

Characterization and expression analysis of a mitochondrial heat-shock protein 70 gene from the Antarctic moss *Pohlia nutans*

Shenghao Liu · Jing Wang · Bailin Cong ·
Xiaohang Huang · Kaoshan Chen · Pengying Zhang

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Abstract Under stress conditions, the mitochondrial respiratory chain generates reactive oxygen species that cause oxidative damage to lipids, proteins and nucleic acids. Mitochondrial chaperones are essential for repairing denatured proteins in plants confronted with diverse stresses. However, their functions have not been well characterized in lower plants such as bryophytes. Here, we cloned a heat-shock 70 protein gene (designated *PnHSP70*) from the Antarctic moss *Pohlia nutans* and investigated its transcription levels under stress conditions. The *PnHSP70* cDNA encoded a polypeptide of 678 amino acids with an ATPase domain, a substrate peptide-binding domain and a C-terminal domain. Sequence alignment and phylogenetic analysis showed that *PnHSP70* was homologous to other known mitochondrial HSP70 proteins. In addition, sub-cellular localization analysis in which the transient expression of *PnHSP70*-green fluorescent protein was observed in *Arabidopsis thaliana* mesophyll protoplasts revealed that *PnHSP70* was targeted to mitochondria. Real-time PCR analysis showed that low or high temperature, salinity, drought or PEG treatments as well as UV-B radiation could induce up-regulation of *PnHSP70* expression levels. The plant hormones abscisic acid and jasmonic acid also up-regulated the expression levels of *PnHSP70*. Together, our results revealed that *PnHSP70*, as a

mitochondrial chaperone, might play an important role in the adaptation of the Antarctic moss *P. nutans* to the polar environment.

Keywords Cold adaptation · *Pohlia nutans* · HSP70 · Psychrophile · Abiotic stress

Introduction

Only about 0.3 % of the Antarctic continent is free of ice. The terrestrial and freshwater ecosystems in this tiny fraction are generally small and isolated, with mosses, lichens and algae constituting the predominant (though sparse) terrestrial vegetation (Morgan-Kiss et al. 2006; Pearce 2008). In the South Shetlands of West Antarctica, the annual mean temperature is around 0 °C, but the region has a wide day-time temperature range. It varies from –5 to 13 °C in summer and falls as low as –30 °C in winter. Furthermore, wind and humidity may significantly decrease the chill temperature (Victoria et al. 2009). Salinity levels can also affect the growth of vegetation, as many species grow near the Antarctic coast. Ozone depletion has caused increased UV-B radiation to reach the surface of Antarctica, particularly in spring, which can also influence the growth of vegetation (Singh et al. 2011; Rautenberger et al. 2013). The isolation of the Antarctic continent began when the supercontinent Gondwana broke up (167 million years ago). Recent biological studies also suggest that most of the terrestrial biota have a long, continuous but isolated, history on the continent (Convey and Stevens 2007; Kato et al. 2013). Therefore, the Antarctic environment represents an exciting place for studying cold adaptation, genetic evolution in Antarctic species, and the threat imposed on species by global climate change (Convey and Smith 2006; Liu et al. 2013).

S. Liu · J. Wang · K. Chen · P. Zhang (✉)
National Glycoengineering Research Center, College of Life
Science, Shandong University, 27 Shandan Road,
Jinan 250100, People's Republic of China
e-mail: zhangpy888@126.com

S. Liu · B. Cong · X. Huang
Key Laboratory of Marine Bioactive Substance, The First
Institute of Oceanography, State Oceanic Administration,
Qingdao 266061, People's Republic of China

Bryophytes, which represent the oldest living clade of land plants, are the most abundant plants in the Arctic and Antarctic (Skotnicki et al. 2000; Gornall et al. 2007). During the course of evolution, they have adopted a variety of strategies that range from physiological changes to stress tolerance under the harsh environmental conditions. For example, the surface temperature of the Antarctic ground is strongly affected by the input of solar radiation and the boundary layer effect (Bramley-Alves et al. 2014). Moss cushions conform to black-body radiation (Newsham 2010), and have, therefore, been found to reach temperatures above 40 °C during the summer months if situated in sun-exposed but wind-sheltered sites (Bramley-Alves et al. 2014). The reversible photoinhibition observed in Antarctic mosses protects them from photoinhibitory damage caused by freeze–thaw stress and high solar radiation levels (Lovelock et al. 1995). Antarctic mosses resist ultraviolet radiation using efficient systems for repairing damage by synthesis of screening compounds such as UV-B absorbing pigments and anthocyanin compounds (Singh et al. 2011). Furthermore, cell wall-bound insoluble phenylpropanoids represent a more passive UV-screening mechanism and improve the tolerance of Antarctic mosses to high ultraviolet radiation (Clarke and Robinson 2008). However, the mechanisms by which Antarctic mosses survive under extreme conditions are still not well documented.

The heat-shock 70 proteins (HSP70s) are essential chaperones and housekeeping proteins whose functional roles have become the focus for studying mechanisms of stress acclimation (Reddy et al. 2010; Montero-Barrientos et al. 2010). The HSP70 family is highly conserved between prokaryotes and eukaryotes (Renner and Waters 2007; Wang et al. 2009; Fu et al. 2009; Reddy et al. 2010). All HSP70s possess three distinct domains: an N-terminal adenosine triphosphatase (ATPase) domain of approximately 400 amino acids, a substrate-binding domain of approximately 200 amino acids, and a highly variable C-terminal domain. Photosynthetic eukaryotes possess four types of HSP70s, each of which localizes to a different subcellular compartment—the cytoplasm, mitochondrion, endoplasmic reticulum or chloroplast—and participates in numerous cellular processes, probably through a common mechanism (De Los et al. 2006; Renner and Waters 2007). Most mitochondria and chloroplast proteins are synthesised in the cytosol as precursors, and post-translationally imported into each organelle. HSP70s located on the receiving side of the membrane (BiP) are recruited to drive the precursor proteins through the Sec61 translocon into the ER lumen (Alder et al. 2005). Moreover, mitochondrial HSP70s are thought to be involved in the translocation of mitochondrial-incoming precursors and subsequent refolding of the substrate (Horst et al. 1997; Shi and Theg 2010). As with the mitochondrion, a recent study on the

model moss *Physcomitrella patens* showed that stromal HSP70 chaperones also play a key role in chloroplast protein import (Shi and Theg 2010). However, to our knowledge, the functions of HSP70 proteins in bryophytes under stress conditions have not been well studied.

During China's 24th Antarctic expedition, we collected 12 moss samples from the Great Wall Station in the Fildes Peninsula of King George Island. These mosses were regularly cultured in an illumination incubator at temperatures of 16 °C and flourished at temperatures down to 4–6 °C, suggesting that they have a strong ability to adapt to low temperatures. To understand the mechanism of its stress acclimation, we performed transcriptome analysis on the Antarctic moss *Pohlia nutans* in response to cold stress using ultra high-throughput sequencing technology. Global profiling of the differentially expressed genes revealed that 3,796 unigenes were significantly up-regulated after cold treatment, while 1,405 unigenes were significantly down-regulated (Liu et al. 2013).

In the present study, to further uncover the mechanisms by which Antarctic mosses respond to environmental stimulation, a full-length mitochondrial HSP70 (PnHSP70) with a high transcript abundance and comparative fold change [expressed as \log_2 (Cold treatment/Control), Ref. Liu et al. 2013] in the *P. nutans* transcriptome was investigated and its gene structure, and phylogenetic characteristics and subcellular localization were characterized. Moreover, the mRNA levels of *PnHSP70* under different stress conditions were investigated using a real-time quantitative RT-PCR approach.

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 (S62°13.260', W58°57.291') during the twenty-fourth Antarctic expedition in 2008. *P. nutans* was then cultivated on a soil medium (a mixture of Base Substrate (Klasmann-Deilmann, Geeste, Germany) and local soil, ratio 1:1) in flowerpots at 16.5 °C, 70 % relative humidity, and 70 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light density with continuous light. Mosses cultivated under the above conditions were used as the control group in all experiments. In the temperature treatment, cultures were kept at different temperatures for different periods of time (4, 10, or 21.5 °C for 0, 3, 6, 12, 24 or 36 h). In the salt treatment, cultures were sprayed with 200 mM NaCl for 0, 3, 6, 12, 24 or 36 h. In the drought treatment, cultures were deprived of water for 5 days, and then re-watered for 6 or 24 h, or were sprayed with 20 % (w/v) PEG6000 solution for 0, 2, 6 or 12 h. In the UV-B radiation treatment, cultures were placed

under an 8 W UV-B lamp (302 nm, reaching 70 $\mu\text{W}/\text{cm}^2$ irradiance) for 0, 2, 6 or 12 h. In the abscisic acid (ABA) treatment, cultures were sprayed with 50 μM ABA for 0, 3, 6, 12, 24 or 36 h. In the methyl jasmonate (MeJA) treatment, cultures were sprayed with 50 μM MeJA for 0, 1, 2, 3, 4 or 6 h. The green parts of the shoots were cut off and frozen immediately in liquid nitrogen after different stress treatments. Furthermore, in each stress treatment, samples at different time points were all taken from the same flowerpot culture to reduce the sample's standard deviation.

Total RNA preparation

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). The CTAB buffer (0.7 mL, 2 % β -ME) was added to the ground powder, incubated at 65 °C for 20 min, and mixed well by vortexing. The moss samples were further extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 13,000 $\times g$ for 10 min at 4 °C. The aqueous phase was transferred to a new tube and precipitated with a 1/4 volume of 10.0 M LiCl overnight at 4 °C and harvested by centrifugation at 13,000 $\times g$ for 20 min at 4 °C. The pellet was washed with 70 % ethanol twice and air-dried for 5 min. The RNA was dissolved in 100 μL diethyl pyrocarbonate (DEPC)-treated water.

Bioinformatics analysis

The *P. nutans* transcriptome was sequenced using the Illumina high-throughput sequencing technology (Liu et al. 2013). The original transcriptome data sets are available at the NCBI Short Read Archive (SRA) with the accession number SRA051595. The HMMER program was used to retrieve HSP70 proteins from the Antarctic moss transcriptome. The full-length *HSP70* gene (GenBank accession number GACA01000497, designated *PnHSP70*) with the highest raw reads and fold change was selected for further analysis. The theoretical molecular weight (MW) and isoelectric point (PI) of the PnHSP70 protein were computed by the ExpASY Compute PI/Mw tool. Multiple alignment of HSP70s was performed using the Clustal W program. A phylogenetic tree was constructed using the Mega 4.0 software (Kumar et al. 2008). In Mega 4.0, 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 the Neighbor-Joining (NJ) method for each replicate. The

bootstrap values reported for each branch reflected the percentage of 1,000 trees containing that branch.

Subcellular localization of the PnHSP70 protein

Subcellular localization analysis was performed according to the methods described by Yoo et al. (2007) with some modifications. The complete coding sequence of *PnHSP70* was amplified using paired PCR primers (Table 1) and integrated into the *Bam*HI and *Hind*III sites of the pBI221 vector. Mesophyll protoplasts were isolated from 4-week-old *Arabidopsis thaliana* ecotype Col-0 plants. Then, 10 μg of the constructed *p35S::PnHSP70::GFP* vector was transformed into 4×10^4 protoplasts and incubated in the dark for 12 h. The transformed protoplasts were observed using a confocal microscope (LSM700, Carl Zeiss, Germany). Green GFP fluorescence, red chlorophyll autofluorescence and bright field images of the *A. thaliana* protoplasts were captured simultaneously and merged together.

Analysis of HSP70 mRNA levels by quantitative real-time PCR analysis

The cDNA for real-time PCR analysis was prepared using 0.5 μg of total RNA with a mixture of oligo(dT)₁₈ and Random 6 primers. The primers targeted to the sequences of the *PnHSP70*, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, GenBank accession number GACA01025145) genes are listed in Table 1. The SYBR[®] PrimeScript[™] RT-PCR Kit (TaKaRa) was used for real-time PCR analysis, following the manufacturer's standard protocol. Real-time PCR was performed on the Stratagene Mx3000P[®] qPCR System for 40 cycles (95 °C for 10 s; 60 °C for 10 s; 72 °C for 20 s). All reactions were carried out in triplicate. The relative quantification of mRNA levels was based on *Ct* (threshold cycle) values. The *Ct* values of *PnHSP70* were normalized using the *Ct* values of the *GAPDH* gene. The efficiency of each real-time PCR was also calculated. Data analysis was performed using the comparative *Ct* ($2^{-\Delta\Delta C_t}$) method (Livak and Schmittgen 2001). Significant differences in the mean

Table 1 Primers used in subcellular localization and real-time PCR analysis

Primers	Sequence (5'–3')
PnHSP70 qPCR-F	GCCTTGCAGATTGAAGAAGTCC
PnHSP70 qPCR-R	CCAGCTTGTCAGACTCAGGAGTG
GAPDH qPCR-F	CCTTGCAAAGGTTATCCAGGACAAC
GAPDH qPCR-R	CACACGGAATGCCATACCAGTCAAC
PnHSP70BamHI	CGCGGATCCGATCCGGGATGGCGTC GCGTGTGC
PnHSP70HindIII	CCCAAGCTTCTTCTTGCCCTCCTTCA CATCCTC

values of HSP70 in different treatments were determined using a one-way ANOVA followed by Tukey's range test. All statistical analysis was performed using SPSS version 19.0 (IBM Corp., Armonk, NY), and significance was set at $p < 0.05$.

Results

Multiple sequence alignment and phylogenetic analysis

The full-length *PnHSP70* cDNA contained an open reading frame (ORF) of 2037 bp encoding a heat-shock protein of 678 amino acids with a deduced molecular weight of 72.82 kDa and a pI of 6.00. The deduced molecular weight

indicated that the isolated *PnHSP70* gene belonged to the HSP70 family. Multiple sequence alignment showed that *PnHSP70* was homologous to HSP70s of other species such as *A. thaliana* (Q9LDZ0), *Oryza sativa* Japonica Group (NP_001068540), *P. patens* subsp. *patens* (EDQ72088), *Vitis vinifera* (XP_002263457) and *Chlamydomonas reinhardtii* (EDP02463). The amino acid sequence deduced from the cDNA shared 93.0 % identity with the HSP70 of *P. patens* subsp. *patens*. However, the percentage identities to the HSP70s of *A. thaliana*, *O. sativa* and *C. reinhardtii* were all below 75.0 % (Fig. 1).

Multiple sequence alignment revealed that the deduced amino acid sequence of *PnHSP70* shared homologous domains with the amino acid sequences of other known mitochondrial HSP70s (Fig. 1). Amino acid residues 1–60

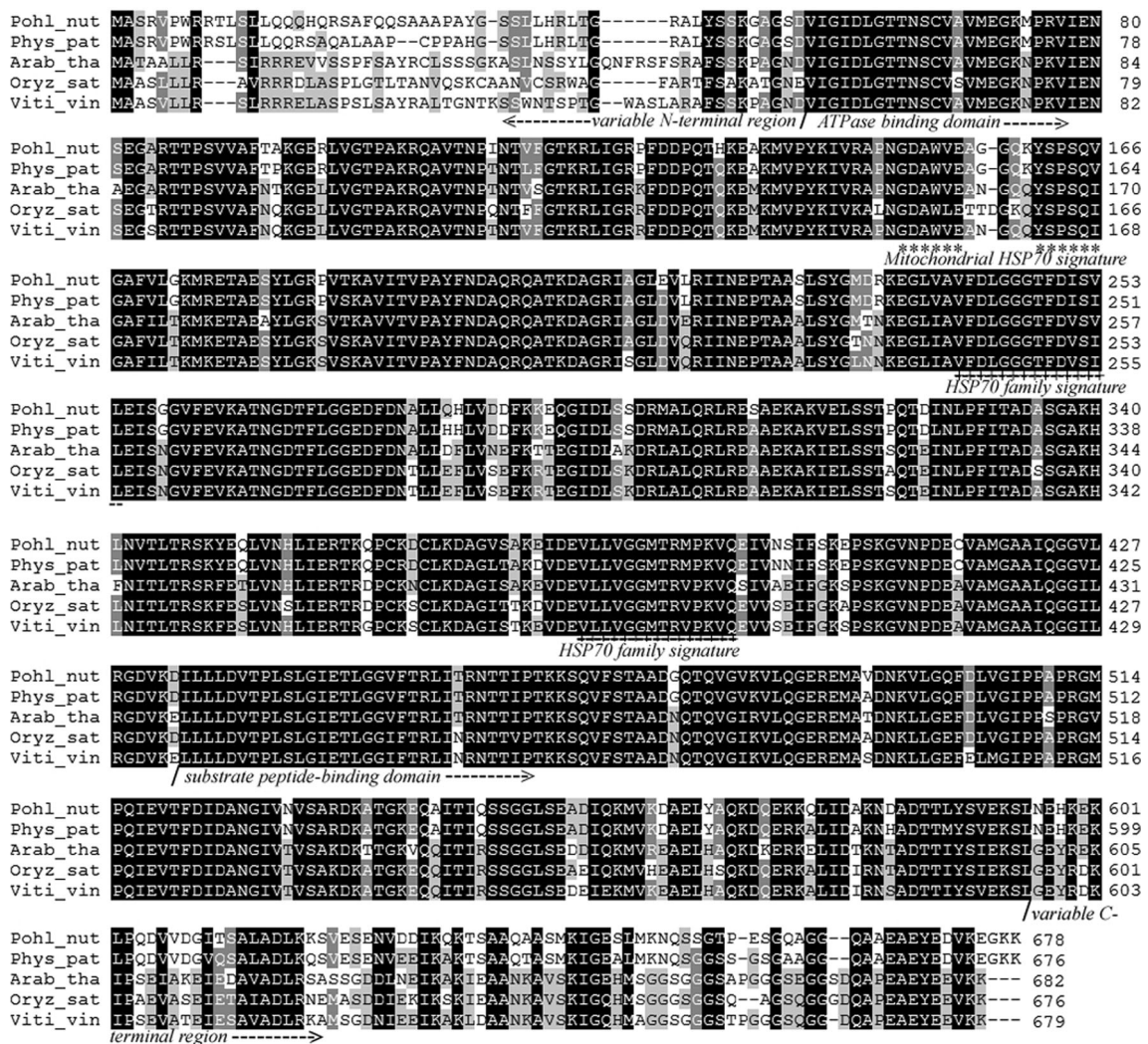


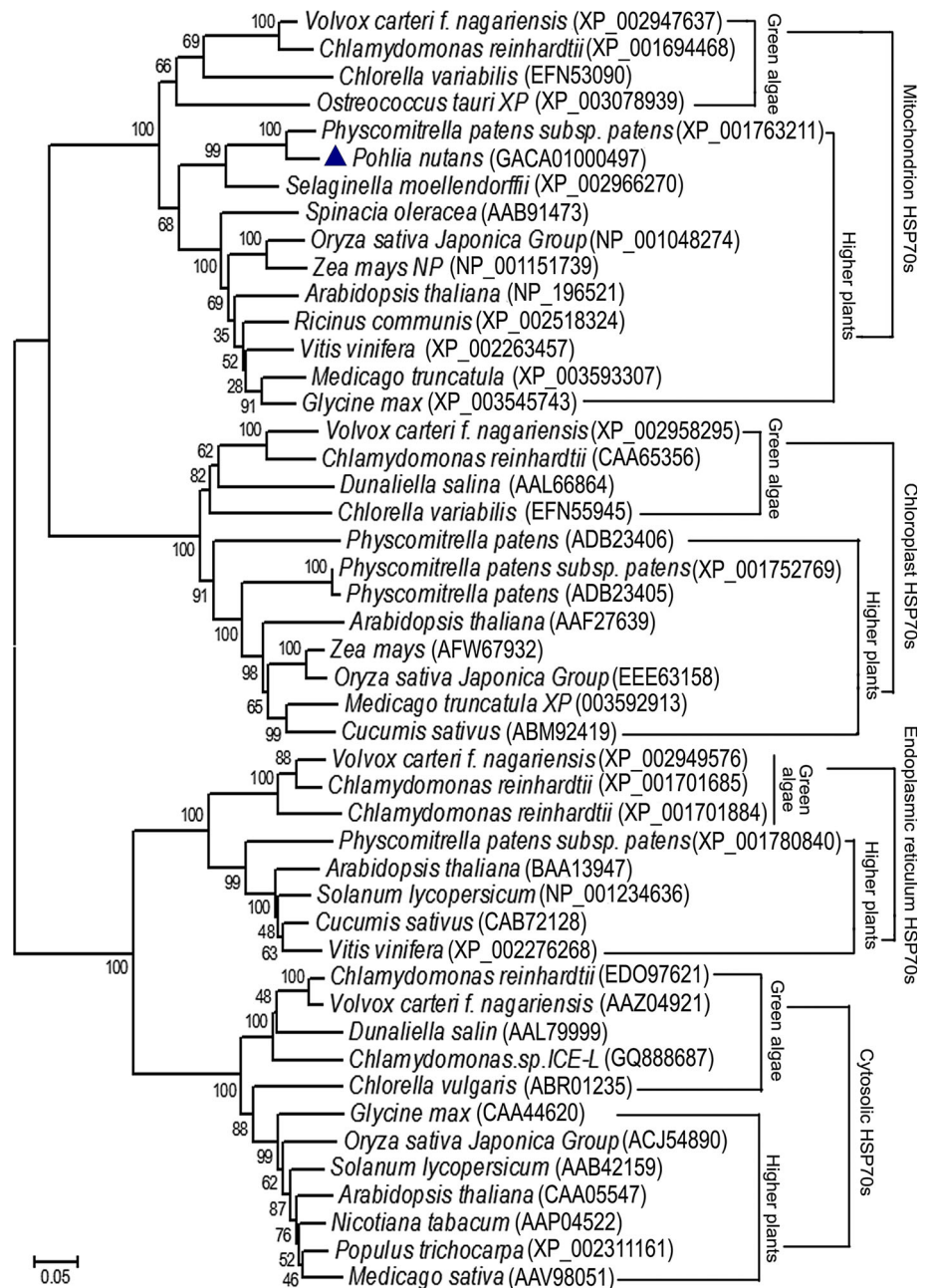
Fig. 1 Alignment and comparison of the Antarctic moss *Pohlia nutans* HSP70 with other plant homologs showing mitochondrial signatures. Arab_tha: *Arabidopsis thaliana* (Q9LDZ0), Oryz_sat: *Oryza sativa* Japonica Group (NP_001068540), Phys_pat: *Physcomitrella patens* subsp. *patens* (EDQ72088), Viti_vin: *Vitis vinifera*

(XP_002263457). The asterisk indicates the GDAWV and YSPSQI signatures found in mitochondrial HSP70 homologs. A plus sign indicates HSP70 family signatures. Black bars show identical amino acid residues. Deletions are indicated by dashes

in the alignment included the variable N-terminal region, which contains sequences for transport into the mitochondrion. The much more highly conserved adenosine triphosphatase (ATPase) binding domain included residues 60–440. This region showed considerable sequence conservation with substitution of individual residues only. The substrate peptide-binding domain (residues 440–600) was extremely well conserved. The variable C-terminal region (600–686) was absent in some proteins and highly variable in others, and its function is not well established. In addition, PnHSP70 possessed the conserved signature sequences GDAWV and YSPSQI, which are only found in mitochondrial HSP70 homologs (Fig. 1).

Representative mitochondrial HSP70 proteins of land plants and green algae were selected to construct a phylogenetic tree using the programs Clustal W and Mega 4.0 (Fig. 2). Additionally, some HSP70s belonging to other groups (chloroplast, cytosolic, and endoplasmic reticulum) were selected and used to construct a phylogenetic tree for studying the phylogenetic relationships among different HSP70 groups. The phylogenetic tree of HSP70 proteins constructed by Neighbor-Joining revealed that the mitochondrial, chloroplast, cytosolic, and endoplasmic reticulum HSP70 groups were clustered in separate clades, which was consistent with earlier published classifications. Moreover, the HSP70 proteins from land plants and green algae were

Fig. 2 A phylogenetic tree of HSP70 family members constructed with the Neighbor-Joining method. Numbers at each branch indicate the percentage of times a node was supported in 1,000 bootstrap pseudoreplications by neighbor-joining. Accession numbers are shown in parentheses



also clustered into separate clades. In the HSP70 phylogenetic tree, the Antarctic moss *P. nutans* (GACA01000497) clustered together with *P. patens* subsp. *patens* (XP_001763211.1), *Selaginella moellendorffii* (XP_002966270.1) and formed a sister group (Fig. 2). However, the PnHSP70 clade consisting of mosses and ferns was relatively close to the mitochondrial HSP70s of green algae, but was relatively distant from higher plants. In general, the relationships displayed in the phylogenetic tree were consistent with the traditional taxonomic classification.

Subcellular localization in *A. thaliana* mesophyll protoplasts

The subcellular localization of PnHSP70 protein was investigated in living *A. thaliana* mesophyll protoplasts (Fig. 3). The protoplasts were transformed with the *p35S::PnHSP70::GFP/pBI221* vector, and the GFP signals were observed by confocal laser-scanning microscopy. When referenced to the morphological characteristics of mitochondria in *A. thaliana* mesophyll protoplasts (Zhang et al. 2009b), we deduced that the green fluorescence was mainly localized in mitochondria (Fig. 3a). Red

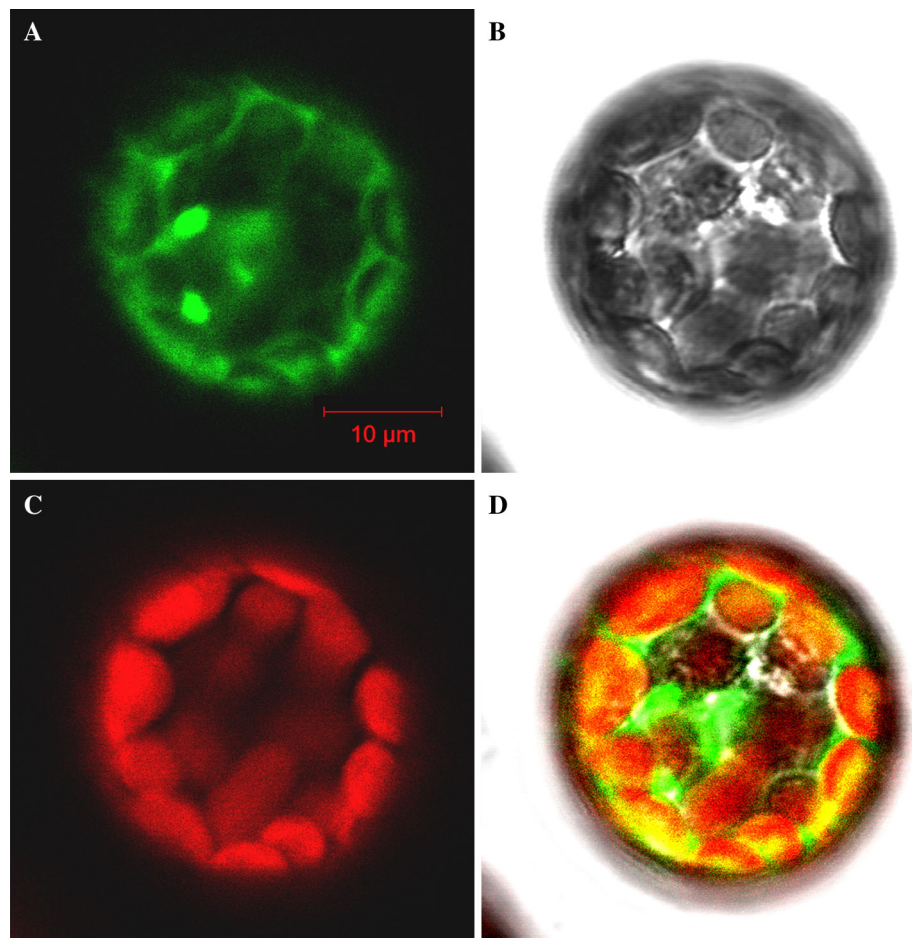
autofluorescence released by chloroplasts surrounded but did not colocalize with the green fluorescence (Fig. 3c). Furthermore, in colocalization analysis involving merging green fluorescence, bright field, and red chlorophyll autofluorescence images, there were no distinct yellow signals in the positions of chloroplasts (Fig. 3d).

Expression levels of *PnHSP70* in response to different stress treatments

In the cold and heat-shock treatments, mosses were kept at 4, 10 or 21.5 °C and incubated for 0, 3, 6, 12, 24 or 36 h. Real-time PCR analysis showed that the expression levels of *PnHSP70* increased 3.34-fold at 24 h after 4 °C treatment and increased 2.74-fold at 24 h after 10 °C treatment. The heat-shock treatment also increased the expression levels of *PnHSP70* (2.30-fold), but the magnitude of increase was lower when compared with the cold-shock treatment (Fig. 4).

In the salt treatment, mosses were sprayed with 200 mM NaCl and incubated for 0, 3, 6, 12, 24 or 36 h to investigate the expression levels of *PnHSP70*. Real-time PCR analysis showed that the expression level of *PnHSP70* reached a

Fig. 3 Intracellular targeting of PnHSP70 in *A. thaliana* protoplasts. Protoplasts were transformed with the *p35S::PnHSP70::GFP/pBI221* vector. The confocal images were taken 12 h after protoplast transformation. **a** Green fluorescence of PnHSP70-GFP fusion proteins; **b** the protoplast in bright field; **c** red chlorophyll autofluorescence; **d** a merged image of **a**, **b**, and **c**. Bar 10 μm



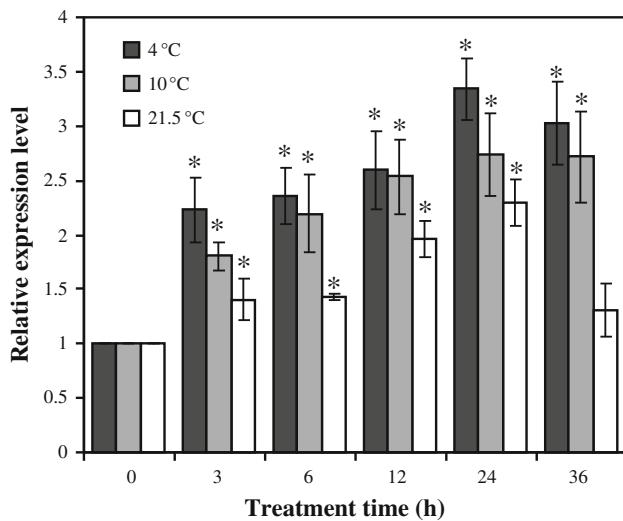


Fig. 4 *PnHSP70* mRNA levels after cold or heat shock stress. Values are expressed as mean ± SE. Standard error bars are shown. Asterisks indicate significant differences in means between the control and treatment groups ($p < 0.05$)

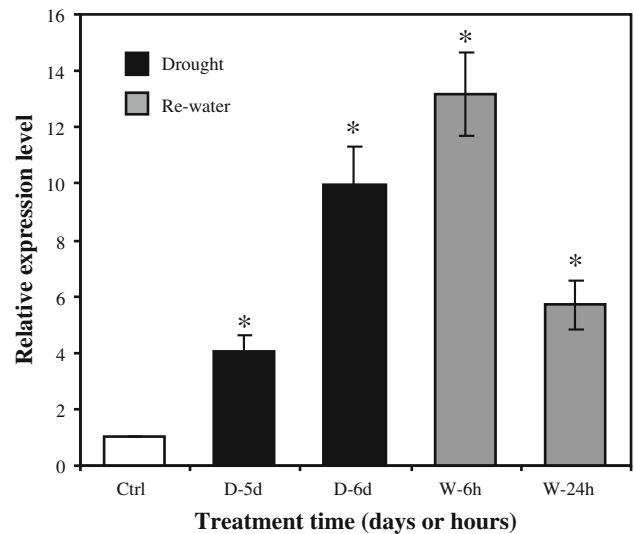


Fig. 6 *PnHSP70* mRNA levels after drought stress and re-watering. D-5d or D-6d: moss deprived of water for 5 or 6 days; W-6 h: re-watering for 6 h; W-24: re-watering for 24 h. Values are expressed as mean ± SE. Standard error bars are shown. Asterisks indicate significant differences in means between the control and treatment groups ($p < 0.05$)

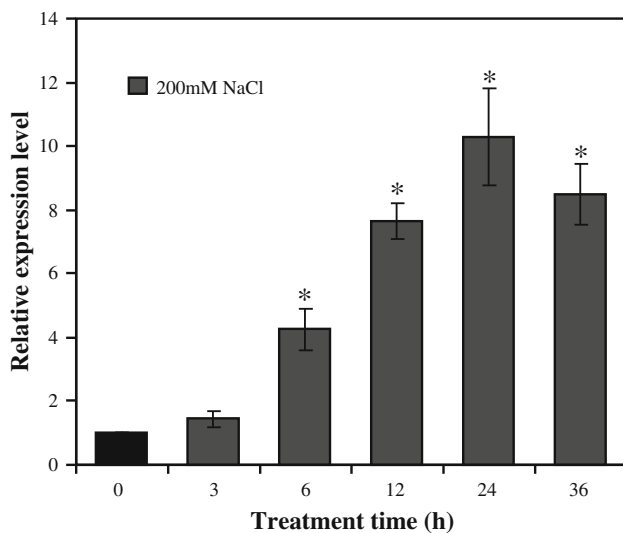


Fig. 5 *PnHSP70* mRNA levels after salt stress. Values are expressed as mean ± SE. Standard error bars are shown. Asterisks indicate significant differences in means between the control and treatment groups ($p < 0.05$)

maximum value of 10.29-fold at 24 h and was increased 8.50-fold at 36 h after 200 mM NaCl treatment (Fig. 5).

In the drought treatment, mosses were deprived of water for 5 or 6 days and then re-watered for 6 or 24 h. Real-time PCR analysis showed that the expression level of *PnHSP70* increased 9.78-fold after water deprivation for 6 days. Furthermore, after re-watering the mosses, the expression levels of *PnHSP70* continually increased to 13.18-fold at 6 h and then gradually decreased to 5.70-fold at 24 h (Fig. 6). Mosses

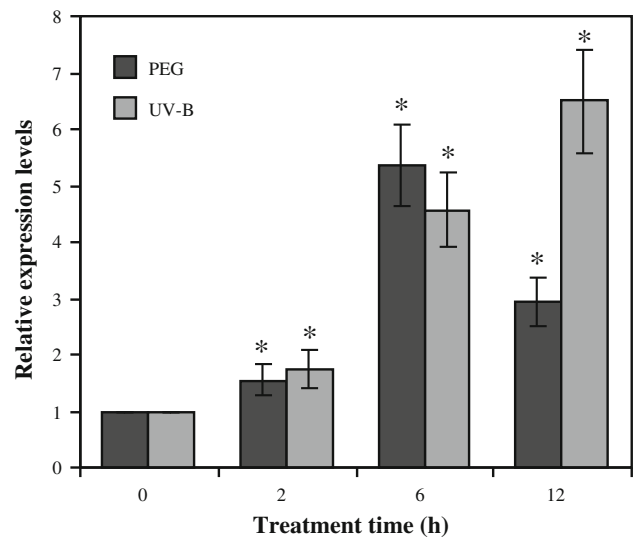


Fig. 7 *PnHSP70* mRNA levels after PEG6000 treatment and UV-B radiation. Values are expressed as mean ± SE. Standard error bars are shown. Asterisks indicate significant differences of means between the control and treatment groups ($p < 0.05$)

were also sprayed with 20 % (w/v) PEG6000 solution for 0, 2, 6 or 12 h to simulate drought stress. Real-time PCR analysis showed that the expression levels of *PnHSP70* increased 5.35-fold after PEG6000 treatment for 6 h (Fig. 7).

In the UV-B radiation treatment, mosses were placed under an 8 W UV-B lamp (302 nm, reaching 70 μW/cm² irradiance) for 0, 2, 6 or 12 h, with mosses cultured under visible light as a control. Real-time PCR analysis showed

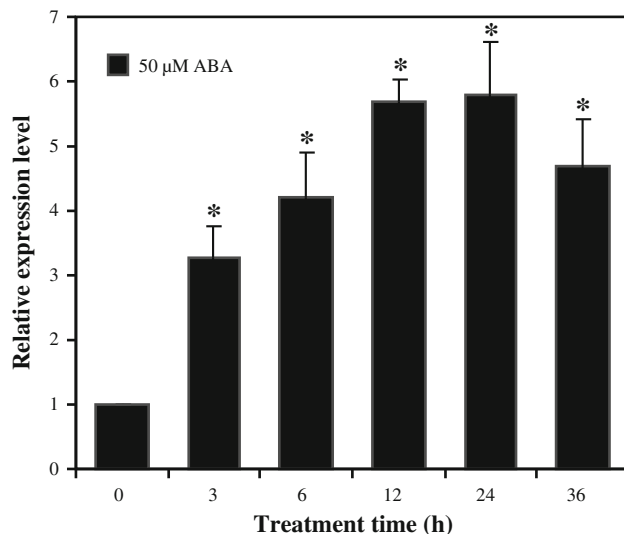


Fig. 8 *PnHSP70* mRNA levels after ABA treatment. Values are expressed as mean \pm SE. Standard error bars are shown. Asterisks indicate significant differences of means between the control and treatment groups ($p < 0.05$)

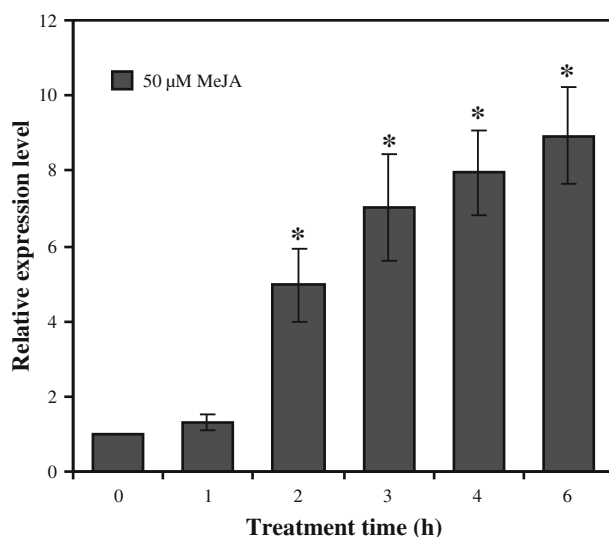


Fig. 9 *PnHSP70* mRNA levels after JA treatment. Values are expressed as mean \pm SE. Standard error bars are shown. Asterisks indicate significant differences of means between the control and treatment groups ($p < 0.05$)

that the expression levels of *PnHSP70* increased 6.50-fold at 12 h after UV-B radiation treatment (Fig. 7).

In the plant hormone treatment, mosses were pretreated with 50 μ M ABA or MeJA and incubated for different times to investigate the expression levels of *PnHSP70*. Real-time PCR analysis showed that the expression level of *PnHSP70* significantly increased by 3.28-fold at 3 h after ABA treatment and reached a maximal level of 5.80-fold at 24 h (Fig. 8). The expression levels of *PnHSP70* increased 8.93-fold at 6 h after 50 μ M MeJA treatment (Fig. 9).

Discussion

HSP70 proteins are central components of the cellular network of molecular chaperones and play a crucial role in maintaining protein homeostasis by reestablishing functional native conformations under diverse environmental stresses (Mayer and Bukau 2005). In the Antarctic moss transcriptome, we found an interesting a mitochondrial heat-shock protein 70 gene (*PnHSP70*) with a significant fold change and relatively high raw reads, implying that the protein had an essential function in cold resistance (Liu et al. 2013). Further study showed that the full-length cDNA encoded a protein of 678 amino acids with a deduced molecular weight of 72.82 kDa. *PnHSP70* contained the conserved mitochondrial HSP70 sequence signatures GDAVV and YSPSQI, suggesting that it was a member of the mitochondrial HSP70 family or lineage (Fig. 1) (Germot et al. 1996).

Recently, because of the highly conserved structure and important function of HSP70s, we proposed that degrees of difference in structure might reflect the phylogenetic relationships across species of algae and land plants (Liu et al. 2010). The phylogenetic tree of HSP70 proteins constructed by Neighbor-Joining revealed that the mitochondrial, chloroplast, cytosolic, and endoplasmic reticulum HSP70 groups were clustered in separate clades, which was consistent with previously published classifications (Renner and Waters 2007; Fu et al. 2009). Within each clade, higher plants and green algae were also clustered together separately (Fig. 2). Moreover, phylogenetic tree analysis showed that *PnHSP70* clustered together with other mitochondrial HSP70s, which was consistent with the results of multiple sequence alignment (Fig. 1). In addition, the subcellular localization was further analyzed by observing the transient expression of *PnHSP70*-GFP in *A. thaliana* mesophyll protoplasts. After comparing the morphological characteristics of *PnHSP70*-GFP fluorescence with mitochondria visualized by MitoTracker Red in *A. thaliana* mesophyll protoplasts (Zhang et al. 2009b), we believe that the fusion *PnHSP70* was targeted to mitochondria (Fig. 3). In the mitochondrial HSP70s clade, *PnHSP70* was relatively close to mitochondrial HSP70s of *P. patens* subsp. *patens* and *S. moellendorffii*, but was relatively distant from green algae and other land plants.

Various abiotic stresses lead to the overproduction of reactive oxygen species (ROS) in plants, which are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates and DNA, ultimately resulting in oxidative stress (Gill and Tuteja 2010). Mitochondria are deeply involved in the production of reactive oxygen species through one-electron carriers in the respiratory chain (Rhoads et al. 2006; Vanlerberghe 2013). Therefore, under environmental stress conditions, the function of

mitochondrial HSP70 proteins in maintaining protein homeostasis by reestablishing functional native conformations is crucial for cell survival. In the Antarctic moss *P. nutans*, real-time PCR analysis showed that the expression levels of *PnHSP70* were increased not only in response to heat-shock stress (2.30-fold) but also cold-shock stress (Fig. 4). In Antarctic *Deschampsia* leaves, Western Blot analysis showed that the optimal temperature for *HSP70* accumulation was 35 °C, with a maximum increase 1.9-fold higher than in control plants (Reyes et al. 2003). In transcriptional analysis of a *HSP70* gene from a haloarchaea, both temperature and osmotic stress could induce expression of the gene, while the expression levels in response to cold-shock were also stronger than under heat shock (Zhang et al. 2009a).

Cellular responses to drought stress include osmotic adjustment, regulation of water circulation (aquaporins), protection or degradation of proteins, and protection against oxidative stress (Timperio et al. 2008). Transgenic tobacco plants constitutively overexpressing a *HSP70* homolog gene were resistant to drought stress, supplying evidence for a direct correlation between the HSP70 and water stress tolerance (Alvim et al. 2001). In the present study, real-time PCR analysis showed that *PnHSP70* mRNA levels increased 9.78-fold after water deprivation for 6 days, and continually increased to 13.18-fold after re-watering for 6 h (Fig. 6). Similarly, *PnHSP70* mRNA levels were also up-regulated by PEG6000 treatment (Fig. 7).

Salinity acts like drought on plants, preventing roots from performing their osmotic activity in which water and nutrients move from an area of low concentration to an area of high concentration. Several reports have suggested that *HSP70* mRNA levels are regulated by osmotic stress (Shim et al. 2002; Dong et al. 2008). In the present study, real-time PCR analysis showed that *PnHSP70* mRNA levels increased 10.29-fold after 200 mM NaCl treatment for 24 h (Fig. 5). In the Antarctic ice algae *Chlamydomonas* sp. ICE-L, the expression of a cytosolic *HSP70* gene was also induced by high salt treatment (Liu et al. 2010). However, in *Laminaria japonica*, it was demonstrated that the expression levels of *LJHSP70* were increased at a low salt concentration rather than at a higher salt concentration (Fu et al. 2009). In the Antarctic bacterium *Psychrobacter* sp. G, the expression of a cold-shock protein (CSP) gene (*Csp1137*) was enhanced by high salinity, whereas *Csp2531* was enhanced by low salinity (Song et al. 2012).

In particular, polar organisms suffer from strong UV radiation, which has been further intensified by the extension of stratospheric ozone depletion (Rautenberger et al. 2013; Huovinen and Gómez 2013). UV radiation stimulates the production of ROS and ABA; the former can cause severe oxidative damage, while the latter triggers downstream stress-responsive pathways (Berli et al. 2011).

Interestingly, our results showed that *PnHSP70* transcripts were increased after UV radiation treatment (6.50-fold), implying it has a positive role in the acclimation of the Antarctic moss *P. nutans* to the polar environment (Fig. 7). In *Deschampsia antarctica*, suppression subtractive hybridization showed that cold and UV radiation mainly induce the expression of genes related to transcription, energy and defense responses, which includes chaperone proteins (approximately 3.50-fold) (Dinamarca et al. 2013).

The plant hormones ABA and JA both play key roles in sensing and signaling numerous environmental conditions (Raghavendra et al. 2010; de Ollas et al. 2013). Recently, research on ABA signaling has been focused largely on the regulation of gene expression. In the present study, real-time PCR analysis showed that ABA treatment also up-regulated the expression levels of *PnHSP70*, which suggested that *PnHSP70* might be a downstream molecule in ABA signaling (Fig. 8). Cold can also cause plant dehydration, which activates the ABA signaling pathway (Chinnusamy et al. 2007). The cold acclimation of bryophytes (*P. patens*) also requires an ABA-dependent signaling process (Bhyan et al. 2012). Furthermore, transient JA accumulation is necessary for the subsequent ABA increase in citrus roots under drought stress conditions (de Ollas et al. 2013). Our results showed that MeJA treatment could also up-regulate the expression levels of *PnHSP70* in *P. nutans* (Fig. 9), suggesting that *PnHSP70* is also involved in the JA signaling pathway.

Heat-shock proteins are well known to be expressed in response to a range of cellular stresses and subsequently protect against protein denaturation (Madden et al. 2008). Although inducible HSP70s are among the most studied of these stress proteins and this study has only examined the accumulation of *PnHSP70* transcripts, to our knowledge, this is the first characterized mitochondrial HSP70 in Antarctic mosses. These findings will help to understand the mechanisms of stress acclimation and to provide a basis for engineering strategies to improve stress tolerance in agricultural crops.

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References

- Alder NN, Shen Y, Brodsky JL, Hendershot LM, Johnson AE (2005) The molecular mechanisms underlying BiP-mediated gating of the Sec61 translocon of the endoplasmic reticulum. *J Cell Biol* 168:389–399
- Alvim FC, Carolino SM, Cascardo JC, Nunes CC, Martinez CA, Otoni WC, Fontes EP (2001) Enhanced accumulation of BiP in transgenic plants confers tolerance to water stress. *Plant Physiol* 126:1042–1054

- Berli FJ, Fanzone M, Piccoli P, Bottini R (2011) Solar UV-B and ABA are involved in phenol metabolism of *Vitis vinifera* L. increasing biosynthesis of berry skin polyphenols. *J Agric Food Chem* 59:4874–4884
- Bhyan SB, Minami A, Kaneko Y, Suzuki S, Arakawa K, Sakata Y, Takezawa D (2012) Cold acclimation in the moss *Physcomitrella patens* involves abscisic acid-dependent signaling. *J Plant Physiol* 169:137–145
- Bramley-Alves J, King DH, Robinson RA, Miller RE (2014) Dominating the Antarctic environment: bryophytes in a time of change. In: Hanson DT, Rice SK (eds) *Photosynthesis in bryophytes and early land plants*. Springer, Dordrecht, pp 309–324
- Chinnusamy V, Zhu JH, Zhu JK (2007) Cold stress regulation of gene expression in plants. *Trends Plant Sci* 12:444–451
- Clarke LJ, Robinson SA (2008) Cell wall-bound ultraviolet-screening compounds explain the high ultraviolet tolerance of the Antarctic moss, *Ceratodon purpureus*. *New Phytol* 179:776–783
- Convey P, Smith RIL (2006) Responses of terrestrial Antarctic ecosystems to climate change. *Plant Ecol* 182:1–10
- Convey P, Stevens MI (2007) Antarctic Biodiversity. *Science* 317:1877–1878
- De Los Rios P, Ben-Zvi A, Slutsky O, Azem A, Goloubinoff P (2006) Hsp70 chaperones accelerate protein translocation and the unfolding of stable protein aggregates by entropic pulling. *Proc Natl Acad Sci USA* 103:6166–6171
- de Ollas C, Hernando B, Arbona V, Gómez-Cadenas A (2013) Jasmonic acid transient accumulation is needed for abscisic acid increase in citrus roots under drought stress conditions. *Physiol Plant* 147:296–306
- Dinamarca J, Sandoval-Alvarez A, Gidekel M, Gutiérrez-Morag A (2013) Differentially expressed genes induced by cold and UV-B in *Deschampsia antarctica* Desv. *Polar Biol* 36:409–418
- Dong Y, Dong S, Meng X (2008) Effects of thermal and osmotic stress on growth, osmoregulation and Hsp70 in sea cucumber (*Apostichopus japonicus* Selenka). *Aquaculture* 276:179–186
- Fu W, Yao J, Wang X, Liu F, Fu G, Duan D (2009) Molecular cloning and expression analysis of a cytosolic Hsp70 gene from *Laminaria japonica* (Laminariaceae, Phaeophyta). *Mar Biotechnol* 11:738–747
- Germot A, Philippe H, Le Guyader H (1996) Presence of a mitochondrial-type 70-kDa heat shock protein in *Trichomonas vaginalis* suggests a very early mitochondrial endosymbiosis in eukaryotes. *Proc Natl Acad Sci USA* 93:14614–14617
- Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem* 48(12):909–930
- Gornall JL, Jónsdóttir IS, Woodin SJ, der Wal RV (2007) Arctic mosses govern below-ground environment and ecosystem processes. *Oecologia* 153:931–941
- Horst M, Oppliger W, Rospert S, Schonfeld HJ, Schatz G, Azem A (1997) Sequential action of two hsp70 complexes during protein import into mitochondria. *EMBO J* 16:1842–1849
- Huovinen P, Gómez I (2013) Photosynthetic characteristics and UV stress tolerance of Antarctic seaweeds along the depth gradient. *Polar Biol* 36:1319–1332
- Kato K, Arikawa T, Imura S, Kanda H (2013) Molecular identification and phylogeny of an aquatic moss species in Antarctic lakes. *Polar Biol* 36:1557–1568
- Kumar S, Nei M, Dudley J, Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* 9:299–306
- Liu S, Zhang P, Cong B, Liu X, Lin X, Shen J, Huang X (2010) Molecular cloning and expression analysis of a cytosolic Hsp70 gene from Antarctic ice algae *Chlamydomonas* sp. *ICE-L*. *Extremophiles* 14:329–337
- Liu S, Wang N, Zhang P, Cong B, Lin X, Wang S, Xia G, Huang X (2013) Next-generation sequencing-based transcriptome profiling analysis of *Pohlia nutans* reveals insight into the stress-relevant genes in Antarctic moss. *Extremophiles* 17:391–403
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25:402–408
- Lovelock CE, Jackson AE, Melick DR, Seppelt RD (1995) Reversible photoinhibition in Antarctic moss during freezing and thawing. *Plant Physiol* 109:955–961
- Madden LA, Sandström ME, Lovell RJ, McNaughton L (2008) Inducible heat shock protein 70 and its role in preconditioning and exercise. *Amino Acids* 34:511–516
- Mayer MP, Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci* 62:670–684
- Montero-Barrientos M, Hermosa R, Cardoza RE, Gutiérrez S, Nicolás C, Monte E (2010) Transgenic expression of the *Trichoderma harzianum* hsp70 gene increases *Arabidopsis* resistance to heat and other abiotic stresses. *J Plant Physiol* 167:659–665
- Morgan-Kiss RM, Prisco JC, Pocock T, Gudynaite-Savitch L, Huner NP (2006) Adaptation and acclimation of photosynthetic microorganisms to permanently cold environments. *Microbiol Mol Biol Rev* 70:222–252
- Newsham KK (2010) The biology and ecology of the liverwort *Cephaloziella varians* in Antarctica. *Antarct Sci* 22:131–143
- Pearce DA (2008) Climate change and the microbiology of the Antarctic Peninsula region. *Sci Prog* 91:203–217
- Raghavendra AS, Gonugunta VK, Christmann A, Grill E (2010) ABA perception and signalling. *Trends Plant Sci* 15:395–401
- Rautenberger R, Wiencke C, Bischof K (2013) Acclimation to UV radiation and antioxidative defence in the endemic Antarctic brown macroalga *Desmarestia anceps* along a depth gradient. *Polar Biol* 36:1779–1789
- Reddy PS, Mallikarjuna G, Kaul T, Chakradhar T, Mishra RN, Sopory SK, Reddy MK (2010) Molecular cloning and characterization of gene encoding for cytoplasmic Hsc70 from *Pennisetum glaucum* may play a protective role against abiotic stresses. *Mol Genet Genomics* 283:243–254
- Renner T, Waters ER (2007) Comparative genomic analysis of the Hsp70s from five diverse photosynthetic eukaryotes. *Cell Stress Chaperon* 12:172–185
- Reyes M, Corcuera LJ, Cardemil L (2003) Accumulation of HSP70 in *Deschampsia antarctica* Desv. leaves under thermal stress. *Antarct Sci* 15:345–352
- Rhoads DM, Umbach AL, Subbaiah CC, Siedow JN (2006) Mitochondrial reactive oxygen species. Contribution to oxidative stress and interorganellar signaling. *Plant Physiol* 141:357–366
- Shi LX, Theg SM (2010) A stromal heat shock protein 70 system functions in protein import into chloroplasts in the moss *Physcomitrella patens*. *Plant Cell* 22:205–220
- Shim EH, Kim JI, Bang ES, Heo JS, Lee JS, Kim EY, Lee JE, Park WY, Kim SH, Kim HS, Smithies O, Jang JJ, Jin DI, Seo JS (2002) Targeted disruption of *hsp70.1* sensitizes to osmotic stress. *EMBO Rep* 3:857–861
- Singh J, Dubey AK, Singh RP (2011) Antarctic terrestrial ecosystem and role of pigments in enhanced UV-B radiations. *Rev Environ Sci Biotechnol* 10:63–77
- Skotnicki ML, Ninham JA, Selkirk PM (2000) Genetic diversity, mutagenesis and dispersal of Antarctic mosses—a review of progress with molecular studies. *Antarct Sci* 12:363–373
- Song W, Lin X, Huang X (2012) Characterization and expression analysis of three cold shock protein (CSP) genes under different stress conditions in the Antarctic bacterium *Psychrobacter* sp. G. *Polar Biol* 35:1515–1524

- Timperio AM, Egidi MG, Zolla L (2008) Proteomics applied on plant abiotic stresses: role of heat shock proteins (HSP). *J Proteomics* 71:391–411
- Vanlerberghe GC (2013) Alternative oxidase: a mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. *Int J Mol Sci* 14:6805–6847
- Victoria FC, Pereira AB, Costa DP (2009) Composition and distribution of moss formations in the ice-free areas adjoining the Arctowski region, Admiralty Bay, King George Island, Antarctica. *Iheringia, Série Botânica* 64:81–91
- Wang S, Zhu X, Yang Y, Yang J, Gu Y, Wei J, Hao R, Boireau P, Cui S (2009) Molecular cloning and characterization of heat shock protein 70 from *Trichinella spiralis*. *Acta Trop* 110:46–51
- Yoo SD, Cho YH, Sheen J (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc* 2:1565–1572
- Zhang H, Cui P, Lin L, Shen P, Tang B, Huang YP (2009a) Transcriptional analysis of the hsp70 gene in a haloarchaeon *Natrinema* sp. J7 under heat and cold stress. *Extremophiles* 13:669–678
- Zhang L, Li Y, Xing D, Gao C (2009b) Characterization of mitochondrial dynamics and subcellular localization of ROS reveal that HsfA2 alleviates oxidative damage caused by heat stress in *Arabidopsis*. *J Exp Bot* 60:2073–2091
- Zhang P, Liu S, Chen K (2013) Characterization and expression analysis of a glutathione reductase gene from Antarctic moss *Pohlia nutans*. *Plant Mol Biol Rep* 31:1068–1076