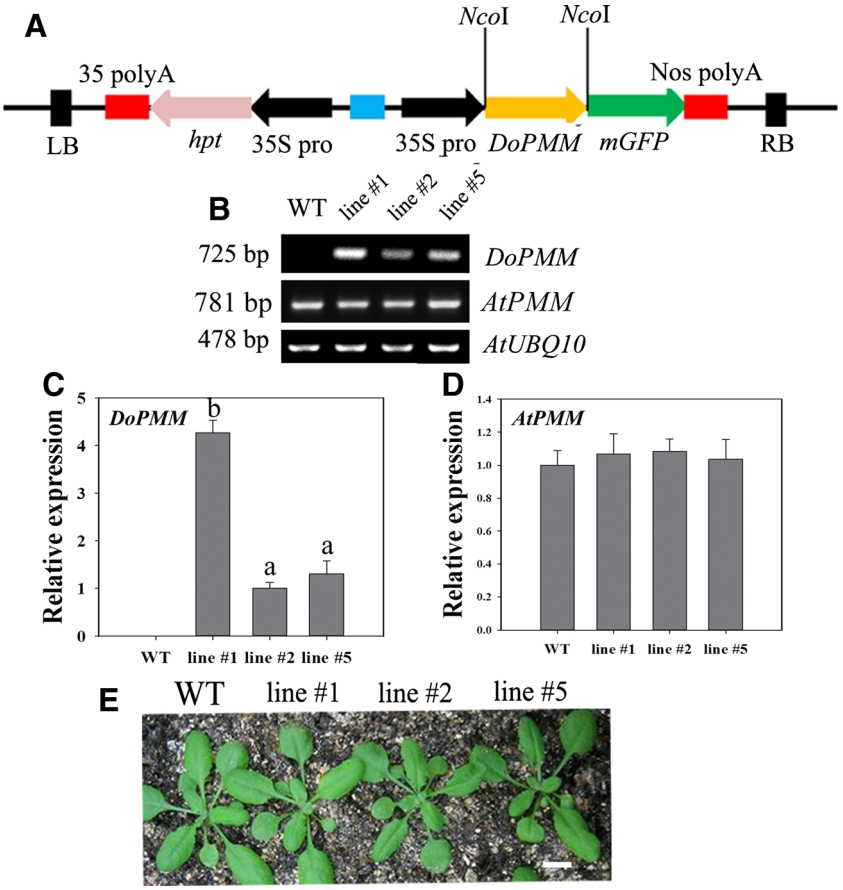


Fig. 6 Overexpression of the *DoPMM* gene in *A. thaliana*. **a** Schematic presentation of *35S*::*DoPMM* overexpression vector. **b** Analysis of the *DoPMM* and *AtPMM* genes in WT and transgenic lines by using semi-quantitative PCR. **c** Analysis of the *DoPMM* gene in WT and transgenic lines by qRT-PCR. **d** Analysis of the *AtPMM* gene in WT and transgenic lines by qRT-PCR. **e** Seedlings of transgenic lines showed no difference with WT (about 1 month old). *WT* wild-type plant; *35S*::*DoPMM* transgenic lines #1, #2, and #5. *Bar* 1 cm

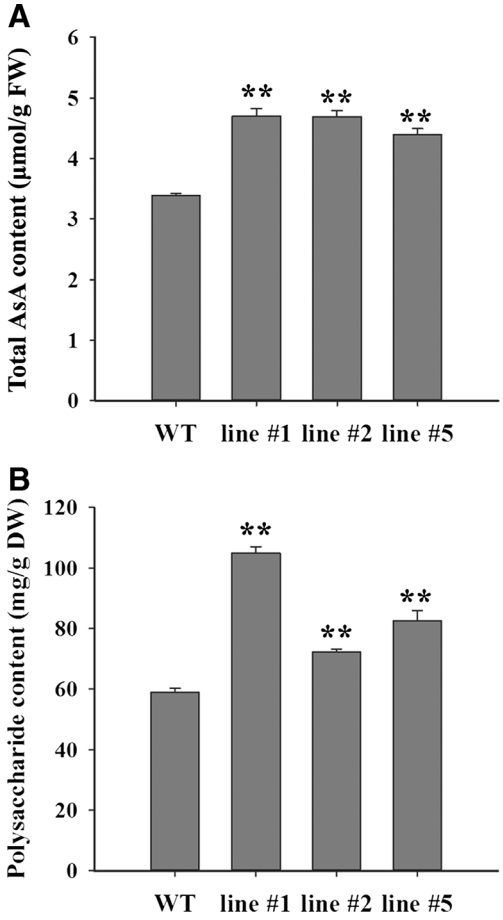


Fig. 7 Analysis of AsA and polysaccharide content in *35S*::*DoPMM* transgenic lines. **a** The AsA content in *35S*::*DoPMM* transgenic lines. **b** The polysaccharide content in *35S*::*DoPMM* transgenic lines. $^{*}P < 0.05$, $^{**}P < 0.01$ between WT and transgenic lines by a *t* test. Each *data bar* represents the mean ± SD ($n = 3$). *WT* wild-type; *35S*::*DoPMM* transgenic lines #1, #2, and #5. *FW* fresh weight; *DW* dry weight

et al. 2001; Badejo et al. 2007). In the present study, we isolated and characterized a *PMM* gene from *D. officinale* named *DoPMM*. Amino acid sequence alignment showed that it shared significant sequence similarity with NtPMM, AtPMM, GmPMM, OsPMM, and TaPMM (Fig. 1), indicating that the DoPMM protein likely has the same catalytic function as other members of the PMM family. DoPMM has four conserved PMM family motifs that play a key role in the catalytic reaction (Silvaggi et al. 2006).

Based on an understanding that *D. officinale* stems are the principal vessel for the storage of polysaccharides (He et al. 2015), the roots, stems, and leaves from *D. officinale* seedlings in the vegetative phase, as well as the roots, stems, leaves, and flowers from adult plants in the reproductive phase were used to determine the relative expression level of the *DoPMM* gene by qRT-PCR. The *DoPMM* gene was expressed in all four organs with tissue-specific abundance in stems in both stages (Fig. 3). This suggests that the *DoPMM* gene is likely to be highly related to the biosynthesis of polysaccharides. The stems of *D. officinale* are used to prepare herbal medicines as they contain an abundance of bioactive mannan polysaccharides, enhancing mouse macrophage cell line (RAW264.7) proliferation, TNF-α secretion, and phagocytosis (Xing et al. 2014; Wei et al. 2016).

The *DoPMM* gene was up-regulated in response to ABA, SA, cold, PEG, and NaCl in the roots of *D. officinale* seedlings (Fig. 5). This suggests that this gene plays important roles in the regulation of abiotic stress responses. Perception of these stress signals often results in a variety of hormones that allows the plant to adapt and respond to various environmental stresses such as ABA and SA (Aimar et al. 2011). Over the past decade or so, studies have shown that ABA and SA can protect plants against abiotic stress factors by inducing a wide range of processes involved in stress tolerance such as up-regulation of antioxidant enzymes and induced stress-responsive genes (Borsani et al. 2001; Shinozaki and Yamaguchi-Shinozaki 2007; Lu et al. 2009). *DoPMM* is an ABA- and SA-inducible gene and a stress-responsive gene (Fig. 5). Drought-inducible genes are also induced by high salinity and/or ABA treatments in other plants (Shinozaki and Yamaguchi-Shinozaki 2007). The *DoPMM* gene responded to cold, PEG and salt at the transcriptional level (Fig. 5) suggesting that its product may be related to cold, drought, or salt tolerance. The downstream *GMP* gene was involved in enhancing salinity stress tolerance and alleviating both low and high temperature stress when it was overexpressed in tobacco (Wang et al. 2011; Kumar et al. 2012).

Genetic evidence shows that GDP-mannose is involved in the AsA biosynthetic pathway (Conklin et al. 1999). Moreover, GDP-mannose is also a known precursor used for the biosynthesis of mannose-containing polysaccharides such as glucomannans and galactoglucomannans (Edwards et al. 1989; Gilbert et al. 2009). The AsA and water-soluble polysaccharide content increased significantly in all three transgenic lines. This result demonstrates that the *DoPMM* gene is involved in AsA and polysaccharide biosynthesis. *MgPMM*, an orthologous gene of *DoPMM* present in *Malpighia glabra*, showed an increase in the AsA content in *MgPMM*-overexpressing transgenic tobacco (Badejo et al. 2009). Downstream *GMP* genes from *A. thaliana* and tomato (*Solanum lycopersicum* Mill.) are involved in AsA synthesis (Keller and Kossmann 1999; Lin et al. 2011). GDP-mannose catalyzed by a downstream enzyme of PMM-GMP is not only related to the synthesis of AsA but is also an important nucleotide sugar that provides mannose for the synthesis of polysaccharides (Keller and Kossmann 1999). From the above discussion and from evidence in recent studies, it can be postulated that the PMM protein catalyzes the formation of mannose-1-phosphate, providing material for the synthesis of GDP-mannose, which is used as a precursor for the biosynthesis of AsA and serves as a donor by providing mannose for the biosynthesis of mannose-containing polysaccharides.

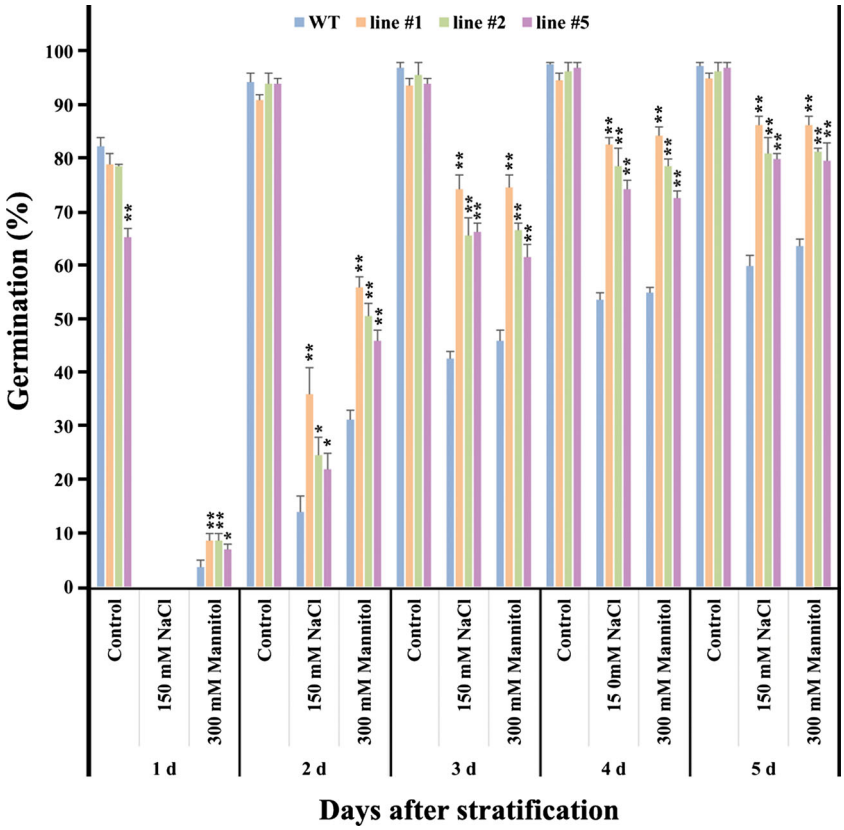


Fig. 8 Analysis of the germination of *35S*::*DoPMM* transgenic seeds under salinity and mannitol stress. **a** The germination of transgenic lines was significantly higher than WT under salinity and mannitol stress. The seeds of WT and transgenic lines were cultured on half-strength MS medium as the control. **b** Seedlings on the seventh day after stratification. Each *data bar* represents the mean ± SD of 100 seeds. *WT* wild-type; three *35S*::*DoPMM* transgenic lines #1, #2, and #5. $^{*}P < 0.05$, $^{**}P < 0.01$ between WT and transgenic lines by a *t* test. Each *data bar* represents the mean ± SD ($n = 3$)

The three *35S*::*DoPMM* transgenic lines had a higher germination percentage than the WT plant under salt and mannitol stress (Fig. 8). This suggests that *DoPMM* from *D. officinale* could increase salt and mannitol stress tolerance in *A. thaliana*. Previous studies also found that the *GMP* gene played an important role in stress tolerance in higher plants. For example, the seed germination percentage of tobacco (*Nicotiana tabacum* L.) decreased significantly under low (4 °C) and high (40 °C) temperature stresses when endogenous *GMP* gene expression in tobacco was blocked using antisense techniques (Wang et al. 2012). The tolerance of potato (*Solanum tuberosum* L.) to low (4 °C) and high (40 °C) temperature improved when the *GMP* gene of tomato was overexpressed (Lin et al. 2011). The *OsGMP* gene from rice (*Oryza sativa* L.) improved salinity stress tolerance in tobacco (Kumar et al. 2012).

Both salt and mannitol can decrease water potential and induce osmotic stress. Plants accumulate compatible solutes to cope with such adverse environments (Krasensky and Jonak 2012). Water-soluble carbohydrates can act as compatible solutes and help plants to absorb water from an osmotically stressed environment in many plant species. For example, *Ziziphus* species accumulated polysaccharides as solutes for osmotic adjustment to uptake water when drought stress was intensified (Clifford et al. 2002) while wheat (*Triticum aestivum* L.) seedlings accumulated water-soluble carbohydrate such as glucose, fructose, sucrose, and fructan under drought and NaCl stress (Kerepesi and Galiba 2000). The *35S*::*DoPMM* plants had a polysaccharide content that were increased to 22–77% of the WT plant levels and may have taken up water more easily than WT plants under both stresses. Taken together, the *35S*::*DoPMM* transgenic lines showed a high germination percentage than the WT plant.

In conclusion, the *DoPMM* gene was cloned from *D. officinale* by RACE and an analysis was conducted. This study demonstrated that the *DoPMM* gene is involved in the biosynthesis of AsA and polysaccharides. This study paves the way for analysis of the relationship between the role of the *DoPMM* gene in the biosynthesis of AsA and polysaccharides. Moreover, our findings provide clues that would allow researchers to understand the mechanism of this plant's responses to abiotic stresses during germination.

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Author contributions JD conceived the experiments and experimental design. CH, SZ, and JT analyzed the data. ZY analyzed the polysaccharides. CH and JATdS collectively interpreted all data and results and wrote all

drafts. All six authors approved the final draft for submission, take full public responsibility for the content, and abide and satisfy the conditions of authorship as defined by the four clauses of the ICMJE.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

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