Expression and interaction of 14-3-3 with plasma membrane H$^+$-ATPase in *Cryptococcus humicola* and application of 14-3-3 in aluminum tolerance

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14-3-3 protein participates in numerous cellular processes, but their roles in abiotic stress tolerance are not well understood. The sequence of the gene encoding the 14-3-3 protein originated from our previous whole genome sequencing. In this paper, the 14-3-3 encoding gene was cloned, the recombinant 14-3-3 protein was expressed and purified, and rabbit antibody against 14-3-3 protein was obtained. The expression level of 14-3-3 protein in *Cryptococcus humicola* increased with increasing aluminum (Al) concentrations and reached the highest level under 20 mM Al. Co-immunoprecipitation between 14-3-3 protein and the plasma membrane (PM) H$^+$-ATPase showed similar trend, as the expression of PM H$^+$-ATPase interacted with 14-3-3 protein. These results suggested that 14-3-3 protein interacted with PM H$^+$-ATPase under Al stress. To verify the role of the 14-3-3 protein in Al-tolerant mechanisms, transgenic yeast overexpressing the 14-3-3 gene were generated. The transgenic yeast could grow well in culture medium containing 2 mM Al, while the yeast with pYES3/CT and wild-type yeast (negative control) could not grow or grew poorly when inoculated at a dilution of 10^{-4} of the original concentration. Therefore, these data showed that over-expression of 14-3-3 protein enhanced the Al tolerance of the yeast. The content of residual active Al in medium cultured by 14-3-3 transgenic yeast is much lower than that of medium cultured by the yeast containing empty vector. These data suggested that over-expression of 14-3-3 protein enhances Al tolerance of yeast, and that Al tolerance may be due to the adsorption of active Al.

Keywords: *Cryptococcus humicola*; 14-3-3; Plasma membrane H$^+$-ATPase; Aluminum resistance.

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Introduction

Aluminum (Al) is one of the most abundant metals in the earth and is also one of the main factors that reduce crop yields in acidic soils. One effective way to overcome Al toxicity in acidic soil is to improve Al tolerance in plants by genetic engineering. Therefore, studies of the mechanism of Al tolerance and the genes involved in Al tolerance are important for agricultural production.

14-3-3 protein is a highly conserved protein family in all higher eukaryotes. 14-3-3 protein binds with over 200 types of proteins involved in almost all biological processes in cells. 14-3-3 protein can bind to the C-terminal autoinhibitory domain of plasma membrane (PM) H$^+$-ATPase, which regulates its enzymatic activity. The activation of PM H$^+$-ATPase by 14-3-3 protein was reported to be involved in various stress responses including cold stress [1], osmotic stress [2], hypersensitive response [3], and low-phosphorus stress [4]. Recently, 14-3-3 was

reported to be involved in Al tolerance in plants. Al stress up-regulated genes encoding plasma membrane (PM) H^+-ATPase (*vha2*) and the 14-3-3 protein (*vf14-3-3b*) in Al-resistant (YD) broad bean roots [5]. Magnesium (Mg) enhanced the phosphorylation levels of VHA2 and its interaction with the vf14-3-3b protein under Al stress, and alleviated the Al toxicity by increasing PM H^+-ATPase activity and Al-induced citrate exudation in YD roots [6].

Cryptococcus humicola BSLL1-1 is an Al-resistant yeast that was isolated from acidic soil [7]. Genes from *C. humicola* may have the potential use to resist Al stress. 14-3-3 gene was found to tolerate Al toxicity in transgenic plants. We wonder if 14-3-3 gene of *C. humicola* has the same characteristic. In this study, the gene encoding 14-3-3 protein of *C. humicola* was cloned. The expression of 14-3-3 and the interaction between 14-3-3 protein and PM H^+-ATPase under Al stress were investigated. To prove the function of 14-3-3 in Al tolerance, the 14-3-3 gene was transferred into an Al-sensitive *Saccharomyces cerevisiae*.

Materials and methods

Strains and culture conditions

C. humicola cells were cultured at 30°C in GM medium at pH 3.0 (glucose, 1.0%; peptone, 0.05%; yeast extract, 0.02%, and $MgSO_4 \cdot 7H_2O$, 0.02%) and supplemented with different concentrations of Al^{3+} when necessary [8]. *Escherichia coli* DH5α (TransGen Biotech, Beijing, China) was used for genetic cloning. The pET-32a (+) vector was used to construct expression vectors. *E. coli* BL21 (*DE3*) (Invitrogen, Carlsbad, CA, USA) was used for recombinant protein expression. The yeast expression vector pYES3/CT and *S. cerevisiae* strain INVSC1 (*MATa his3D1 leu2 trp1-289 ura3-52*) were used to generate transgenic yeast (Invitrogen, Carlsbad, CA, USA).

Purification of 14-3-3 recombinant proteins and preparation of the antibodies

Total RNA was extracted from *C. humicola* using Trizol (Invitrogen, Carlsbad, CA, USA). DNase I (Promega, Madison, WS, USA) was used to remove residual genomic DNA in the RNA. The quality of RNA was examined by gel electrophoresis. cDNA was synthesized using M-MLV reverse transcriptase (Fermentas, Lithuania). The gene encoding the 14-3-3 protein was obtained by PCR amplification and cloned into a pMD-18T vector (TaKaRa, Japan) to generate pMD-14-3-3. The primers were 14-3-3-F with the *Xho*I site (GGATCCATGTCTACTGTTTCG CGTGAAGACT, the enzyme site is underlined) and 14-3-3-R with the *Bam*HI site (GAATTCTTA GGCCGCAGCGGGCTCCTCCTTG, the enzyme site is underlined). Constructed plasmids were subsequently used for gene sequencing.

The 14-3-3 encoding gene was obtained from pMD-14-3-3 digested with *Xho*I and *Bam*HI. The fragment was then ligated into the pET-32a (+) vector between the *Xho*I and *Bam*HI sites to generate pET-32a-14-3-3. The prokaryotic expression vector was transformed into *E. coli* BL21 (DE3). For recombinant protein expression, cells were grown at 37℃ in LB medium with 100 μg/mL ampicillin for 2 h. Then, 1 mM IPTG was added. After growing for an additional 8 h at 37℃ with shaking at 80 rpm, cells were collected by centrifugation (5,000 rpm for 5 min). Cell pellets were resuspended in cold PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4). The cell suspension was sonicated on ice for 7 min and then centrifuged at 12,000 rpm for 15 min at 4℃. The supernatant and the precipitate were subjected to SDS-PAGE. Proteins were visualized by staining with Coomassie blue. His-tagged 14-3-3 recombinant protein was purified by affinity chromatography. A rabbit was immunized with the purified 14-3-3 recombinant protein, and the anti-14-3-3 antibodies were prepared from the immunized rabbit serum. The specificity of the antibody raised against the 14-3-3 protein was confirmed by Western blot analysis and was used for western blot experiments.

Isolation of plasma membrane and analysis of PM H^+-ATPase activity

Cells treated with different concentrations of Al for 12 h were collected by centrifugation and then quickly ground with liquid nitrogen. The membrane protein was extracted according to the method described by Yan *et al*. [9]. The plasma membrane protein concentration was assayed using the Bradford method [10], and the activity of the plasma membrane H^+-ATPase was measured according to the method described by Shen *et al*. [11]. The plasma membrane H^+-ATPase activity was measured in a 0.5 ml reaction volume containing 50 mM BTP/MES, 5 mM $MgSO_4$, 50 mM KCl, 0.02% Brij (w/v), 50 mM KNO_3, 1 mM $(NH_4)_2MoO_4$, 1 mM NaN_3, 4 mM ATP-Na_2, and 500 µg of plasma membrane protein. After the reaction mixture was heated at 30°C for 30 min, 1 ml of reaction stop solution (containing 2% H_2SO_4 (v/v), 5% SDS (w/v), and 0.7% $(NH_4)_2MoO_4$ (w/v)) was added, and then immediately 50 µl of Vc was added and placed at room temperature for approximately 20 min. The absorbance at a wavelength of 700 nm was determined. The release of inorganic phosphorus was calculated according to the inorganic phosphorus standard curve. One unit of PM H^+-ATPase activity is defined as the molar number of inorganic phosphoric acid released from ATP decomposition catalyzed per milligram of protein within 1 min at 30℃.

Assay of H^+-pump activity

The plasma membrane H^+-pump activity was measured using method described by Yan *et al*. [12]. The reaction mixture contained 5 mM BTP/MES (pH 6.0), 12 µM acridine orange (AO), 300 mM KCl, 250 mM Suc, 0.5 mM EGTA (adjusted to pH 6.0 with BTP), 1 mM NaN_3, 1 mM Na_2MoO_4, 50 mM KNO_3, 0.05%(w/v) Brij 58, and 50 µg of membrane protein in a final volume of 1.5 ml. After equilibration of the membrane vesicles with reaction medium for 15 min, proton transport was initiated by the addition of 2 mM ATP-Na_2. The proton gradient was recorded by a spectrophotometer as the change in AO absorbance at 495 nm. After the 30-min measurement, the established pH gradient was completely collapsed by addition of 5 mM gramicidine (Gram).

CoIP

The interaction of the 14-3-3 protein with PM H^+-ATPase was studied according to a method described by Chen *et al*. [5]. The cells were treated with 50 mM $AlCl_3$ for 0 (the control), 4, 8, 12, and 24 h and collected for protein extraction. First, 200 µg of total plasma membrane proteins plus 2 µg of polyclonal antibodies against the 14-3-3 protein were incubated in cold phosphate buffer (10 mM, pH 7.4) containing 0.02% Brij 58 (w/v) and 10 mM NaF with a total volume of 1 mL at 4℃ for 6 h with occasional shaking (40 rpm/min). Then, 20 µL of protein A/G plus agarose (Santa Cruz Biotech, Santa Cruz, CA, USA) was added to the protein solution, which was continuously incubated at 4℃ for 12 h. The protein samples were centrifuged (12, 000 rpm) for 2 min to precipitate the proteins. The protein pellets were washed 5 times with 1 mL of ice-cold phosphate buffer (10 mM, pH 7.4) containing 0.02% Brij 58 (w/v) and 10 mM NaF. The pellets were then resuspended in 40 µL of 1×electrophoresis sample buffer, and 20 µL aliquots were subsequently separated by 10% SDS-PAGE for western blot analysis. The separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with rabbit antibodies against the phosphorylated Thr of VHA2 [5] or the 14-3-3 protein, followed by goat anti-rabbit IgG conjugated with peroxidase.

As negative control, the activated agarose was incubated without 14-3-3 protein, blocked as described above, and incubated with total plasma membrane proteins.

Construction of transgenic yeast of 14-3-3

To analyze the role of 14-3-3 in Al tolerance, the pYES3/CT yeast expression vector and the *S. cerevisiae* strain INVSC1 (*MATa his3D1 leu2 trp1-289 ura3-52*) were used to generate transgenic yeast. The coding region of 14-3-3 was digested from pMD-14-3-3 with *Bam*HI and *Eco*RI, and the fragments were purified and cloned into the

*Bam*HI/*Eco*RI site of pYES3/CT, which carries the *TRP*1 selection marker. The inducible GAL1 promoter controlled 14-3-3 expression. Sequence analysis was used to confirm the insertion of 14-3-3 into the pYES3/CT. The resulting pYES3-14-3-3 and pYES3/CT empty vector were transformed into the *S. cerevisiae* strain INVSC1 using the lithium acetate method. The presence of the pYES3/CT-14-3-3 plasmid in transgenic yeast was confirmed by PCR analysis and by western blot using 14-3-3 antibodies.

Al tolerance and residual Al content analysis of transgenic yeast

The INVSC1 strain was inoculated from colonies on yeast extract peptone dextrose (YPD) medium plates and grown for 3 days at 30°C. The transformants were grown on selective culture medium that lacked tryptophan (SC-trp: 0.67% yeast nitrogen base without amino acids but containing ammonium sulfate; 2% glucose; 0.01% adenine, arginine, cysteine, leucine, lysine, threonine, and uracil; 0.005% aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, and valine; and 2% agar) for 2 days at 30°C, and the resulting trp^+ colonies were selected for further use.

Yeast cells harboring pYES3/CT-14-3-3 and pYES3/CT were incubated overnight in SC-liquid medium containing 2% (w/v) glucose and were then transferred to YPD medium containing 0.2 mM or 2 mM Al^{3+} with an initial OD_{600} of 0.1. After 24 h of incubation, the culture was centrifuged at 12,000 rpm for 10 min, and the supernatant was filtered using a sterilized filter with 0.2 µm pores. The levels of inorganic monomeric Al and total Al in the filtered supernatant were determined according to the method described by Nian *et al*. [7]. Each analysis was performed in duplicate.

Results

Cloning and sequencing of the 14-3-3 gene

The 14-3-3 gene was amplified using cDNA as a template and ligated into pMD-18T to obtain pMD-14-3-3. The correct pMD-14-3-3 was used for sequencing and the 14-3-3 gene is a fragment of 774 bp. The resulting sequence was submitted to GenBank under accession number KU203774.

Expression and purification of 14-3-3 protein

The pMD-14-3-3 plasmid was digested with *Xho*I and *Bam*HI to obtain the 14-3-3 cDNA fragment. The gene was cloned into the prokaryotic expression vector pET32a (+) to generate the recombinant plasmid pET32a-14-3-3. To obtain the recombinant 14-3-3 protein, the pET32a-14-3-3 recombinant plasmid was transformed into *E. coli* BL21. From the figure 1A, 1B, and 1C, it was found that the expression level of the protein was maximum at 20℃. Therefore, 20℃ is the optimum temperature to induce the protein expression.

After being induced for 2h, 4h, 6h, 8h, 10h, and 12h using 1 mM IPTG, the cells were collected, and SDS-PAGE analysis was performed. The results showed that there was an obvious protein band around the expected protein molecular weight of approximately 48 kDa (including the protein tag), whereas the strain containing the pET32a (+) did not show this protein band, indicating that pET32a-14-3-3 successfully expressed 14-3-3 protein in the BL21 strain. The protein content was the highest when the cells were induced for 8 h. Thus, induction for 8 h at 20℃ and using 1mM IPTG was the best expression condition.

The prokaryotic expression vector pET32a (+) contains the 6X-His protein tag. Therefore, the target protein can be isolated and purified using a Ni agarose gel affinity chromatography column. The cells were collected after induction and were ultrasonically broken. The 14-3-3 protein was purified using the 6X-His protein tag and eluted using 300 mM imidazole (Figure 1D). The 14-3-3 recombinant protein was used to prepare the antibodies. The specificity of the antibodies was confirmed using the purified 14-3-3 protein by western blot analysis. The

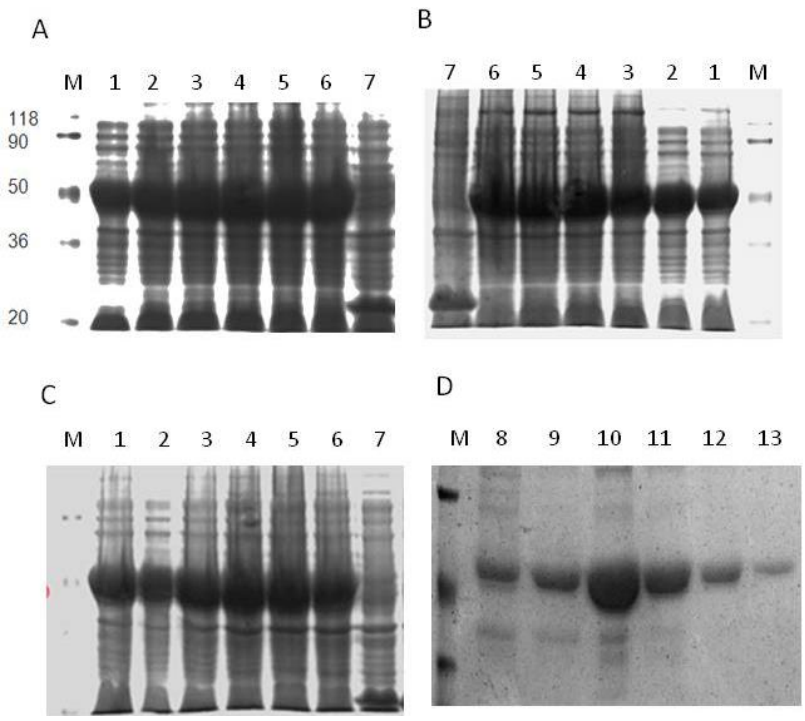

Figure 1. Expression of recombinant 14-3-3 protein at 20℃ (A), 30℃ (B), and 37℃ (C). M: Protein ladder. Lines 1-6 are IPTG inductions with (1) for 2 h; (2) for 4 h; (3) for 6 h; (4) for 8 h; (5) for 10 h; (6) for 12 h. Line 7 is *E. coli* BL21 with pET32a (+). Lines 8-13 (D) are elution of 14-3-3 protein using different concentrations of imidazole with (8) 50 mM; (9) 100 mM; (10) 200 mM; (11) 300 mM; (12) 400 mM; (13) 500 mM.

antibodies were specific and could be used for further experiments (data not shown).

Interaction of 14-3-3 protein with plasma membrane H^+-ATPase

To elucidate the relationship between 14-3-3 protein and plasma membrane H^+-ATPase under Al stress, the expression levels of proteins and their interactions under Al stress were detected by 14-3-3 antibody and plasma membrane H^+-ATPase antibody. The results showed that with an increase in Al concentration, the protein levels of 14-3-3 were elevated under lower Al concentrations and expression was decreased at higher Al concentrations (Figure 2A). The expression reached the highest level at 20 mM Al.

The interaction analysis of 14-3-3 protein and plasma membrane H^+-ATPase was performed by Co-immunoprecipitation. We found that the expression of plasma membrane H^+-ATPase showed the highest level at 20 mM Al, and the levels of 14-3-3 protein interacting with phosphorylated plasma membrane H^+-ATPase also increased with the increase in Al concentration (Figure 2B), suggesting that the interaction between 14-3-3 protein and plasma membrane H^+-ATPase existed under Al stress.

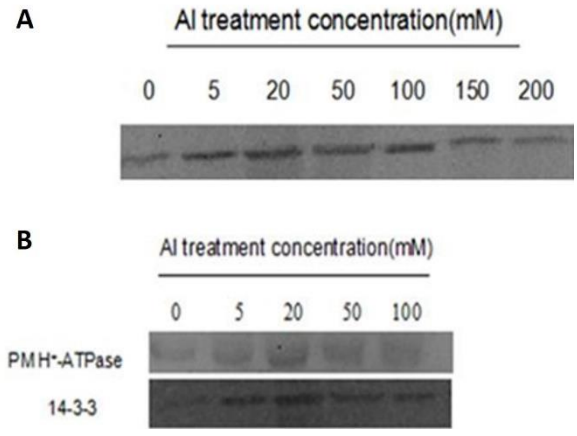

Figure 2. Expression level of 14-3-3 protein (A) and interaction of 14-3-3 with plasma membrane H^+-ATPase (B) in the presence of different concentrations of Al.

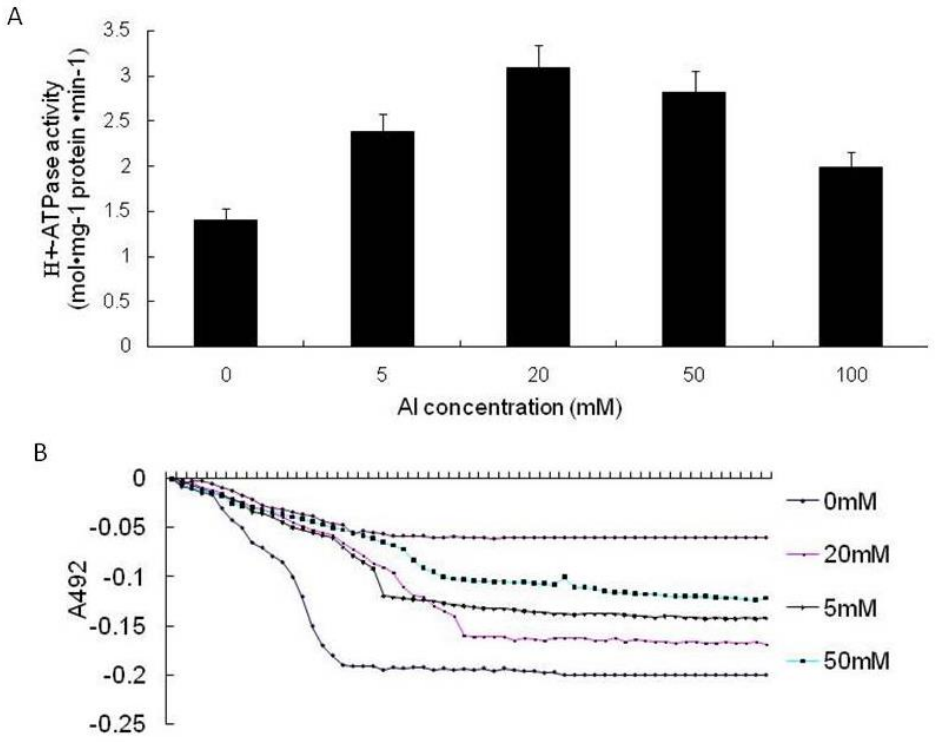

Figure 3. The activity of plasma membrane H^+-ATPase (A) and the activity of the hydrogen pump (B) under Al stresses. The cells were treated with 5, 20, 50, and 100 mM Al for 6 h. The experiments were conducted three times.

Activity of plasma membrane H^+-ATPase and H^+ pump

To characterize the role of plasma membrane H^+-ATPase under Al stress, the activities of the plasma membrane H^+-ATPase and hydrogen pump were measured. As shown in figure 3A, the activity of the plasma membrane H^+-ATPase increased with increased Al concentrations, while the activity of plasma membrane H^+-ATPase reached maximum at 20 mM Al treatment, and then began to decrease. As seen in figure 3B, the hydrogen pump reached the highest activity when treated with 20 mM Al, and the trend of hydrogen pump activity was consistent with that of plasma H^+-ATPase activity. These results suggest that Al stress can increase the activity of the plasma membrane H^+-ATPase and hydrogen pump, which is consistent with the expression of 14-3-3 protein and plasma membrane H^+-ATPase. These data indicate the correlation between 14-3-3 protein and plasma membrane H^+-ATPase under Al stress.

Determination of the Al tolerance of transgenic yeast

To obtain the eukaryotic expression vector containing the 14-3-3 gene, the correct pMD-14-3-3 recombinant plasmid and pYES3/CT empty vector were digested with *Bam*HI and *Eco*RI. A 14-3-3 cDNA fragment containing the *Bam*HI/*Eco*RI restriction site and a linear eukaryotic expression vector containing the cleavage sites were obtained. The 14-3-3 fragment was ligated into pYES3/CT to obtain a recombinant eukaryotic expression vector pYES3/CT-14-3-3. The plasmid was transferred into *S. cerevisiae* strain INVSC1. The cells were plated on tyrosine-deficient selective medium and cultured at 30℃ for 3 days. A single colony was inoculated in GM medium containing galactose as a carbon source, and the positive clone was verified by PCR amplification.

To compare the Al tolerance of transgenic yeast (INVSC1 contained pYES3/CT-14-3-3) with negative control yeast (INVSC1 contained

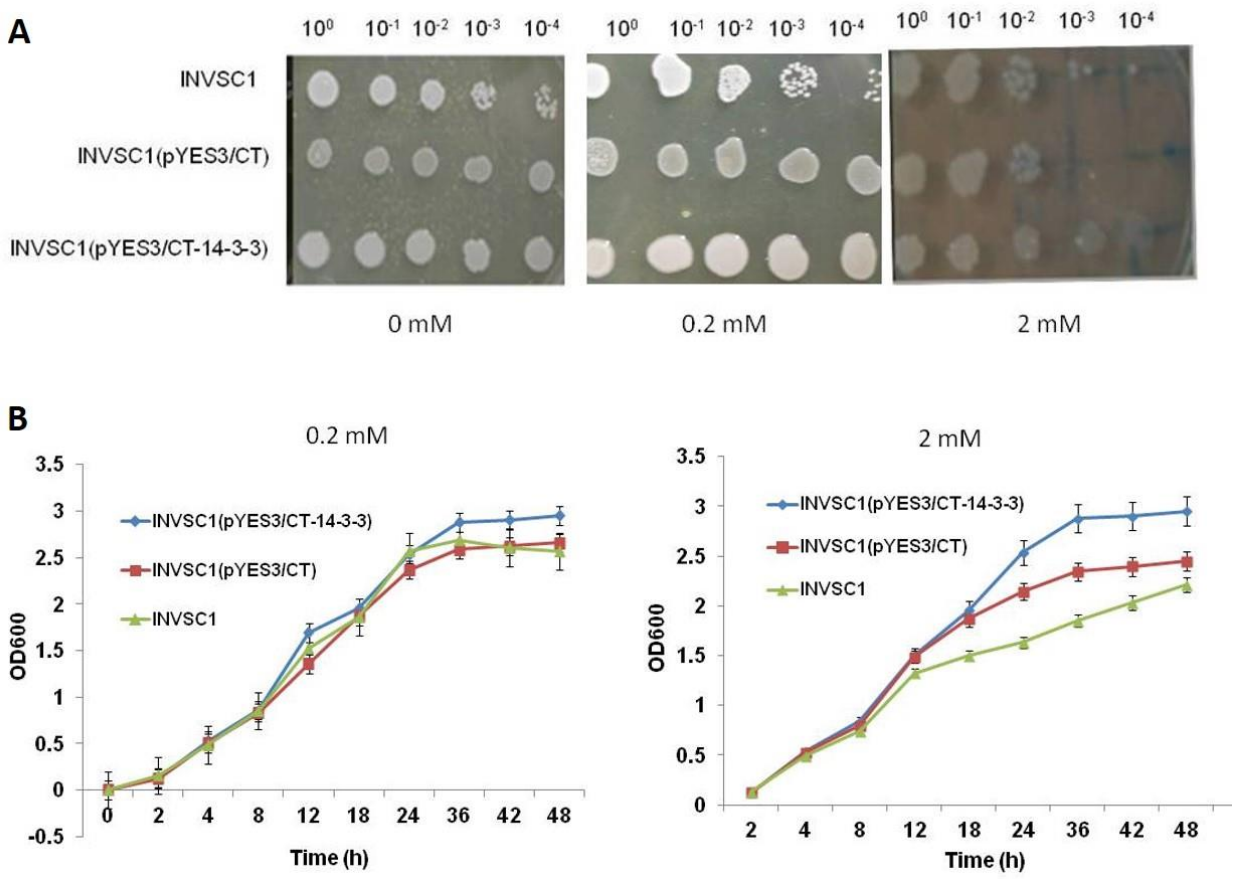

Figure 4. Growth of transgenic yeast on solid media (A) and liquid medium (B) under Al stress. Yeast cells were pre-incubated in SC-liquid medium containing 2% (w/v) glucose and reached an OD_{600} of 2.0. A. Ten-fold serial dilutions were prepared (1:10, 1:100, 1:1000, and 1:10,000), and 5 µl of each dilution was spotted onto SC-trp medium supplemented with 2% (w/v) galactose and 0, 0.2, and 2 mM Al. The results were recorded after the cultures were incubated at 30°C for 3 days. Each sample was spotted in triplicate, and three independent experiments were conducted. B. The initial OD_{600} of each culture was adjusted to 0.05 in liquid medium with 0.2 mM and 2 mM Al. The cultures were then incubated at 30°C while shaking at 200 rpm. The OD_{600} was measured every 2 h.

pYES3/CT) and wild-type strain (INVSC1), the growth of these strains under Al stress was determined. It can be seen from figure 4A that the colonies of the wild-type strain INVSC1, the control strain INVSC1(pYES3/CT), and the transgenic yeast strains INVSC1(pYES3/CT-14-3-3) were decreased with an increase in Al concentration, while the growth of the transgenic yeast INVSC1(pYES3/CT-14-3-3) was much better than that of the negative control strain INVSC1(pYES3/CT) and the wild-type strain INVSC1. On the medium containing 2 mM Al, the negative control strain INVSC1 (pYES3/CT) and the wild-type yeast INVSC1 grew poorly or did not grow at the concentration of 10^{-4}, while the transgenic yeast grew much better, indicating that overexpression of 14-3-3 protein enhances the Al tolerance of yeast.

Growth of transgenic yeast in liquid medium containing Al

To verify the Al tolerance of the transgenic yeast in the liquid medium, the growth of the strains in the Al-containing liquid medium was determined. As in figure 4B, there was no significant difference in the growth of the control yeast INVSC1 (pYES3/CT), the wild-type yeast INVSC1, and the transgenic yeast INVSC1(pYES3/CT-14-3-3) at 0.2 mM Al. When cultured in broth containing 2 mM Al, the tolerance of transgenic yeast INVSC1(pYES3/CT-14-3-3) to Al was higher than that of the control

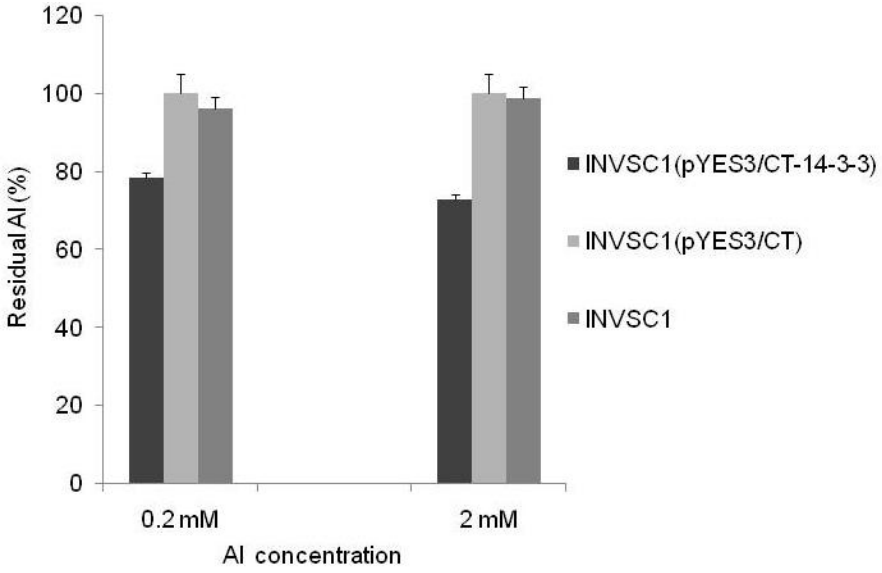

Figure 5. Assay of residual Al in culture after incubating transgenic yeast in the presence of 0.2 mM Al and 2 mM Al. Uninoculated media was shaken and used as a negative control. The residual Al in the negative control was designated as 100%. All values are the means of three replicates. Asterisks represent significant differences when compared with controls ($P < 0.05$).

yeast INVSC1(pYES3/CT) and wild-type yeast INVSC1. The results further demonstrated that 14-3-3 protein could improve the ability of yeast to resist Al stress.

Residual active Al content in the culture medium

To investigate the role of 14-3-3 protein in Al resistance, we determined the content of residual active Al in the liquid medium after culture of the strain. As in figure 5, when the Al concentration was 0.2 mM, the residual active Al in the broth cultured with the transgenic yeast INVSC1(pYES3/CT-14-3-3) was 75% of the initial addition. When cultured in the broth containing 2 mM Al, the residual active Al content in the medium cultured by the transgenic yeast INVSC1(pYES3/CT-14-3-3) was 72% of the initial addition, indicating that the transgenic yeast had a certain adsorption of Al, resulting in a decrease in active Al content in the culture medium.

Discussion

14-3-3 proteins are conserved and identified in all eukaryotic cells. They have functions in nearly all cellular processes by interacting with a large number of proteins. In recent years, an increasing number of studies have reported that 14-3-3 plays an important role in responding to a variety of biotic and abiotic stresses [13-18]. The 14-3-3 gene *AtGF14l* conferred cotton and *Arabidopsis thaliana* with drought tolerance [12], and AtGRF9-overexpressing *A. thaliana* plants showed enhanced drought tolerance [14]. The 14-3-3l and 14-3-3k double *A. thaliana* mutant showed enhanced salt resistance [18]. Potato overexpressing 14-3-3 genes showed higher antioxidant activity than wild-type plants [19]. However, their exact roles in the microbes have not yet been explored. In the case of *C. humicola*, the roles of 14-3-3 protein in stress responses are less explored. In this study, 14-3-3 protein interacted with PM H^+-ATPase under Al treatment, and Al stress upregulated expression of 14-3-3 and the interaction between PM H^+-ATPase and 14-3-3. These results were consistent with the results reported in plants by Chen *et al*. [5]. Although 14-3-3 protein from *C. humicola* conferring Al tolerance to yeast was proved in this work, the mechanism of 14-3-3 protein enhancement of Al tolerance needs to be further studied.

Acknowledgements

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