Characterization and expression analysis of two mitogen-activated protein kinase kinase kinase genes from Antarctic moss Pohlia nutans

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Research Article

Keywords: Abiotic stress, Cold adaptation, Expression analysis, Mitogen activated protein kinase

Posted Date: July 13th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3151982/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Polar Biology on February 6th, 2025. See the published version at https://doi.org/10.1007/s00300-025-03352-2.

Abstract

Mitogen activated protein kinase (MAPK) signaling plays essential roles in plant growth, development and responses to environmental stresses. However, only limited information is available on the MAPK signaling genes in the bryophytes. Two full-length of mitogen-activated protein kinase kinase kinase genes (designated as *PnMEKK1* and *PnMEKK2*) were identified from Antarctic moss *Pohlia nutans*. The full length cDNA of *PnMEKK1* and *PnMEKK2* were 3012 bp and 3096 bp, encoding the receptor-like kinases of 804 and 576 amino acids, respectively. Multiple sequence alignment showed that PnMEKK1 and PnMEKK2 possessed the conserved STKc_MAP3K-like domains, but they had relatively low identities with other protein kinases. Phylogenetic analysis showed that they clustered together with the protein kinases of ferns and mosses but not higher plants. In addition, the subcellular localization analysis by observing the transient expression of PnMEKK-green fluorescence protein in *Arabidopsis* mesophyll protoplasts revealed that PnMEKK1 and PnMEKK2 were cytoplasm-localized protein kinases. Meanwhile, the mRNA expression profile of *PnMEKK1* and *PnMEKK2* were quantified by quantitative RT-PCR. Results showed that cold, salinity, drought and UV-B radiation could motivate the up-regulation of *PnMEKK1* and *PnMEKK2* mRNA expression. In addition, the application of plant hormone abscisic acid (ABA) and methyl jasmonate (MeJA) also could up-regulate the mRNA expression level. Taken together, we purposed that these two isolated PnMEKKs might involve in Antarctic mosses *Pohlia nutans* adapting to the polar extreme environments.

Introduction

Mitogen-activated protein kinase (MAPK) cascades are ubiquitous signaling module in eukaryotes, and involved in transducing extracellular signals to the nucleus (Sinha et al. 2011; Zhang et al. 2022). MAPK cascades consist essentially of three components, namely the MAP kinase kinase kinase (MAPKKK, MEKK), MAP kinase kinase (MAPKK, MKK, MEK) and MAP kinase (MAPK, MPK) (Cai et al. 2014; Xu and zhang 2015). MEKK receives signal from receptors/sensors either directly or indirectly (Rodriguez et al. 2010). Through serial phosphorylation, MEKKs, MKKs and MAPKs can transduce signals received from upstream factors to a variety of downstream substrates including transcription factors and other kinases (Taj et al. 2010). The foundation of MAPK-mediated signal transduction is highly organized protein-protein interactions (PPIs) (Jiang et al. 2022). In *Arabidopsis*, there are approximately 20 MAPKs, 10 MKKs, 80 MEKKs (Zhou et al. 2017). Compared with MAPKs and MKKs, MEKKs have more complex and variable primary structures and domain compositions (Taj et al. 2010). Among these putative MEKKs, 12 belong to the MEKK subgroup and the rest belong to the CTR1 (Constitutive Triple Response 1) subgroup. However, it is still controversial whether members in the CTR1 group are true MEKKs (Xu and Zhang 2015). Crystallographic studies showed that MAPK family share similar three dimensional structures. The N-terminal domain (135 residues) is composed largely of beta-sheets and a glycine-rich loop that is termed the phosphate anchor ribbon, which acts as ATP binding pocket. The C-terminal domain (225 residues) contains a catalytic base, magnesium binding sites and the phosphorylation lip (activation loop) (Taj et al. 2010).

MAPK cascades play a critical role in transduction of diverse extracellular stimuli such as biotic and abiotic stresses as well as a range of developmental responses including differentiation, proliferation and death (Taj et al. 2010; Zhang et al. 2018). For example, through the modification of *Agrobacterium*-triggered plant immunity, the MKK4/5-MPK3/6 cascade is a crucial signaling route regulating *Agrobacterium*-mediated transformation (Liu et al. 2021). MAPK cascades are regulated by various biotic and abiotic stress stimuli such as pathogen infections, heavy metal, wounding, high and low temperatures, high salinity, UV radiation, ozone, reactive oxygen species, drought and high or low osmolarity (Taj et al. 2010; Sinha et al. 2011). However, the involvement and significance of MAPK cascades in plants responsive to biotic and abiotic stresses are still not fully elucidated. Recent studies reveal that they also play essential roles in plant growth and development by phosphorylating different substrates downstream of receptor-like protein kinases (RLKs) (He et al. 2018). The extracellular domains of RLKs are quite divergent and enable them to perceive highly variable, rapidly evolving stimuli (Lehti-Shiu et al. 2009; Gao et al. 2012). Over the course of evolution, there was a rapid expansion in the number of RLKs from lower to higher plants (Shiu et al. 2003); however, the number of downstream components such as MAPK components did not increase correspondingly (Xu and Zhang 2015). Recently, a crucial question in biology is how MAPKcascades regulate different outputs with specificity (Ma et al. 2023). Most MEKKs have large, undefined functional domains (Xu and Zhang 2015). Therefore, to predict MEKK functions would be extremely useful for studying the mechanism of plant stress-resistance.

Bryophytes, which represent the oldest living clade of land plants, diverged from the ancestors of vascular plants approximately 450 million years ago (Morris et al. 2018; Patiño et al. 2018). Antarctic mosses were among the pioneer plants to inhabit on the land, which have become the highest plant forms occurring naturally in Antarctic continent (Skotnicki et al. 2000; Convey et al. 2007). To successfully colonize Antarctic low-temperature environments, they have evolved a number of strategies that range from molecular to whole cell or even to ecosystem levels to achieve their survival and propagation (An et al. 2021). For example, the Antarctic mosses resist ultraviolet radiation using efficient systems of synthesis of screening compounds such as UV-B absorbing pigments and anthocyanin compounds for repairing damage (Singh et al. 2011; Singh et al. 2012). Therefore, Antarctic mosses are the ideal species for discovering new mechanisms of stress resistance as well as genetic evolution, under extreme conditions. Now, only limited information is available on the MEKKs in the relative lower plants such as liverworts and mosses. In the present study, two novel receptor-like protein kinase genes were isolated from Antarctic moss *Pohlia nutans* and their gene structure, phylogenetic evolution, subcellular localization and expression profile were investigated in this study.

Materials and methods

Plant materials and stress treatments

The Antarctic moss *Pohlia nutans* was collected from the terrene near the Great Wall Station of China in Antarctica (S62°13.260', W58°57.291') during the 24th Antarctic expedition of China in 2008. The

samples were then grown in soil plant pots (a mixture of Base Substrate (Klasmann-Deilmann, Geeste, Germany) and local soil, ratio 1:1) in a growth chamber at 16°C, 70 μmolquantam^{-2}s^{-1} light and 70% relative humidity. In this study, mosses cultivated under above conditions were used for experimental control. For the cold treatments, mosses were transferred to 4°C and incubated for 1h, 3h, 6h, 12h and 24h. For the salt, abscisic acid (ABA) or methyl jasmonate (MeJA) treatment, mosses were sprayed with 200 mM NaCl or 50 μM ABA or 50 μM MeJA and incubated at 16°C for 0.5h, 1h, 3h, 6h and 12h. For drought or UV-B radiation treatment, mosses were transfer to 20% (w/v) polyethylene glycol 6000 (PEG6000) solution or placed under 8W UV-B lamp (302nm, reaching 70 μW/cm^2 irradiance) for 2 h, 6 h and 12 h. Green parts of shoots were frozen by liquid nitrogen immediately after different stress treatment.

Total RNA preparation

The moss samples were ground in liquid nitrogen with mortar and pestle and then the ground powders were transferred into 1.5 ml tubes. Total RNA was extracted from liquid nitrogen ground moss powder using cetyltrimethylammonium bromide (CTAB) extraction buffer comprising 2% CTAB, 1% polyvinylpyrrolidone (PVP) K-30 (soluble), 100 mM Tris HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 0.5 g/L spermidine (free acid) and 2% β-mercaptoethanol (added just before use) (Zhang et al. 2013). 0.5 mL CTAB buffer (2% β-ME) was added to the ground powder and incubated at 60ºC for 10 min, mixed well by vortexing. The moss samples were further extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1) centrifuged at 13,000×g for 10 min at 4ºC and the aqueous phase was transferred to a new tube. The supernatant was precipitated with 1/4 volume of 10 M LiCl for overnight at 4ºC and harvested by centrifugation at 13,000×g for 30 min at 4ºC. Pellet was washed with 70% ethanol twice and air-dried for 5 min. RNA was dissolved in 100 μL Diethyl pyrocarbonate (DEPC)-treated water.

Bioinformatics analysis

The transcriptome of Antarctic moss *P. nutans* was sequenced by Illumina ultra high-throughput sequencing technology (Liu et al. 2013). The original transcriptome data sets were available at the NCBI Short Read Archive (SRA) with the accession number SRA051595. The HMMER program was used to retrieve the protein kinase from Antarctic moss transcriptome. Two full-length of MAPK genes (GenBank accession number GACA01000194 and GACA01042117, designated as PnMEKK1 and PnMEKK2, respectively) with the relatively high raw reads and fold change which implied the important role in cold resistance were selected for further analysis. The theoretical molecular weight (MW) and isoelectronic point (PI) of PnMEKK1 and PnMEKK2 protein were computed by ExPASy Compute PI/Mw tool. Multiple alignments of the PnMEKK1 and PnMEKK2 were performed by the ClustalW. The phylogenetic tree was constructed using the MEGA v5.2 software (Tamura et al. 2011). In MEGA, distance matrices were generated using the Pairwise deletion option with the Poisson Correction amino acid matrix. One thousand bootstrap replicates were created and trees were generated using neighbor-joining (NJ) method for each replicate. The bootstrap values reported for each branch reflect the percentage of 1000 trees which contained that branch.

Subcellular localization of PnMEKK1 and PnMEKK2 proteins

The subcellular localization analysis was performed according to the methods described by (Yoo et al. 2007) with some modifications. The paired PCR primers for amplifying the full-length sequence of the *PnMEKK1* and *PnMEKK2* were listed in Table 1. The complete coding region of *PnMEKK1* and *PnMEKK2* were integrated into the *Bam*H I and *Kpn* I sites of pBI221 vector which contains a cauliflower mosaic virus 35S promoter and a green fluorescence protein (GFP) tag. Mesophyll protoplasts were isolated from 4-week old *Arabidopsis thaliana* ecotype Col-0 plants. 10 μg of PnMEKK1-GFP or PnMEKK2-GFP recombinant vector was transfected into 4×10^4 protoplasts and incubated in dark for 12 h. GFP fusion proteins were examined by the confocal laser scanning microscopy (LSM700, Carl Zeiss, Germany). The green GFP fluorescence, red autofluorescence of chloroplast and bright field image released from *Arabidopsis* protoplasts were imaged simultaneously and merged together.

Table 1
Primers used in subcellular localization and qRT-PCR analysis.

Primers	Sequence (5'-3')
PnMEKK1 BamHI	CG*GGATCC*ATGGAAGAGGCCAACGCCCGCGAG
PnMEKK1 KpnI	GG*GGTACC*AACTCCACTGCCTGCTCTTGAAGGAG
PnMEKK2 BamHI	CG*GGATCC*ATGGCAAGAATTGGGTGTATGGAGG
PnMEKK2 KpnI	GG*GGTACC*AGTAAGGTAACGAATTATACAAGCTCTGG
PnMEKK1 qPCR-F	ACCGCTCTGGAGCTTTCCAACAC
PnMEKK1 qPCR-R	GCATCCTTGACTGATCCTCCGAC
PnMEKK2 qPCR-F	GGAGAAAGAGGAGGCGATTGATAG
PnMEKK2 qPCR-R	CTTTGCATGTAAAAAACTCACCTCG
tubulin qPCR-F	AGCGGTGTGACTTGTTGCTTACG
tubulin qPCR-R	TCTGCTGGGTAAGTTCTGGGACG

Analysis of mRNA expression level by real-time PCR

The cDNA for qRT-PCR was prepared using 0.5 μg of total RNA with the mixture of oligo$(dT)_{18}$ and Random 6 primers. The primers targeted against sequences of Antarctic moss *PnMEKK1*, *PnMEKK2* and tubulin (GenBank accession number GACA01042200) genes were listed in Table 1. Bestar® SybrGreen qRT-PCR mastermix (DBI Bioscience) was used for qRT-PCR analysis. qRT-PCR was performed on the PTC-200 Real Time PCR system for 40 cycles (95°C for 15 s; 60°C for 20 s; 72°C for 20 s). All reactions were carried out at least three duplicates. Quantification of mRNA level was based on Ct (threshold cycle) values. The Ct values of *PnMEKK1* and *PnMEKK2* were normalized using the Ct value of tubulin

gene. Data analysis was executed using comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak et al. 2001). Data are presented as the mean ± SE (n = 3) and subjected to a one-way ANOVA followed by Student's *t* test to determine the differences among treatments. Differences were considered statistically significant at $P < 0.05$.

Results

Multiple sequence alignment and phylogenetic analysis

To obtain the protein kinase genes from Antarctic moss *P. nutans*, we analyzed the transcriptome data with HMMER software for searching the protein kinase domain (PF00069). In Antarctic moss transcriptome, two full-length of *PnMEKK1* and *PnMEKK2* genes with highest raw reads and fold change were selected for further analysis. The raw reads of *PnMEKK1* gene in cold-treatment sample and control sample were 603 and 230, with a normalized fold change 2.50, while the raw reads of *PnMEKK2* gene in cold-treatment sample and control sample are 1861 and 903, with a normalized fold change 2.16. These two genes were both up-regulated after cold treatment, suggesting that they might involve in cold resistance. The full length of *PnMEKK1* and *PnMEKK1* cDNA contained an open reading frame (ORF) of 2415 bp and 1783 bp, encoding the receptor-like kinases of 804 and 576 amino acids with a deduced molecular weight of 90.76 and 68.09 kDa and pI of 5.60 and 7.78. Several protein kinase with high similarity with PnMEKK1 or PnMEKK2 were selected for sequence alignment analysis. Results showed that PnMEKK1 were homologous to *P. patens* Pp1s3_556V6.1 and Pp1s296_5V6.1, while PnMEKK2 were homologous to *P. patens* Pp1s366_33V6.1, Pp1s366_28V6.1. However, the identity percent of PnMEKK1 or PnMEKK2 with *A. thaliana* AAG51718.1 and *Vitis vinifera* XP_002280598.2 were both lower than 11%. Furthermore, PnMEKK1 and PnMEKK2 possessed the conserved the PX domain, STKc_MAP3K-like, Serine/Threonine protein kinases, Protein kinase domains, suggesting that they were the mitogen-activated protein kinase kinase kinase (MEKKs).

Representative protein kinases of dicots, monocots, ferns and bryophytes were selected to construct a phylogenetic tree using the programs of ClustalW and MEGA v5.2. Several reported receptor-like protein kinases from *A. thaliana*, were also selected to construct the phylogenetic tree. The first characteristic of the constructed phylogenetic tree was that all gymnosperm and angiosperm were grouped in separate clusters according to their protein sequence distance. Furthermore, PnMEKK1 (GACA01000194) clustered together with *Physcomitrella patens subsp. patens* (Pp1s296_5V6.1 and Pp1s3_556V6.1) and *Selaginella moellendorffii* (EFJ15825), while PnMEKK2 (GACA01042117) clustered together with *Physcomitrella patens subsp. patens* (Pp1s366_33V6.1 and Pp1s366_28V6.1) and *Selaginella moellendorffii* (EFJ15950) (Fig. 2). Mitogen-activated protein kinase and receptor-like protein kinase both have the protein kinase domain. The phylogenetic tree showed that PnMEKK1 and PnMEKK2 were relatively close to mitogen-activated protein kinase kinase kinase 7 (*Vitis vinifera* XP_002280598.2 and CAN60248, *Phoenix dactylifera* XP_008791458.1), but not clustered together with these selected receptor-like protein kinases of *A. thaliana*.

Subcellular localization in Arabidopsis protoplasts

The subcellular localizations of PnMEKK1 and PnMEKK2 were investigated in *Arabidopsis* protoplasts *in vivo* (Fig. 3). When protoplasts were transfected with recombinant vector of PnMEKK1-GFP/pBI221 or PnMEKK2-GFP/pBI221, green fluorescence would be emitted by the continuously expressed GFP fusion protein. The positions of green fluorescence indicated that these two proteins were both mainly localized in cytoplasm (Fig. 3a and 3e). The control vector of pBI221 was also transformation and expressed in all the parts of protoplasts (Fig. 3i). Meanwhile, the red fluorescence released by chloroplasts was imbedded, but not colocalized with green fluorescence (Fig. 3c and 3g). Furthermore, the colocalization analysis by merging green fluorescence, bright field, and red fluorescence also didn't present the distinct yellow signals in the positions of chloroplasts (Fig. 3d and 3h). Therefore, PnMEKK1 and PnMEKK2 should be the cytoplasm-localized protein kinases.

Expression Levels of PnMEKK1 and PnMEKK2 in Response to stress treatment

Real-time fluorescence quantitative PCR (qRT-PCR) was used to examine the time-dependent expression pattern of *PnMEKK1* and *PnMEKK2* in Antarctic moss *Pohlia nutans* under cold stress at the time point of 1, 3, 6, 12 and 24h (Fig. 4). The results showed that *PnMEKK1* mRNA expression levels increased 3.71-fold at the time point of 3h and increased 2.86-fold at the time point of 12h at 4°C. Meanwhile, *PnMEKK2* mRNA expression levels increased 3.67-fold at 1h and increased 3.10-fold at 3h after cold stress.

Antarctic mosses were sprayed with 200 mM NaCl and incubated at 15°C for 0.5, 1, 3, 6 and 12h. The results showed that *PnMEKK1* and *PnMEKK2* transcription rose markedly at an early stage (Fig. 4). *PnMEKK1* mRNA expression levels reached the maximal level of 8.94-fold at 0.5h, declining thereafter to 3.78-fold at 1h after NaCl treatment. However, *PnMEKK2* mRNA expression levels increased 8.91-fold at 0.5h and constantly increased 9.51-fold at 1h after salt stress.

In the presence of 20% PEG6000 to simulate drought stress, *PnMEKK1* and *PnMEKK2* transcription were both significantly increased, but the magnitude of *PnMEKK2* transcript was obviously larger than that of *PnMEKK1* transcript in each time point (Fig. 4). In detail, the levels of *PnMEKK1* and *PnMEKK2* transcript increased 2.03-fold and 3.93-fold respectively after PEG6000 treatment for 2h.

In UV radiation treatment, Antarctic mosses were placed under different times of UV-B radiation, while cultured at visible light as control. qRT-PCR analysis indicated that the level of *PnMEKK1* transcription increased nearly 3-fold after UV-B radiation for 6h and the level of *PnMEKK2* transcription increased 2.89-fold after UV-B radiation for 2h. Therefore, the response of PnMEKK2 transcript was earlier than that of *PnMEKK1* in terms of UV-B radiation (Fig. 4).

Antarctic mosses were sprayed with 50 μM ABA or MeJA and incubated for 0.5h, 1h, 3h, 6h and 12h to investigate the mRNA expression levels of *PnMEKK1* and *PnMEKK2*. The results of qRT-PCR analysis

showed that *PnMEKK1* mRNA expression levels significantly increased 3.28-fold at 3h after ABA treatment and reached the maximal level of 5.80-fold at 24h (Fig. 5). Meanwhile, *PnMEKK2* mRNA expression levels increased 4.33-fold at 0.5h and reached the maximal level of 11.11-fold at 1h after ABA treatment. The expression levels of *PnMEKK1* and *PnMEKK2* under 50μM MeJA treatment were similar to those under ABA stress. *PnMEKK2* had stronger responsive patterns than *PnMEKK1* after MeJA treatment. *PnMEKK1* mRNA expression levels increased 2.65-fold at 3h after MeJA treatment, while *PnMEKK2* mRNA reached the maximal expression level of 10.41-fold at 0.5h and constantly increased 9.55-fold at 1h after MeJA treatment (Fig. 5).

Discussion

In nature, plants are continually challenged by diverse stress stimuli, including pathogen infection, drought and high salinity (Sinha et al. 2011; Nair et al. 2022). To cope with unfavorable environmental conditions, plants have evolved different mechanisms to protect themselves from adverse environmental conditions by transducing extracellular stimuli into appropriate intracellular responses (Pitzschke et al. 2009). MAPK cascades are universal signaling pathways involved in response to external stimuli in eukaryotes, and they play essential roles in developmental and environmental signal transduction (Chen et al. 2021). Previously, we performed the transcriptome analysis of the Antarctic moss, *Pohlia nutans*, in response to cold stress using the Illumina ultra high-throughput sequencing technology (Liu et al. 2013). Two full-length of mitogen-activated protein kinase kinase kinase genes (designated as *PnMEKK1* and *PnMEKK2*) with higher expression abundance and fold change were identified from Antarctic moss *Pohlia nutans* transcriptome. Multiple sequence alignment showed that PnMEKK1 and PnMEKK2 possessed the conserved STKc_MAP3K-like domains, Serine/Threonine protein kinases and Protein kinase domains, but they had relatively low identities with other protein kinases from higher plants (Figs. 1 and 2). In addition, the subcellular localization analysis by observing the transient expression of MEKK-green fluorescence protein in *Arabidopsis* mesophyll protoplasts revealed that PnMEKK1 and PnMEKK2 were mainly localized in cytoplasm (Fig. 3). Therefore, the structural features of the conserved kinase domain and subcellular localization of plasma membrane strongly suggest that these two proteins function as mitogen-activated protein kinase kinase kinase.

In Antarctic, moss species growing in areas in which temperatures fall below zero are likely to have tolerance to freezing stress. However, little is known about the physiological changes induced by cold acclimation in non-vascular plants such as bryophytes (Minami et al. 2005). Several studies carried out in in different plant genera indicated that MAPKs involved in temperature stress tolerance. The *StMAPK3* responded to cold stress and chilling temperature stress (Majeed et al. 2022). In *Arabidopsis*, *AtMEKK1*, *AtMKK2*, *AtMPK3* and *AtMPK4/6* were upregulated by cold stress (Rodriguez et al. 2010; Sinha et al. 2011). In the cold-tolerant *S. acaule*, *SaMKK2* and *SaMAPK16* expression rose notably (Chen et al. 2022). The transcript level of *ZmMPK3* increased markedly within 30 min and remained high during a 4 h period (Wang et al. 2010). Cold stress also induced the expression and activity of *ZmMKK4* (Kong et al. 2011). MEKK1 functions upstream of MKK1, MKK2 and MPK4, and it has been confirmed the MAPK cascades consisting of MEKK1-MKK2-MPK4/6 play a critical role in cold stress (Sinha et al. 2011).

Previous transcriptome sequencing indicated that the expression levels of *PnMEKK1* and *PnMEKK2* were increased 2.50-fold and 2.16-fold respectively after cold stress for 6h (Liu et al. 2013). In this study, qRT-PCR analysis confirmed that *PnMEKK1* and *PnMEKK2* transcript accumulated significantly after cold stress and increased 2.86-fold and 1.95-fold at 6h (Fig. 4a), which was consistent with the results in Antarctic moss transcriptome sequencing.

Several reports suggested that the MAPK cascades play a vital role in drought and salinity stress (Lin et al. 2021). In *Arabidopsis*, previous study demonstrated that the MEKK1 mRNA accumulated in response to environmental stresses, including drought stress (Mizoguchi et al. 1996). The drought-hypersensitive mutant (drought-hypersensitive mutant1 [dsm1]) of a putative MEKK gene in rice was induced by salt, drought, and abscisic acid. Overexpression of DSM1 in rice increased the tolerance to dehydration stress at the seedling stage (Ning et al. 2010). In maize, ZmMKK1 might act as an ABA- and ROS-dependent protein kinase in positive modulation of salt and drought tolerance in transgenic *Arabidopsis* (Cai et al. 2014)., ZmSIMK1, ZmMKK4, ZmMPK17, GhMPK2 and GhMPK17 improved tolerance to salt stress by controlling salt marker genes (Lin et al. 2021).In the present study, our results showed that both salinity and drought could quickly motivate the up-regulation of *PnMEKK1* and *PnMEKK2* mRNA expression in Antarctic moss (Fig. 4). This indicates that they may be essential for the rapid drought or salinity response.

Particularly, polar organisms suffer from strong seasonal changes in the radiation climate (Karentz and Bosch 2001). This is further intensified by the fact that the extending of stratospheric ozone depletion and the increasing of UV radiation on the Earth's surface coincides with spring conditions, intensifying the additional stress upon the organisms (Karentz 1989). UV radiation will stimulate the production of ROS and ABA: the former could cause severe oxidative damage, and the latter triggers downstream stress-responsive pathways (Berli et al. 2011). Interestingly, our results showed that *PnMEKK1* and *PnMEKK2* mRNA transcripts were both increased when Antarctic moss *Pohlia nutans* were treated with the UV radiation, implying their positive role in acclimatizing to polar environment (Fig. 4).

Plant hormones are a group of small molecules which are central to signalling during developmental regulation and environmental adaptation ((Santner and Estelle 2009). ABA and JA are the main hormonal determinants of plant enhancing tolerance to abiotic stresses (Danquah et al. 2014). ABA is dramatically produced under water deficit conditions and regulates the expression of various genes ((Tuteja and Sopory 2008). ABA induces protein kinases of the SnRK family to mediate a number of its responses (Fujita et al. 2009). Study showed that the basal land plant *Physcomitrella patens* and the model plant *Arabidopsis thaliana* share a conserved ABA signaling pathway mediated through ABI1-related type 2C protein phosphatases (Sakata et al. 2009). In *P. patens*, results indicate that cold acclimation of bryophytes requires an ABA-dependent signaling process (Bhyan et al. 2012). In the present study, the application of plant hormone ABA and MeJA also could up-regulate the mRNA expression level of *PnMEKK1* and *PnMEKK2*, which suggested that they involved in plant homone signal transduction (Fig. 5). Collectively, we purposed that these two isolated MAPKs might involve in plant homone

signalling and in acclimatizing to polar environment in Antarctic mosses. Further studies should be therefore performed to analyze the stress-related phenotype of transgenic plant.

Declarations

Acknowledgments

This work was supported by the Natural Science Foundation of Shandong Province (ZR2022MC075) and the National Natural Science Foundation of China (41976225).

Author contributions

ZP is the experimental designer of this study. LC is the executor of experiments. LS and KQ participated in data collation. ZP was in charge of the project and supervised the experimental design. LC was responsible for writing and revising the paper. All the authors read and agreed to the final text.

Conflict of interest The authors declare no conflict of interest.

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Figures

Fig. 1

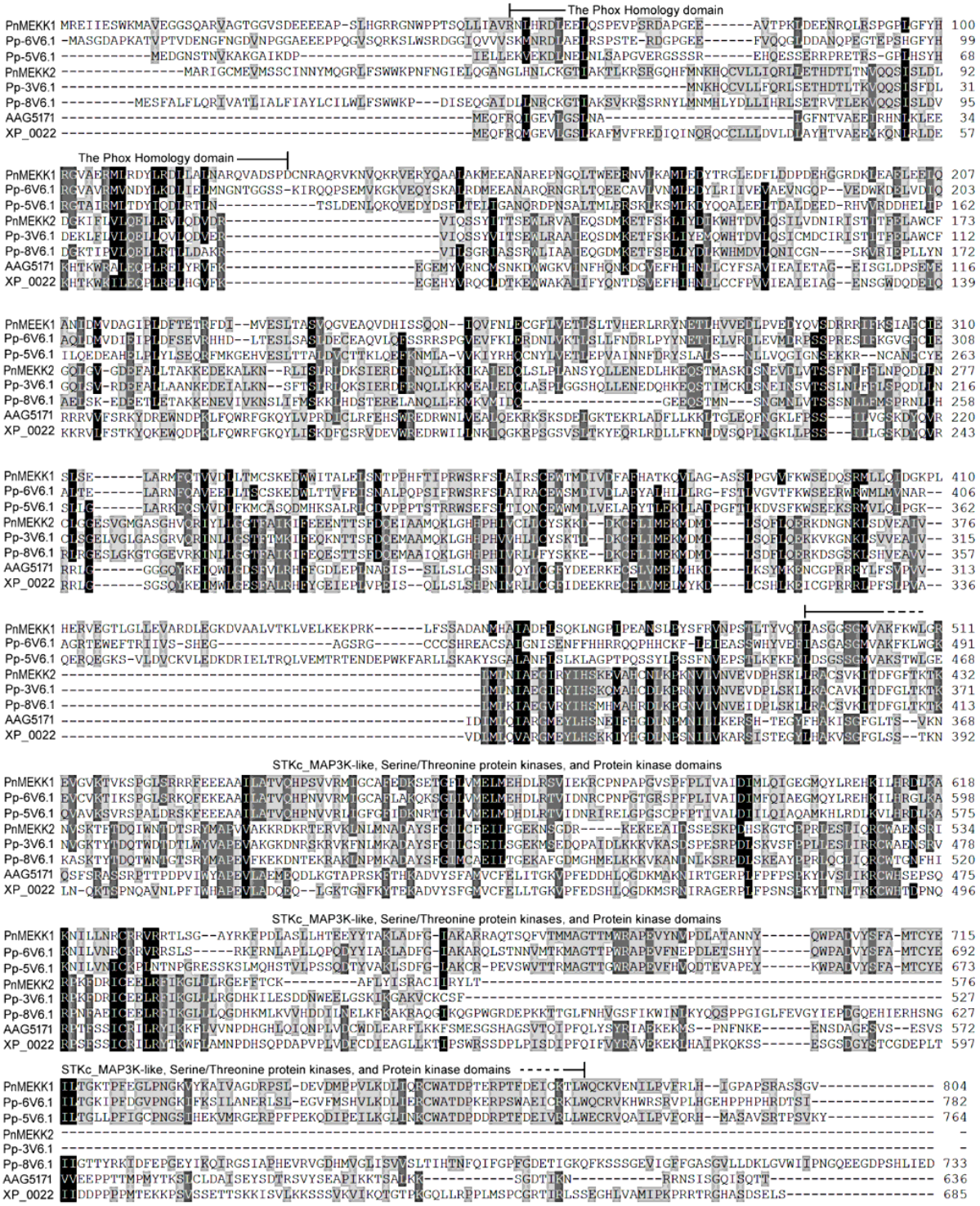

Figure 1

Sequence alignment of PnMEKK1 and PnMEKK2 with other homologs of *Physcomitrella patens*Pp1s3_556V6.1 (abbr. Pp-6V6.1), Pp1s296_5V6.1 (abbr. Pp-5V6.1), Pp1s366_33V6.1 (abbr. Pp-3V6.1), Pp1s366_28V6.1 (abbr. Pp-8V6.1), *Arabidopsis thaliana* AAG51718.1 (abbr. AAG5171) and *Vitis vinifera* XP_002280598.2 (abbr. XP_0022). Multiple sequence alignments were conducted using ClustalW. Black boxes show identical amino acid residues, and gray shades show similar residues.

Deletions are indicated by dashes to allow maximum alignment. The predicted domains were labeled above the sequence.

Fig. 2

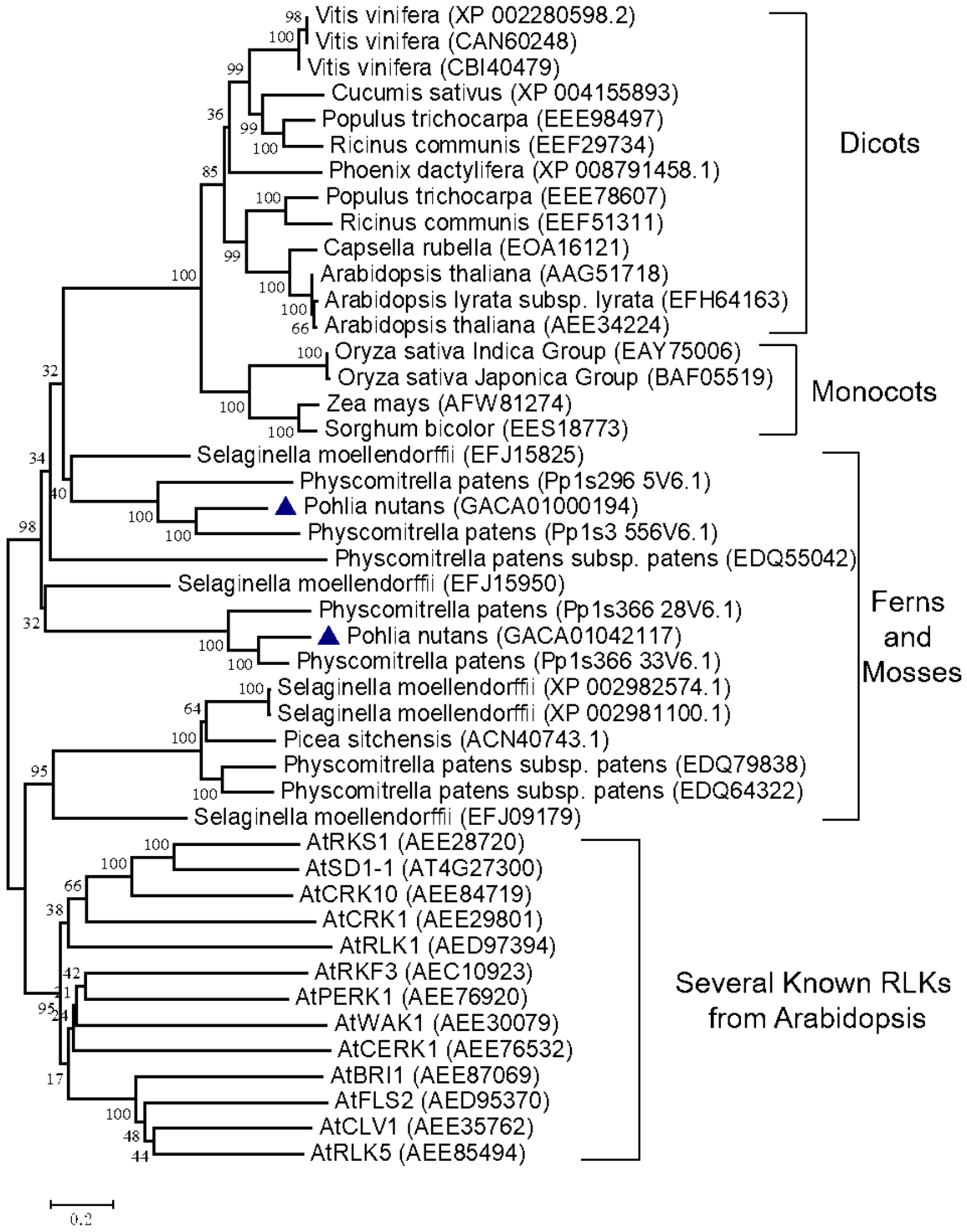

Figure 2

Phylogenetic relationship between PnMEKK1 and PnMEKK2 from Antarctic moss Pohlia nutans with the other protein kinases from different plants. The tree was constructed with the neighbour-joining method.

Numbers at each branch indicated the percentage of times a node was supported in 1000 bootstrap pseudoreplication.

Fig. 3

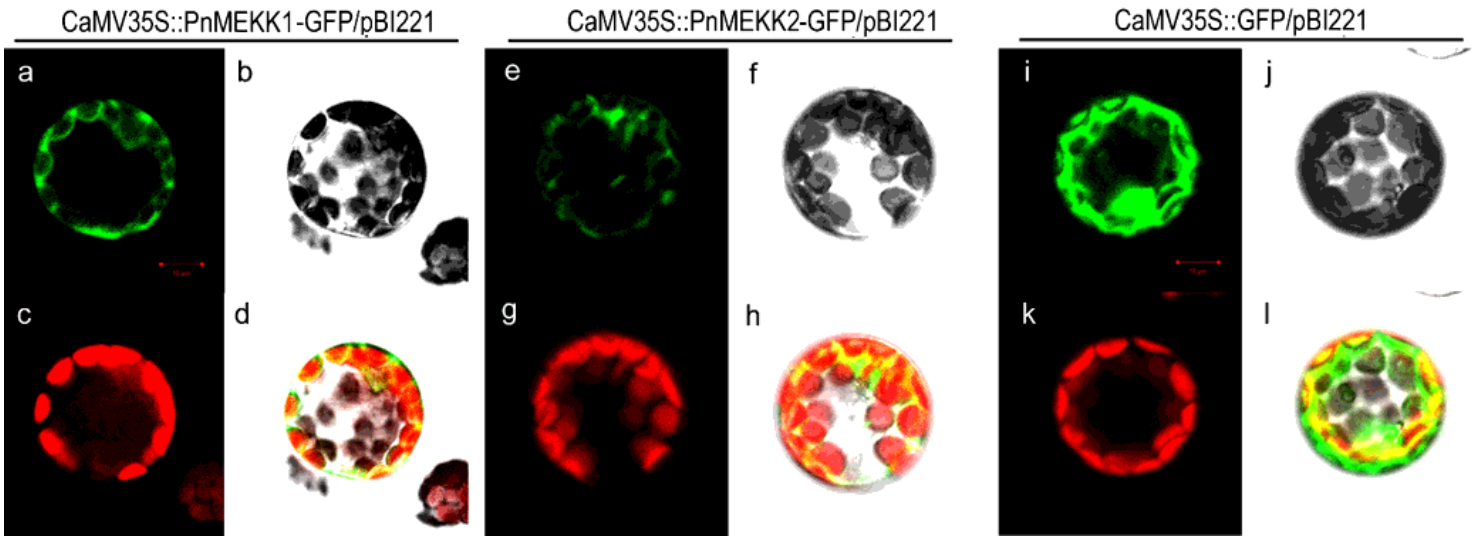

Figure 3

Intracellular targeting of PnMEKK1 and PnMEKK2 in *Arabidopsis thaliana* protoplasts. The full-length PnMEKK1 and PnMEKK2 were inserted between cauliflower mosaic virus 35S promoter and GFP in pBI221 vector. The confocal images were taken after 12 h of protoplast transfection. a or i or e, green fluorescence of PnMEKK1 or PnMEKK2 or 35S:GFP fusion protein; b or f or j, protoplast in bright field; c or g or k, red autofluorescence of chloroplasts; d or g or k, merged image of green fluorescence, bright field, and red autofluorescence.

Fig. 4

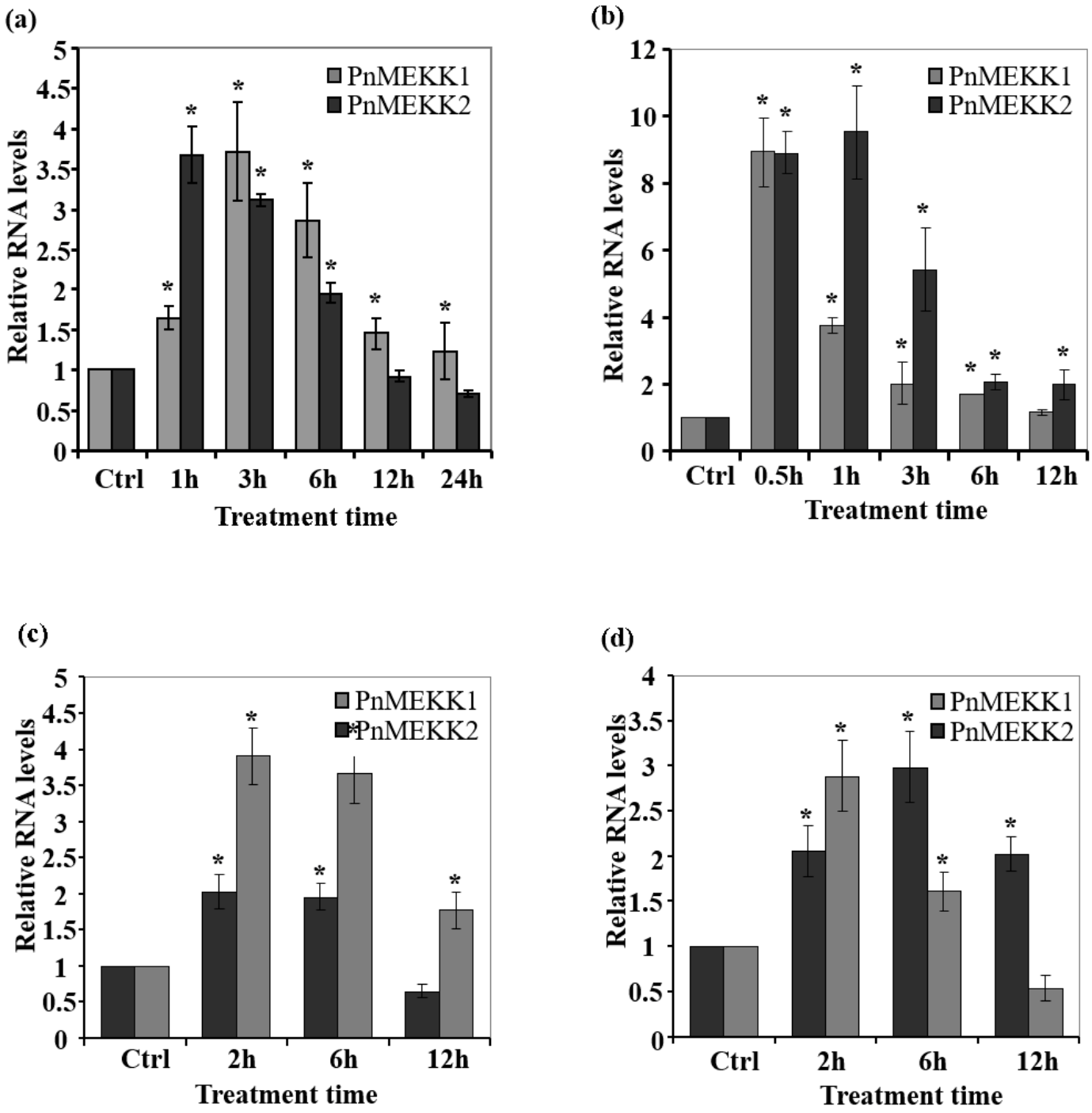

Figure 4

Transcript levels of PnMEKK1 and PnMEKK2 genes in Antarctic moss after abiotic stress. (a) Cold stress; (b) salinity stress; (c) drought stress; (d) UV-B radiation. Values were expressed as means±SE. Standard error bars are shown. *$P < 0.05$.

Fig. 5

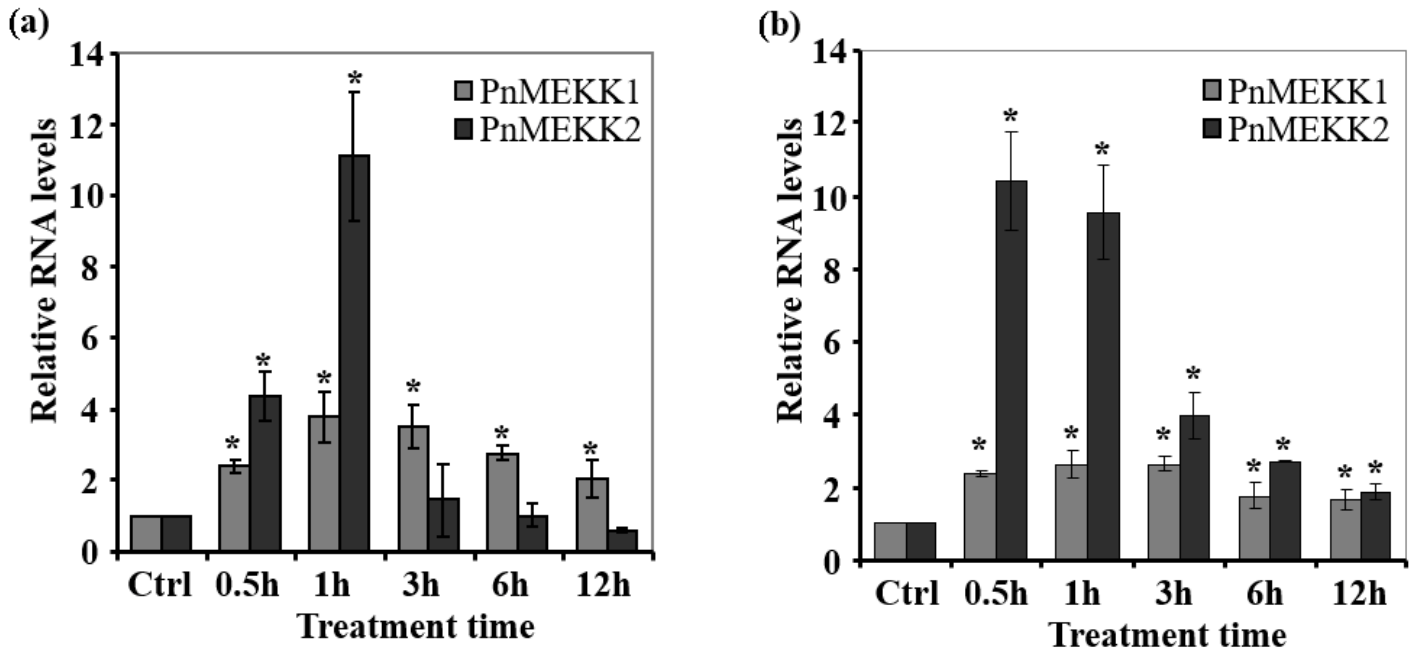

Figure 5

Transcript levels of PnMEKK1 and PnMEKK2 genes in Antarctic moss after ABA and MeJA treatment. (a) ABA treatment; (b) MeJA treatment. Values were expressed as means±SE. Standard error bars are shown. *$P < 0.05$.