Molecular Cloning and Sequence Analysis of a Mitogen-Activated Protein Kinase Gene in the Antarctic Yeast Rhodotorula Mucilaginosa AN5

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Molecular Cloning and sequence analysis of a mitogen-activated protein kinase gene in the Antarctic yeast *Rhodotorula mucilaginosa* AN5

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Declarations

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The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and material
All data generated or analysed during this study are included in this published article and are available from the corresponding author.

Code availability
Not applicable.

Authors' contributions
Cuijuan Shi and Yun Ju conducted experiments. Hong Zhang，Kai Yu and Yingying Wang collected and analyzed the data. Jie Jiang and Guangfeng Kan designed and supervised the project. Chuanzhou Zhang and Ziyi Cheng wrote and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT
The mitogen-activated protein kinase (MAPK) cascades play important roles in various signaling transduction networks of biotic and abiotic stress responses. However, MAPK signaling pathways in cold-active yeast *Rhodotorula mucilaginosa* have not been reported comprehensively. In the present study, MAPK gene (*RmMAPK*) was first cloned and characterized from Antarctic sea ice yeast *R. mucilaginosa* AN5. The full length of the *RmMAPK* gene is 1086 bp and encodes a 361 amino acids protein with a predicted molecular mass of 40.9 kDa and a pI of 5.25. The RmMAPK contains 11 MAPK conserved subdomains and the phosphorylation motif TGY located in the activation loop of the kinase. Quantitative real-time PCR assay revealed that the expression of *RmMAPK* up-regulated rapidly and significantly when yeast cells were subjected to low temperature (4 °C), high salinity (120‰ NaCl) and heavy metal (2 mmol/L $CuCl_2$), which suggested that the *RmMAPK* might act as a key function in response to extreme stresses, such as low temperature, high salinity and heavy metal.

1. Introduction

All living organisms are capable of perceiving and adapting external environment changes by activation of some signal transduction pathways, which eventually lead to changes of intracellular activities. In several cases, a family of serine/threonine protein kinase known as the mitogen-activated protein kinase (MAPK) is involved. MAPK was first identified as microtubule-associated protein kinase by Sturgill and Ray [1], and thus was named microtubule-associated protein kinases. After that, MAPKs are found extensively in a variety of eukaryotic organism, ranging from fungi, plants to human [2-4].

In some fungi and yeasts, the most studied MAPK pathway is usually named the high osmolarity glycerol (HOG) signaling pathway, because of its activation by exposing in a high level of osmotic shock [5]. The HOG MAPK signaling pathway is a three-kinase module composed of MAPK Hog1p, MAPKK Pbs2p and MAPKKK Ssk2p. When cells are exposed to osmotic stress, the transmembrane osmosensors Sho1p and Sln1p transmit the signal to their respective downstream MAPK kinase kinase (MAPKKK) Ssk2p, then to MAPK kinase (MAPKK) Pbs2p, which phosphorylates and activates ScHog1. The activated Hog1 enhances the transcription of glycerol phosphate dehydrogenase gene to increase intracellular glycerol production and adjust the osmotic balance [6-8]. Apart from hyperosmotic adaptability response, HOG signaling pathway also plays similar roles in fungi response to other extracellular challenges, including low temperature, nutrient limitation, UV irradiation, heat shock, heavy metals or oxidative stress [9-13].

Antarctic yeast *Rhodotorula mucilaginosa* AN5 is a unicellular pigmented eukaryote isolated from sea ice of Antarctica and stored at our laboratory at -80 °C [14]. Surviving and thriving in permanent low temperature environments, the yeast AN5 was tested to be tolerant at 0 °C with the optimum growth temperature at 20 °C [14]. Furthermore, in polar sea ice, the only liquid that yeasts lives is pocketed of concentrated brines [15]. Our experiments also indicated that yeast AN5 could survive and reproduce in high salinity of 120‰, and was more resistant compared to mesophilic yeasts (data not shown). Moreover, in the Antarctic ecosystems, besides the geochemical characteristics, the human activity and industrial emissions of heavy metals to the atmosphere, give a significant increase of some metals [16,17].The yeast strain AN5 has been observed strong tolerant to different concentrations of heavy metals, such as Cd, Pb, Mn, Cu, Cr and Hg [14]. All of these investigations prove that Antarctic yeast *Rhodotorula mucilaginosa* AN5 is cold-active yeast and behave some special properties that adapt to polar sea ice habitats.

To explain the adaption mechanisms of polar yeast to abiotic stresses and research the involvement of MAPK in extreme environments resistance, a *MAPK* homologue gene *RmMAPK* was isolated, identified and characterized from Antarctic yeast *Rhodotorula mucilaginosa* AN5. In addition, the expression variation of *RmMAPK* gene under low temperature, high salinity and copper stresses were quantified by quantitative real-time PCR (qPCR) assay.

2. Materials and methods

2.1 Microorganisms and culture conditions

The yeast strain *R. mucilaginosa* AN5 was isolated from Antarctic sea-ice samples collected by the 23th Chinese Antarctic scientific expedition in 2007.

The yeast was cultivated overnight from frozen glycerol stock in 10 ml YEPD medium (1.0% yeast extract, 2.0% peptone, 2.0% dextrose in sterilized sea water) at 20 °C on an orbital shaker of 120 rpm. And then the culture was diluted in 250 ml YEPD medium and grown to mid-log phase (A_{600} = 0.5). For low temperature treatments, yeast was transferred to 4 °C and further culture for 0 h, 4 h, 12 h and 24 h respectively. For high salinity treatments, yeast was cultured in 120‰ salinity by the adding of NaCl. For heavy metal treatments, yeast was cultured in YEPD medium by adding of $CuCl_2$ to the final concentration of 2 mmol/L. The cells were collected by centrifugation at 8 000 rpm for 5 min, and then frozen in liquid nitrogen and stored in -80 °C for further analysis.

E. coli DH5α and BL21 were grown in LB medium (0.5% yeast extract, 1.0% peptone and 1.0% NaCl, pH 7.0) at 37 °C.

2.2 Cloning and sequencing of *RmMAPK* gene

The collected yeast cells were ground with a mortar and pestle in liquid nitrogen. Total RNA was extracted following the instruction of total RNA extractor (Trizol) (Sangon, China) and then treated with gDNA eraser (Takara, Dalian, China) for 2 min at 42 °C to remove the possible DNA contamination. The integrity of purified RNA was examined in 1.0% (w/v) agarose gel. The concentration and quality of RNA were measured by a NAS-99 micro-volume spectrophotometer (ATCGene, USA).

The first strand cDNA was synthesized following the manufacturer's instruction of the PrimeScript RT reagent kit (Takara, China). The gene-specific primers F1 and R1 (Tab. 1), designed according to

the *MAPK* gene sequence obtained by RNA-Seq (PRJNA379637), were used for PCR amplification with cDNA. The product was purified using SanPrep column DNA gel extraction kit (Sangon, China) and inserted into pGM-T vector (Sangon, China). The ligation product was transformed into competent DH5α cells and plated onto LB agar plate containing ampicillin (50 μg/ml). The positive clones, confirmed by direct bacterium PCR amplification with primers F1 and R1, were sequenced by Sangon Biotech (Shanghai, China).

2.3 Bioinformatic analysis of the *RmMAPK* gene and deduced protein

The *MAPK* gene homologous sequence was searched by running the blastx program of NCBI. The amino acid sequences homologous to MAPK protein were retrieved from GenBank. The localization of MAPK protein was predicted by PSORT (https://psort.hgc.jp/form2.html). Multiple sequence alignment was performed by ClustalW in BioEdit version 7.0. The evolutionary tree was constructed in MEGA 6.0 using the neighbor-joining method with 1 000 bootstrap calculations. The molecular weight and isoelectric point were calculated with Expasy Prot Param tool.

2.4 qPCR analysis of *MAPK* transcription under abiotic stress

Total RNA was isolated from the control and treated yeast cells using total RNA extractor (Trizol) (Sangon, China) following the manufacturer's instruction. qPCR was performed using the SYBR *Premix Ex Taq*™ II kit (TaKaRa, China) with an ABI 7500 real-time PCR system (Applied Biosystems, USA). The designed primers for *RmMAPK* amplification were F2 and R2 (Tab. 1). β-actin, which was amplified with primers ACT-F and ACT-R (Tab. 1) was applied as an internal standard to control the variations in product abundance. Amplification was achieved with the thermal profile of 30 s at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 60 °C, followed by a melting-curve analysis. The relative differences of RNA expression in samples with different treatments and exposure times were determined by the ΔΔCt relative quantification method.

3. Results

3.1 Bioinformatic analysis of *MAPK* gene and deduced amino acid sequence

The sequence of amplified *MAPK* cDNA, referred to as *RmMAPK*, was 1273 bp long, containing an ORF of 1086 bp, which encoded a polypeptide of 361 amino acid residues (Fig. 1). The estimated molecular mass of polypeptide was 40910.1 Da and the pI was 5.25, which were in good accordance with those of MAPK protein. The nucleotide sequence data for *RmMAPK* had been deposited in GenBank under the accession number KX987161, and the corresponding protein number was APB88859. Additionally, using the PSORT online tool, the deduced RmMAPK protein was predicted to be localized to the cytoplasm, generally matching with cytoplasmic location of other MAPKs (Xu et al., 2008).

Multiple alignment analysis of amino acid sequences of MAPKs from *R. mucilaginosa* AN5, *Saccharomyces cerevisiae*, *Hortaea werneckii* and *Schizosaccharomyces pombe* revealed that the RmMAPK domains were highly conserved and contained all 11 conserved subdomains that were characteristic of serine/threonine protein kinases (Fig. 2). The amino acid sequence 28–36 (VGMGAFGLVCS) located in subdomain I was the conserved ATP-binding signature of protein kinases. Similarly, an active site with Asp141 and its surrounding amino acids were conservative in all the four sequences. The activation loop was highly conserved with two differences at positions 165 and 166, which in RmMAPK were leucine and alanine, whereas in the other three kinases, they were isoleucine and glutamine. And as the putative phosphorylation site of MAPK, TxY motif was found at amino acids 173–175. The common docking (CD) domain at the C-terminus was evolutionary-conserved with a DxxDEPxx (304–311) motif that functions as a docking site for MAPK kinases. It was also highly conserved in RmMAPK, only one difference with Sty1 at position 312, two differences with HwHog1 at positions 310 and 312, and three differences with ScHog1 at positions 299, 312 and 313. However, the important negatively charged residues at positions 304 (Aspartate), 307 (Aspartate) and 308 (Glutamate) remained unchanged [5,18](Tanoue and Nishida, 2003; Konte and Plementias, 2013). The last conserved domain was the PDB-2 region which was the Pbs2 kinase and Ptp2 phosphatase binding site in *S. cerevisiae* [19]. Following the PDB-2 region, there were only 14 amino acid residues towards the C-terminus in RmMAPK, but 85 residues in ScHog1.

The deduced amino acid sequence of RmMAPK was compared with the known 17 MAPK amino acid sequences from other fungi and yeasts available from GenBank database, and a neighbor-joining phylogenetic tree was constructed (Fig. 3). The amino acid sequence of RmMAPK was approximately 82% homologous to that of *S. cerevisiae* HOG1p. Moreover, RmMAPK showed the highest homology to mitogen-activated protein kinase HOG1 of *Rhodotorula graminis* WP1 (96% identity, GenBank: XP018268102) and p38 MAP kinase of *Rhodosporidium toruloides* NP11 (95% identity, GenBank: XP016271521). The phylogenetic analysis indicated that the RmMAPK belonged to the HOG1-related group of MAPKs.

3.2 The effect of low temperature on *MAPK* gene transcription

To detect the expression level of *RmMAPK* under low temperature, polar yeast *R. mucilaginosa* AN5 was incubated at 4 °C for 4, 12 and 24 h (Fig. 4A). The results of qPCR showed that the mRNA expression of *RmMAPK* increased 1.89-fold after 4 h cold shock, and reached to maximum value of 4.44-fold at 12 hours. Then, *RmMAPK* expression level decreased at 24 h, and was 2.94-fold of the control.

3.3 The effect of high salinity on *MAPK* gene transcription

To illustrate the response of RmMAPK gene to high salinity, qPCR was applied to analyzed the expression level of RmMAPK mRNA in Antarctic yeast *R. mucilaginosa* AN5 stressed by 120‰ salinity for 4 h, 12 h and 24 h (Fig. 4B). Research results suggested that after 4 h stress, the expression level of RmMAPK mRNA increased 5.5-fold in 120‰ salinity, reaching the maximum value. With extended stress time, RmMAPK expression level down-regulated at 12 h and 24 h after salinity stress.

3.4 The effect of copper on *MAPK* gene transcription

To investigate the putative involvement of RmMAPK in the adaptation of heavy metal copper ion, qPCR was applied to measure the alteration of MAPK mRNA in response to copper exposure at different times of 4 h, 12 h and 24 h (Fig. 4C). MAPK transcription of non-treated yeast cells showed no obvious changes in detected 24 h. In copper ion stress cells, the mRNA expression of *MAPK* was up-regulated immediately at 4 h. With extended treatment time, the *MAPK* expression of copper-induced cells continued to increase and enhanced about 2-fold after 24 h compared with yeast strain without copper stress. This suggested that *MAPK* gene was induced and time-dependently increased under Cu^{2+} stress.

4. Discussions

MAPKs are found universally in fungi, animals and plants [20], and have a function in the transmission of environmental signals to intercellular targets, and regulate a variety of cellular responses [8,21]. NCBI data research and literature reports show that, until now, only 6 MAPK candidate proteins are found in 4 *Rhodotorula* species, namely *R. mucilaginosa* (KAG0659125), *R. graminis* WP1 (XP_018271420 and XP_018268102), *R. toruloides* NP11 (XP_016277038, XP_016273893 and XP_016271878) and *Rhodotorula* sp. JG-1b (KWU43806). However, these proteins are just mentioned in NCBI database, and no protein characterization is described. In this work, from Antarctic yeast *R. mucilaginosa* AN5, a sequence of 1273 bp is amplified, which contains an ORF of 1086 bp encoding a polypeptide of 361 amino acid residues with calculated molecular mass of 40.9 kDa and pI of 5.25. The mass and pI value are in accord with known members of the MAPK family of a variety of organisms, such as TmHog1 (46.5 kDa pI 5.25) from yeast *Trichosporonoides megachiliensis* (AB621550) [22], CsNMAPK (42.7 kDa, pI 5.56) from Cucumber *Cucumis sativus* (DQ812086) [23], and Kmhog1 (52.8 kDa, pI 5.21) from yeast *Kluyveromyces marxianus* (EU625288) [24], *etc*.

The MAPK-like protein RmMAPK from *R. mucilaginosa* AN5 contains all the conserved domains and motifs as found in the other yeast (Fig. 2), which means that RmMAPK might possess the same role in changing cellular processes in response to environmental stress. Sequence analysis indicated that RmMAPK contained the 11 evolutionarily conserved kinase subdomains like those in other eukaryotic organisms of *Poncirus trifoliata* [25] and *Cucumis sativus* [23]. Those conserved amino acids residues may be considered to be involved in substrate specificity or protein interaction [26]. The ATP-binding site of RmMAPK starts closely to the N-terminus at amino acids 26-34, and is completely identical with those of the other MAPKs [27]. Additionally, RmMAPK contains the consensus sequence of TGY at amino acids 171-173 close to subdomain VIII. As the characteristic of hyperosmolarity-activated MAP kinases, TGY activated MAPKs via double phosphorylation of T (threonine) and Y (tyrosine) [28]. The activated MAPKs are able to phosphorylate the downstream substrates of other kinases and/or transcription factors [2]. CD domain (DxxDEPxx) is at the C-terminus of RmMAPK, and usually thought to be binding site of MAPKKs [29]. The C-terminal region of RmMAPK is estimated to be significantly shorter than that of ScHog1, which also found in other known fungi [30]. Konte and Plemenitas confirm that the C-terminus 70 residues of ScHog1 from *Wallemia ichthyophaga* are not necessary for its function [5].

Antarctic sea ice, the habitats of polar microorganisms, possess fundamental traits of low temperature, high salinity and heavy metals [15]. The extreme environments interfere the physiological and biochemical metabolisms of sea-ice organisms. Long-term living in extreme environments, cold-active organisms have developed varieties of mechanisms to survive, colonize and thrive in the habitats. To avoid or relive abiotic stresses, signal transduction pathways must be perceived and responded firstly. MAPK cascade, as one of the earliest signaling pathways to perceive extracellular stimuli

among sophisticated defense networks, is able to regulate cell division, growth and development to respond to a variety of abiotic stressors [31,32].

Antarctica represents one of the coldest environments, and low temperature is the fundamental property in Antarctic area. MAPKs are widely known to act as vital functions in stress responses of plants [33], animals [34] and yeasts [35], and then activate other functional proteins to accommodate low temperature stress. In yeast *Dekkera bruxellensis*, HOG-MAPK signaling pathway was activated in cold stress response [35]. Here, qPCR assay showed that the expression level of RmMAPK in polar yeast AN5 was obviously up-regulated under cold shock, and attained the maximal value of 4.44-fold at 12 h (Fig. 4A). This variation trend of RmMAPK was consistent with the cold-shock response of MAPK Hog1p in *S. cerevisiae* treated with low temperature [36]. Likewise, in Antarctic notothenioid fish *Dissostichus mawsoni*, MAPK/p38 signaling pathway was activated by low temperature exposure [34]. Our results primarily provide insight into the roles of MAPKs in regulating Antarctic yeast cold shock responses.

In Antarctica, as seawater is frozen, salts are expelled from seawater into brine channels and pores where sea-ice microorganisms live. With the decrease of air temperature, the pore space and size decreases, and salinity of brines in the pores rises [15]. Salt stress can cause a variety of physiological response such as high osmotic stress, osmolytes accumulation, ionic toxicity and oxidative stress, *etc* [37]. Some studies have reported that the expression level of RmMAPK was altered under salt stress [37,38]. Our results indicated that after 4 h salinity treatment RmMAPK expression level were increased immediately with the maximum of 5.5-fold when compared to the control (Fig. 4B). In soybean *Glycine max*, MAPK (GMK1) was induced after 5 min treatment of 300 mmol/L NaCl [39]. In spotted sea bass *Lateolabrax maculatus*, significant up-regulations were observed in the expressions of *mapk* after salinity challenge [40].

Heavy metals are enriched in Antarctic sea ice owing to atmospheric circulations and anthropogenic impacts [16,17]. There are some reports about the participation of yeast MAPK pathway in metal stress. In *S. cerevisiae*, MAPK Hog1p is phosphorylated in response to arsenite, and then activated Hog1p phosphorylates aquaglyceroporin Fps1p and down-regulates its transport activity, thereby reducing arsenite influx [41]. Apart from that, Hog1p can also be phosphorylated to high levels upon cadmium and zinc stress [42]. Now, a number of arsenite and cadmium-activated MAPK signaling pathways are identified in the fission yeast *S. pombe* [43], pathogenic fungi *Cryptococcus neoformans* [44], opportunistic yeast *Candida albicans* [45] and *Candida lusitaniae* [46]. Meanwhile, Exposure of alfalfa (*Medicago sativa*) seedlings to excess copper rapidly activates four distinct MAPKs: SIMK, MMK2, MMK3, and SAMK [21]. Here, we found that *RmMAPK* mRNA of *R. mucilaginosa* AN5 was up-regulated within 24 h in response to 2 mmol/L copper treatment, which meant the possible involvement of MAPK pathway in yeast copper stress.

In conclusion, in the current study, we present the MAPK-like kinase in the yeast *R. mucilaginosa* AN5 from Antarctic sea ice. The protein sequence is conservative and quite similar to the Hog homologs of other yeasts such as *S. cerevisiae* and *H. werneckii*. qPCR shows that the transcription of *RmMAPK* gene is up-regulated in response to cold, salinity and copper exposure, which indicates the participation of MAPK pathway in adaptation to abiotic challenge. Further research on the components upstream and downstream *RmMAPK* in this pathway may shed light on our understanding about the response mechanisms to environmental stress in Antarctic yeast *R. mucilaginosa*.

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Figures

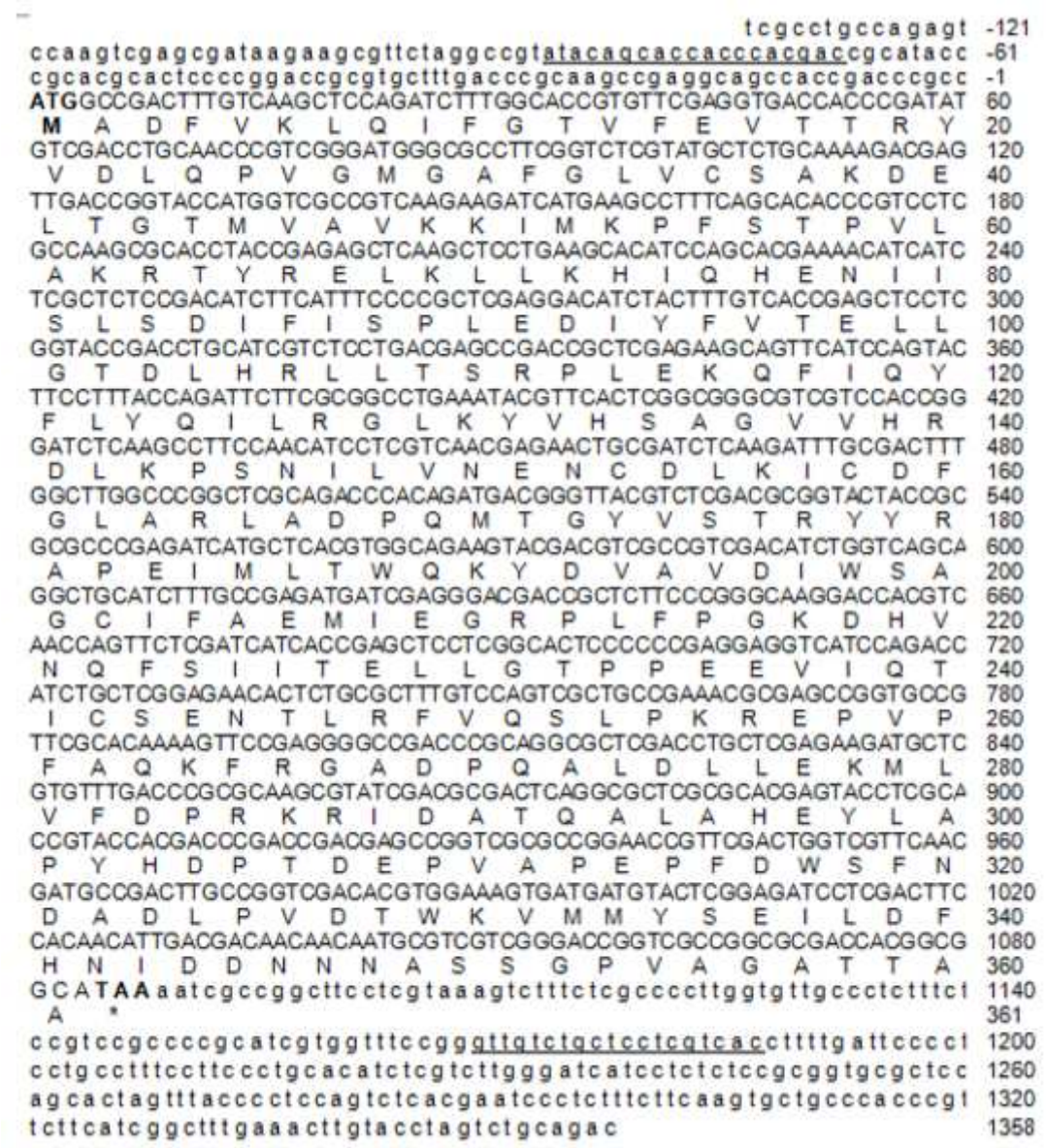

Figure 1

Partial nucleotide sequence and deduced amino acid sequence of the MAPK gene from R. mucilaginosa AN5. The amino acid sequence is shown below the nucleotide sequence. Forward and reverse primers for initial cDNA fragment amplification are underlined. The DNA sequence includes the putative coding region (upper case) and the 5’ and 3’ noncoding regions (lower case). The initiation codon is bold, and the stop codon is bold and marked by an asterisk.

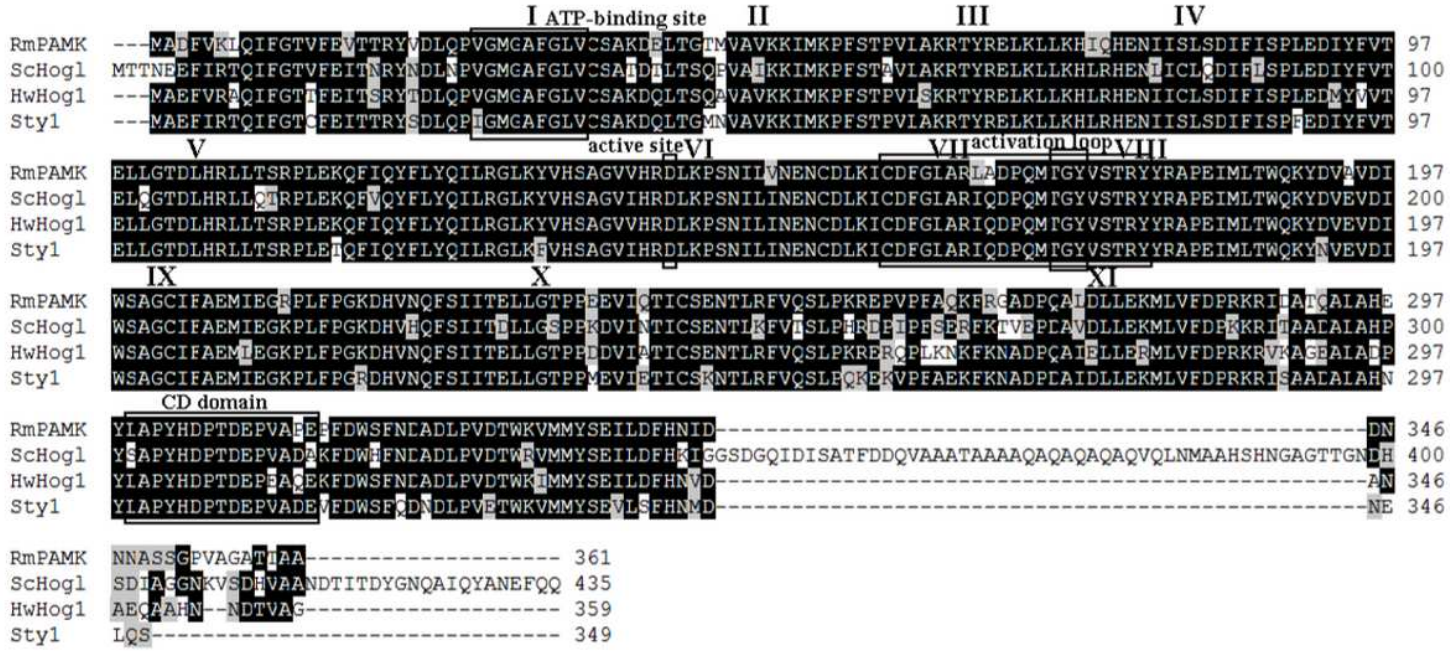

Figure 2

Multiple alignment of amino acid sequences of MAPK from R. mucilaginosa AN5 (RmMAPK, GenBank: APB88859), S. cerevisiae (ScHogl, GenBank: NP013214), H. werneckii (HwHog1, GenBank: AAM64214), and S. pombe (Sty1, GenBank: NP592843). The 11 major conserved subdomains of serine/threonine protein kinases are marked by Roman numerals (I-XI). ATP-binding site, active site, activation loop containing the TGY motif and common docking (CD) domain are indicated with framed boxes.

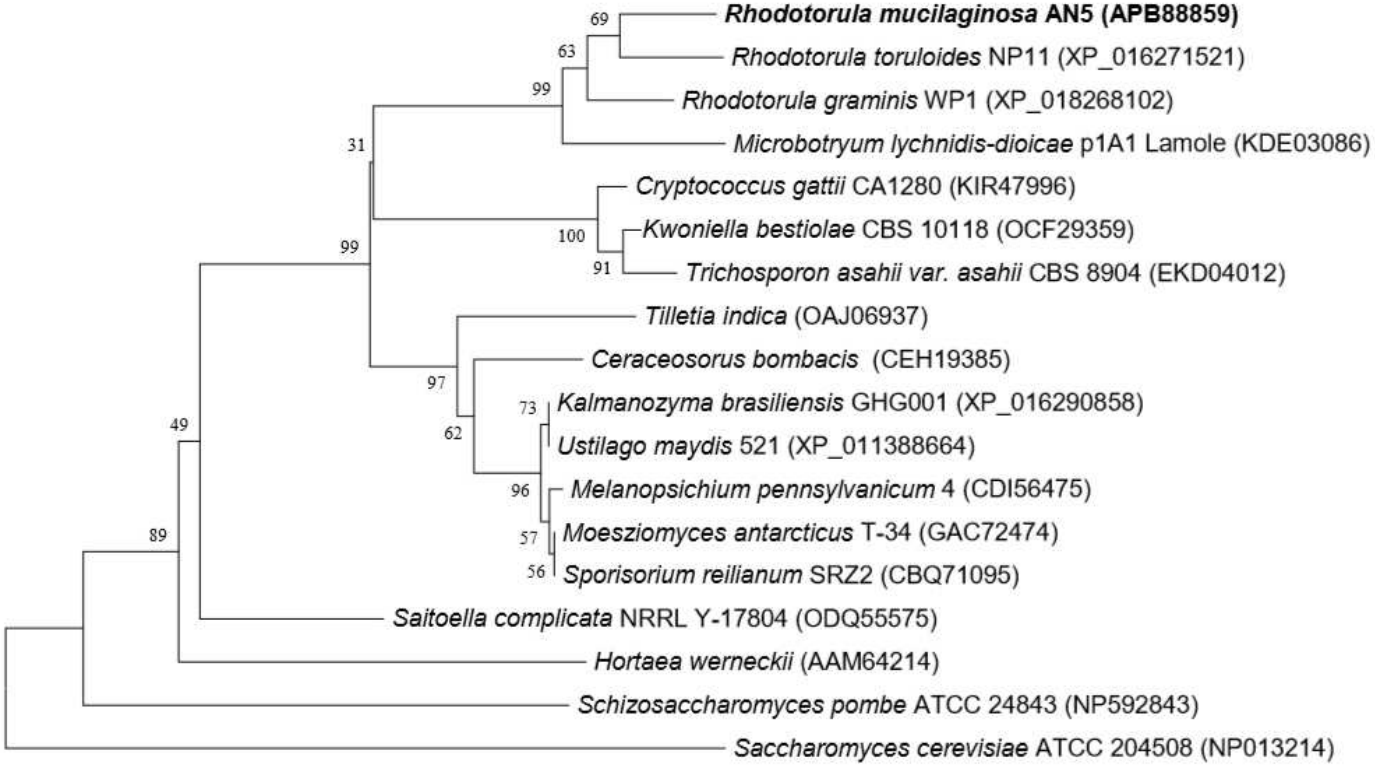

Figure 3

The neighbor-joining phylogenetic tree of deduced MAPK-like proteins from yeasts and fungi species. The protein alignment was carried out with BioEdit 7.0, and MEGA 6.0 was used for construction of the tree.

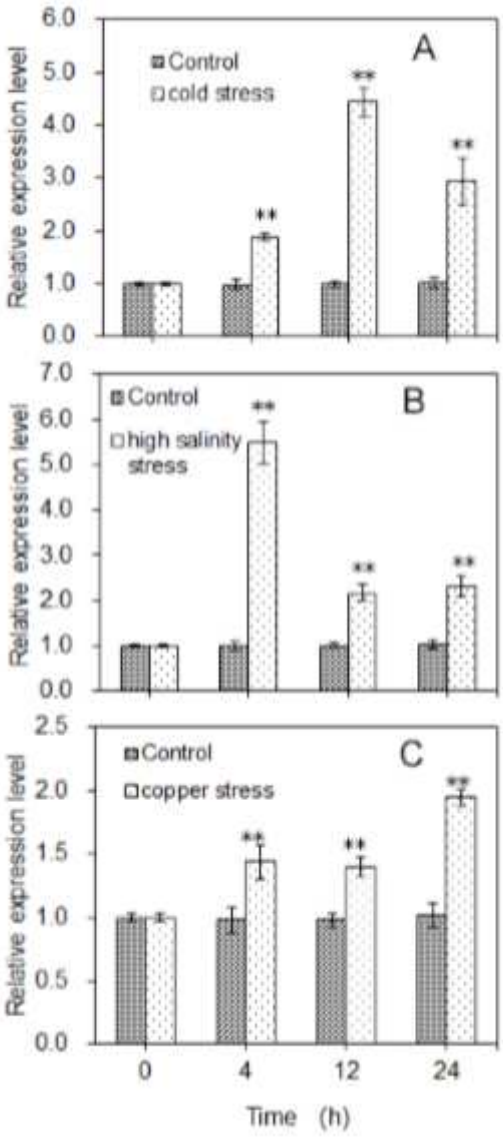

Figure 4

Transcription of mapk gene in R. mucilaginosa AN5 exposed to 4 °C (a), 120‰ NaCl (b) and 2 mmol/L Cu2+ (c) analyzed by qPCR. Bars represent the mean values of three replicates ± standard deviation (SD). Double asterisks (**) indicate significant differences from control at $p<0.001$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.pdf