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ECTOPIC EXPRESSION OF *CU/ZN-SOD* GENE FROM *CERIOPS TAGAL* ENHANCES SALINITY TOLERANCE OF YEAST

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Abstract

Ceriops tagal is a typical non-salt secretion mangrove plant while it has a strong salt resistance. However, the mechanism of *C. tagal* underlying salt stress remains largely unknown. Superoxide dismutase (SODs) is the first line of defense system against active oxygen in plants. In order to investigate whether SOD involves in salt resistance in *C. tagal*, an antioxidant enzyme related gene (*Cu/Zn-SOD*) was isolated from the root of *C. tagal* and expressed in yeast in this study. The transgenic yeast INVSc1 could effectively express the Cu/Zn-SOD protein and improve the salt tolerance of yeast significantly. This is the first study about cloning a *Cu/Zn-SOD* gene from the root of *C. tagal* and revealing its role in salt stress resistance in yeast.

Key words: *Ceriops tagal*, Cu/Zn-superoxide dismutase, Hetero-expression, Salt tolerance.

Introduction

The *Ceriops tagal* is a salt-tolerant mangrove plant growing in mudflats and marshes in tropical and subtropical areas. It does not secretes salt but shows a strong salt tolerance (Aziz & Khan, 2001). It formed its own unique salt tolerance mechanism during the long-term evolution process with the greatly increased activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) under salt stress. The activities of these enzymes were positively correlated with the degree of salt stress (Benavídes *et al*., 2000; Hernández *et al*., 2009). SOD, CAT and POD are defense enzymes which can eliminate reactive oxygen species (ROS) and maintain the redox balance (Ge *et al*., 2013). The relative level of ROS in plants is low and keep in balance under normal conditions, but this kind of dynamic balance could be broken and the ROS will be largely produced under environmental stresses (Mittova *et al*., 2003). Overproduction of ROS can lead to DNA damage, protein oxidation and lipid oxidation which results in dysfunction of plant cells (Polle, 2001). Therefore, plant cells have evolved effective reactive oxygen species defense system during the long process of evolution, including enzymatic system and non-enzymatic system to resist oxidative stress (Alscher *et al*., 2002).

Superoxide dismutase (SODs) is the first line of cell defenses against active oxygen which converts superoxide anion (O^{2-}) and water (H_2O) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Bowler *et al*., 1992; Bowler *et al*., 1994). Most plants contain a series of superoxide dismutase isoenzyme. According to the different metal co-factors, SOD is divided into four types: iron ions SOD (Fe-SOD), manganese ions SOD (Mn-SOD), copper and zinc SOD (Cu/Zn-SOD) and nickel SOD (Ni-SOD) (Marček *et al*., 2016) and they locate in different components of cells. Cu/Zn-SOD and Mn-SOD could effectively eliminate ROS and maintain the redox balance of immune system (Lu *et al*., 2015). The transgenic lines of cucumber with high activities of SOD and POD under low temperature could enhance the cold resistance (Liu *et al*., 2013). The resistance of *Arabidopsis* to salt was improved when the *Mn-SOD* was overexpressed in *Arabidopsis* (Wang *et al*., 2004). Overexpression of *Cu/Zn-SOD* gene in plant could improve its resistance to oxidative stress (Gupta *et al*., 1993). Transgenic sweet potato (*Ipomoea batatas* (L.) Lam. cv. Yulmi) plants showed an enhanced tolerance to multiple environmental stresses by expressing the genes of both Cu/Zn SOD and ascorbate peroxidase (APX) under the control of an oxidative stress-inducible SWPA2 promoter in the chloroplasts of sweetpotato plants (Lim *et al*., 2007). Previously, our laboratory has assessed the biochemical and transcriptional responses of *C. tagal* to high salt treatment (500 mmol/L NaCl) via hydroponic experiments and Illumina RNA-seq (Xiao *et al*., 2016). To investigate transcriptional responses of *C. tagal* to salt stress, RNA-seq data was generated from seedling root tissues after 0, 1, 3, 9, 12, and 24 h of salt treatment. Digital gene expression analysis was conducted by comparing transcripts from the untreated control to the salt treated samples, and 7,330 differentially expressed transcripts were identified. Several genes encoding the superoxide dismutase expressed higher under the high salt treatment compared to the untreated sample.

In this study, a cDNA sequence, designated *CtCu/Zn-SOD*, putatively encoding the Cu/Zn superoxide dismutase gene was isolated from the root of the *C. tagal* and its function to improve the salt tolerance was identified via heterologous expression in auxotrophic *Saccharomyces cerevisiae* strain INVSc1. Based on the obtained data, it may lay a great fundamental comprehension to the salt tolerance of mangrove plants and provide a new perspective of crop breeding via transmitting this gene to many crops to obtain high salt tolerance crops.

Materials and Methods

Plant materials and growth conditions: Mature viviparous seedlings of well-developed *C. tagal* (Dongzhaigang mangrove, Hainan province, China) were cultured with Hoagland nutrient solution (Benavídes *et al*., 2000). When the second pair of leaves grew and began budding, the seedlings were transferred into 500 mmol/L NaCl solution. The roots were collected after salt treatment of 0 h, 1 h, 3 h, 6 h, 9 h, 12 h, and 24 h and immediately frozen in nitrogen and then stored at -80℃ for the extraction of total RNA.

RNA isolation and the amplification of Cu/Zn-SOD gene: The roots of the *C. tagal* were ground to powder in liquid nitrogen and the total RNA was extracted using the developed-CTAB method (Hernández *et al*., 2000). The first strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The primers for the amplification of *Cu/Zn-SOD* were designed base on the sequence of the *Cu/Zn-SOD* screening by transcriptome and digital gene expression profiling (Xiao *et al*., 2016):

PCL5229Contig-F: 5′-GGGGGTACCAGGGGTGCT CTGAGATCACAGAA-3′, PCL5229Contig-R: 5′-GGG TCTAGAATGCAATGGGAGACCTGAGAAA-3′.

The above primers and first chain cDNA were used for PCR amplification and the PCR products were detected by 1.0% agarose gel electrophoresis and the spectrophotometry. The purified products were cloned into pEASY-Blunt cloning vector (TransGen Biotech, Beijing, China), then transformed into *Trans5α* chemically competent cells and sequenced.

Bioinformatics analysis: The NCBI Blast server and the ProtParam of the ExPASy (http://web.expasy.org /protparam/) were used to search the homologous gene and analyze the theoretical physicochemical property of the deduced polypeptide of *CtCu/Zn-SOD*. The online prediction tool LocTree3 (https://www.rostlab.org/ services/loctree2/) was used to predict the protein subcellular localization of the *CtCu/Zn-SOD* (Goldberg *et al*., 2014). The deduced amino acid sequences were aligned using the ClustalX program. The phylogenetic tree was conducted by the software MEGA5 using the neighbor-joining (NJ) method. Bootstrap testing with 1000 repeats was used to examine the significance level of the NJ analysis.

RNA-Seq and Quantitative real-time PCR: The total RNA from the roots of *C. tagal* treated with 500 mmol/L salt solution in different time was extracted and used for transcriptome sequencing via Illumina sequencing technology in our previous study (Xiao *et al*., 2016).

The SYBR® Premix Ex Taq™ Ⅱ (TaKaRa, Dalian, China) was used to perform the quantitative real-time PCR (qRT-PCR) following the manufacturer's instructions. The cDNA of *C. tagal*'s young roots treated with 500 mmol/L NaCl for 0, 1, 9, and 24 hours were used as the templates. The relative transcript quantity of the *CtCu/Zn-SOD* was quantified in terms of comparative threshold cycle (Ct) using the $2^{-\Delta\Delta Ct}$ method. The *β-actin* gene was used as the reference gene. All data were given in terms of relative mRNA expression as means ± s.d. The primers used in quantitative real-time PCR were shown as follows:

actin-F: 5′-TGGAAGCTGCTGGAATCCAT-3′;
actin-R: 5′-TCCTCCACTGAGCACAACGTT-3′;
Cu/Zn-SOD-F*:* 5′-TGGCTGAAGCAACAATTGTG-3′*;*
Cu/Zn-SOD-R: 5′-GTCAGACTAAGTTCATGTCC-3′.

Plasmid construction, yeast transformation: The yeast expression vector pYES2 is a high-copy, autonomously replicated *S. cerevisiae-E. coli* shuttle vector which confers ampicillin resistance to *E. coli* and uracil prototrophy to *ura3* yeast (Gao *et al*., 2014). The target gene in pEASY-Blunt cloning vector was digested by an *Xba*I-*Kpn*I double digestion and cloned into the expression vector pYES2 under the control of the inducible promoter GAL1. The recombinant colonies were detected by PCR and enzyme digestion and the positive clone was named p*CtCu/Zn-SOD*. The recombinant plasmid p*CtCu/Zn-SOD* was introduced into the auxotrophic *S. cerevisiae* strain INVSc1 (His^-, Leu^-, Trp^-, Ura^-) by lithium acetate/single-stranded carrier DNA/PEG method (Gietz & Schiestl, 2007), then the yeast expression strain was cultured on the synthetic minimal medium plates lacking uracil (SC-Ura) solid medium with the temperature at 30℃.

Hetero-expression of *CtCu/Zn-SOD* in yeast and SOD activity analysis: For SOD activity analysis, the yeast strains INVSc1 (p*CtCu/Zn-SOD*) were cultivated on SC-U medium containing glucose at 30℃ until the OD_{600} (optical densities at 600 nm) to about 0.5, then the new yeast stain was diluted by 1:1000 proportion dilution in SC-U induced medium with 2% lactose by adding 1 mL of 0.5, 1, 2, 3, 4, and 5 mol/L concentration salt for about 20 h, respectively. The untreated strains were as a control. After induction, the yeast strains were harvested into micro centrifuge tube after OD_{600} to 2.5 by centrifugation, washed three times with sterile double distilled water to eliminate the influence of the residual medium and metabolites. Thereafter, the yeast strains were suspended with 100 μL ddH_2O and 100 μL 0.2 mol/L NaOH was added to crack cells for 5 min. The supernatant containing proteins was collected for SOD activity analysis using SOD Assay Kit (Sigma) according to the manufacturer's instructions. All experiments were performed in triplicate.

Assay of salt stress resistance for transgenic yeast: For salt stress assay, the transgenic yeast stains of INVSc1 containing p*CtCu/Zn-SOD* were grown in SC-U liquid medium with 0, 1, and 3 mol/L NaCl at 30℃ for 72 h, the strains containing empty pYES2 was used as a negative control. Then the same volume of yeast liquid was diluted 10^5 times and inoculated on the SC-U solid medium. The medium was cultivated at 30℃ and the number of a yeast single colony was counted after salt stress to the transgenic yeast strains about 72 h. All experiments were performed in triplicate.

Statistical analysis: The average of three experiments was used for statistical analysis. All results showed in this study were presented as the mean ± s.d. The student's *t*-test was used to analyse significant differences between the transgenic and non-transgenic (with empty vector) groups. A difference with $p<0.05$ or $p\leq0.01$ was regarded as statistically significant or extremely significant.

Results

Isolation and bioinformation analysis of *CtCu/Zn-SOD* gene: Based on the transcriptomes of *C. tagal* to salt stress, the Contig5229 putatively encode a superoxide dismutase gene. Therefore, the primers PCL5229.Contig-F/R with *Xba*I and *Kpn*I restriction sites were used for PCR amplification.

A plastid-derived superoxide dismutase (SOD) gene, designated *CtCu/Zn-SOD*, was isolated from the roots of *C. tagal*. The full-length of the *CtCu/Zn-SOD* ORF was 687 bp encoding 228 aa. The online blast searching showed that the amino acids sequence of the CtCu/Zn-SOD had a great homology to several known plant plastid-Cu/Zn-SOD. The prediction results of LocTree3 indicated that the subcellular location of the CtCu/Zn-SOD was in the chloroplast. These results were consistent with the homology analysis. The online program ProtParam showed that the molecular weight of CtCu/Zn-SOD was 23 KDa. The aliphatic index was 91.58 and the grand average of hydropathicity was 0.040.

The putative polypeptide sequence of the CtCu/Zn-SOD and several various plant superoxide dismutase were compared (Fig. 1), including *Kandelia candel* (100% identity; Genebank: AJK90567.1); *Jatropha curcas* (85% identity; Genebank: XP_012064680.1); *Hevea brasiliensis* (85% identity; Genebank: XP_021692107.1); *Manihot esculenta* (87% identity; Genebank: XP_021609177.1); *Pyrus x bretschneideri* (85% identity; Genebank: XP_009372495.1); *Fragaria vesca subsp. vesca* (84% identity; Genebank: XP_004287550.1); *Malus domestica* (84% identity; Genebank: XP_008385499.1); *Juglans regia* (87% identity; Genebank: XP_018818935.1); *Quercus suber* (83% identity; Genebank: XP_023875972.1). The homology analysis showed that CtCu/Zn-SOD was completely accordant with the Cu/Zn-SOD from *Kandelia candel* (100% identity) which belonged to the mangroves. This result indicated that, in evolutionary, the Cu/Zn superoxide dismutase may be highly conserved in mangroves. In fact, there has been at least 80% identity with the majority of the plant Cu/Zn superoxide dismutase. Phylogenetic tree of the putatively amino acids has also proved that CtCu/Zn-SOD was most likely a plastid-derived Cu/Zn superoxide dismutase and evolutionarily conserved in many plants (Fig. 2).

Expression pattern of *CtCu/Zn-SOD* under the salt stress: In order to characterize the effects of salt shock on *C. tagal*, a series of microarray experiments to monitor the transcript profiles using young roots, treated with 500 mmol/L NaCl for 0, 1, 9, and 24 hours were performed in our previous work (Xiao *et al*., 2016). High-salinity-induced differentially expressed genes (DEGs) were identified and functional annotated via bioinformation analysis. Among all these DEGs, the candidate full-length CDS of *Cu/Zn-SOD* was chosen as candidate gene, because the expression profile of *Cu/Zn-SOD* via RNA-seq was in line with the qRT-PCR (Fig. 3A, 3B). The candidate gene could be induced by the salinity of 500 mmol/L NaCl. Furthermore, qRT-PCR also showed that the expression level of the *CtCu/Zn-SOD* increased quickly with the increasement of the processing time treated with 500 mmol/L NaCl (Fig. 3B). These showed that the gene *CtCu/Zn-SOD* was induced by the salinity.

Ectopic expression of *CtCu/Zn-SOD* gene in yeast: In order to investigate the function of *CtCu/Zn-SOD* genes to salt stress, the recombinant plasmid p*CtCu/Zn-SOD* was transformed into yeast INVSc1 strains. For SOD activity analysis in yeast cells, the proteins were extracted from yeast cells ((INVSc1 (pYES2) and INVSc1 (p*CtCu/Zn-SOD*)) after induction with different concentration salt solution treated. The results showed that the SOD activity of yeast cells ((INVSc1 (pYES2) and INVSc1 (p*CtCu/Zn-SOD*)) with salt treatment were higher than the untreated yeast strains and the latter was obviously higher than the former with various concentration salt treatment. This indicated that the *CtCu/Zn-SOD* gene expressed successfully in yeast strains. Furthermore, the SOD activity rose firstly, then reduced, and rose next, reduced finally with the increasement of the salt solution's concentration (Fig. 4).

Salt resistance analysis of transgenic yeast with *CtCu/Zn-SOD*: Colony count results showed that transgenic yeast strains with *CtCu/Zn-SOD* or non-transgenic yeast strains both could grow under salt stress, but the growth of transgenic yeast strains were significantly better than the yeast with empty vector pYES2. The transgenic yeast strains can bear higher salt stress concentration (Fig. 5A).

The yeast colonies were further counted and analyzed. The results showed that the growth of yeast was inhibited by salt stress. With the increase of salt concentration, the inhibitory effect was gradually increased. Without treated, the number of two yeasts was not significantly different. But with the increase of salt concentration, the number of transgenic yeast strains were significantly higher than the non-transgenic strains ($p<0.05$) (Fig.5B). This showed that exogenous *Cu/Zn-SOD* gene could improve the salt tolerance of INVSc1 (p*CtCu/Zn-SOD*) yeast under the same salt stress.

Discussion

For plants, some protecting enzymes such as antioxidant enzymes were involved in the response to abiotic stress, such as salt, drought, low temperature and heat stress. The superoxide dismutase (SOD) protein including copper-zinc and manganese superoxide dismutase (Cu/Zn-SOD and Mn-SOD) is one of the important antioxidases in protection against oxidative tissue injury caused by various abiotic factors (Tanaka *et al*., 1999; Zhu *et al*., 2008). Previous studies have indicated that lanthanum might stimulate the plant defense system (POD, SOD and CAT), promote the accumulation of secondary metabolites (soluble sugars and proteins) of plant cells and prevent the decline of the relative chlorophyll content and chlorophyll fluorescence parameters under different stresses (Gao *et al*., 2003; Liu *et al*., 2010). In recent years, the researchers paid more and more attentions to plant stress responses mechanism, especially the salt stress responses. The continuous development of various research methods has accumulated more and more salt stress response data.

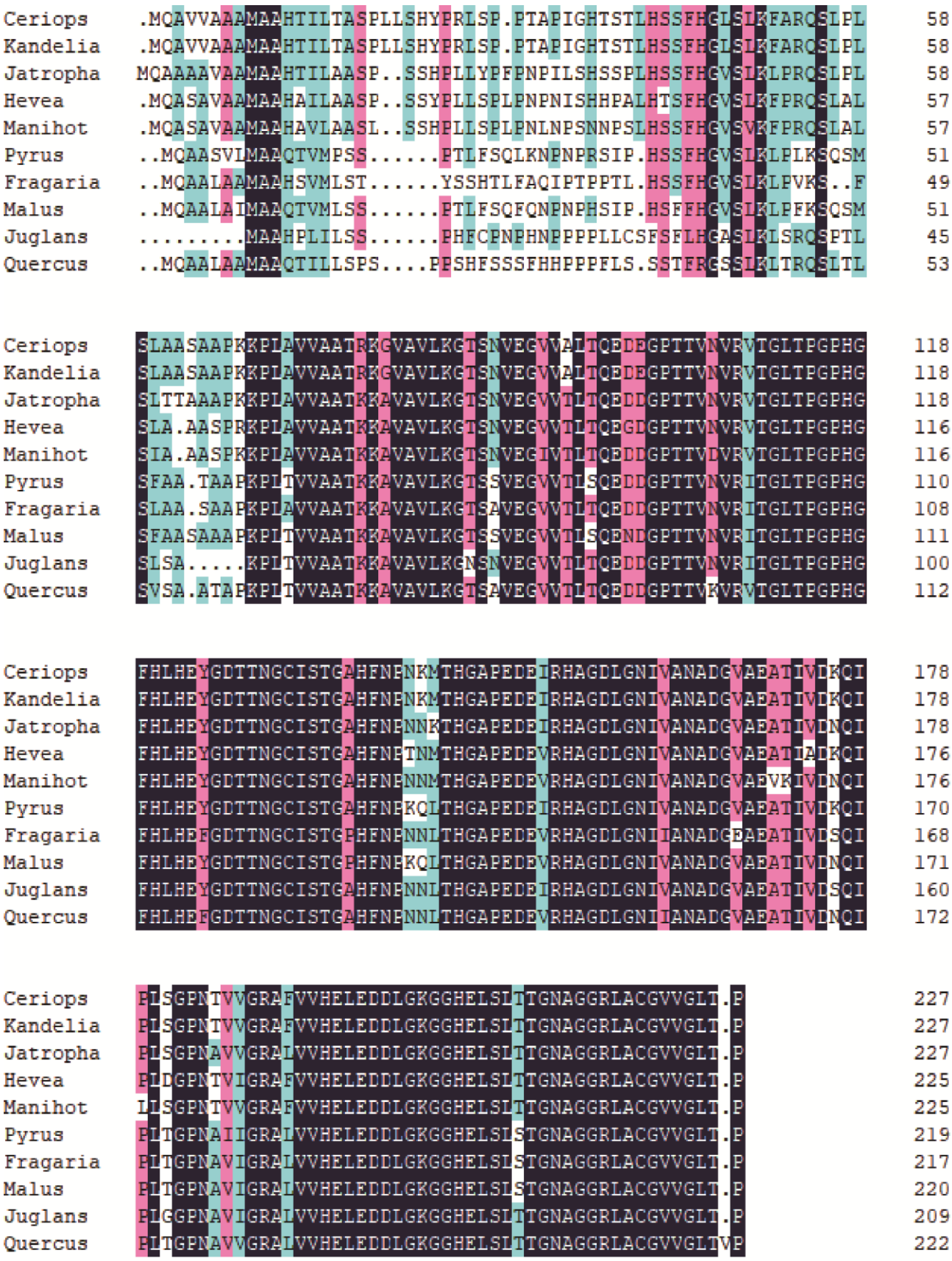

Fig. 1. Multiple sequence alignment of the deduced amino acid sequence of CtCu/Zn-SOD and other plant superoxide dismutase including *Kandelia candel* (100% identity; Genebank: AJK90567.1); *Jatropha curcas* (85% identity; Genebank: XP_012064680.1); *Hevea brasiliensis* (85% identity; Genebank: XP_021692107.1); *Manihot esculenta* (87% identity; Genebank: XP_021609177.1); *Pyrus x bretschneideri* (85% identity; Genebank: XP_009372495.1); *Fragaria vesca subsp. vesca* (84% identity; Genebank: XP_004287550.1); *Malus domestica* (84% identity; Genebank: XP_008385499.1); *Juglans regia* (87% identity; Genebank: XP_018818935.1); *Quercus suber* (83% identity; Genebank: XP_023875972.1).

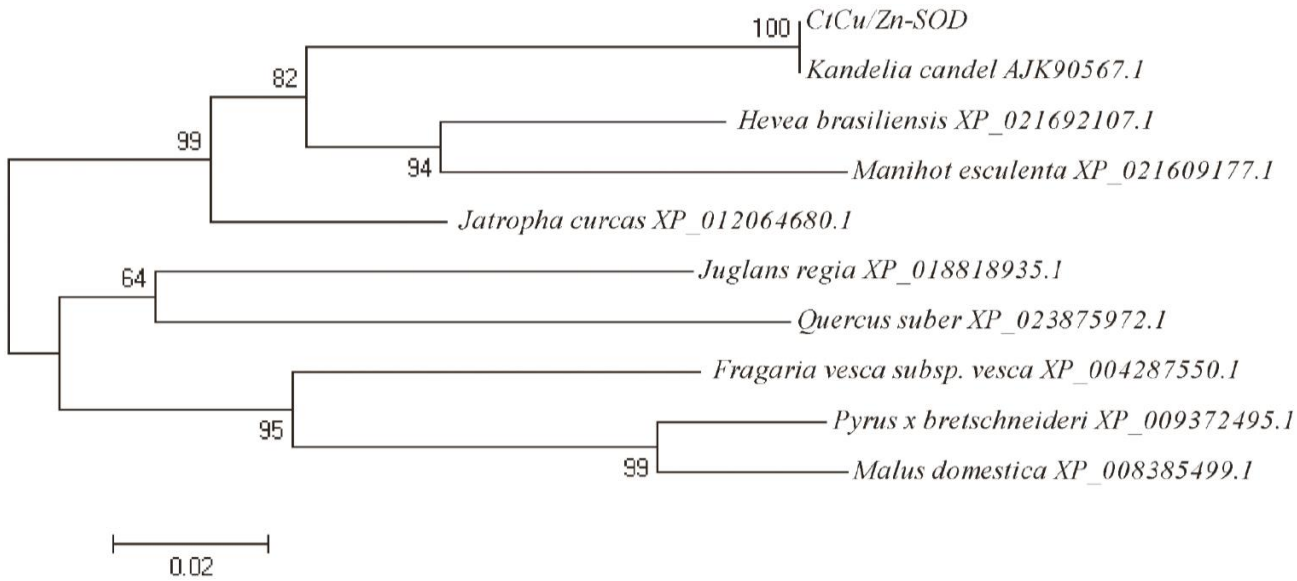

Fig. 2. Phylogenetic tree was generated by MEGA5.0 with the neighbor-joining method.

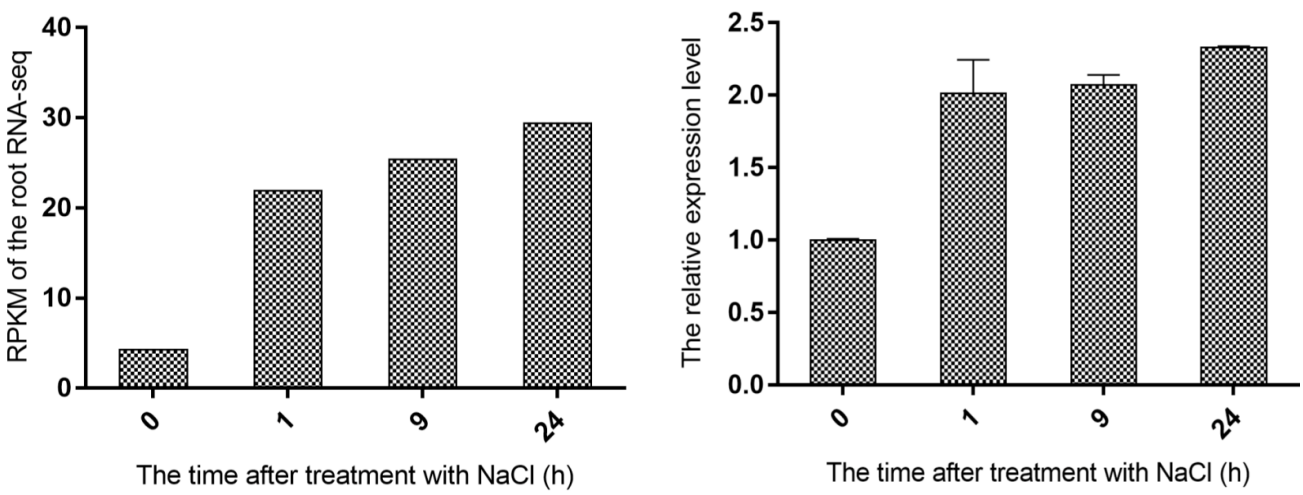

Fig.3. Expression level of *CtCu/Zn-SOD* under salt stress.
A.The RPKM (*CtCu/Zn-SOD*) of the root RNA-seq in *C. tagal* treated by 500 mM NaCl for 0 h, 1 h, 9 h, and 24 h
B.The relative expression level of *CtCu/Zn-SOD* in *C. tagal* treated by 500 mM NaCl for 0 h, 1 h, 9 h, and 24 h

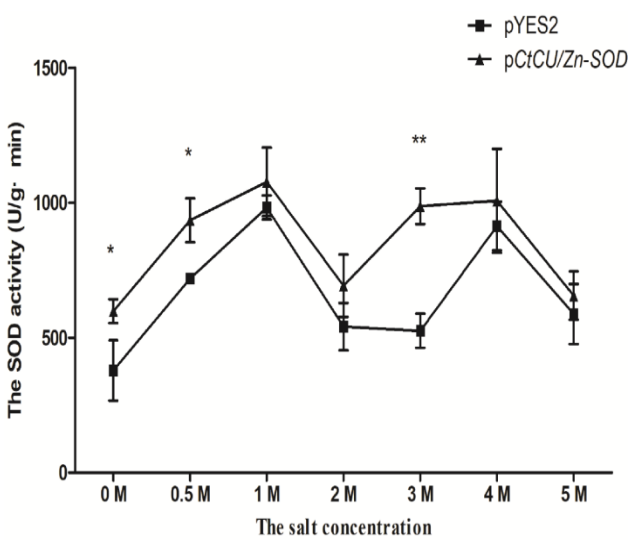

Fig. 4. The SOD activity of yeast strains (INVSc1 (pYES2) and INVSc1 (p*CtCu/Zn-SOD*)) treated with different concentration salt solution.

The burst of active oxygen species in the high salt environment causes the dynamic imbalance, and then causes damage to the biological membrane system of the cell (Hoagland & Arnon, 1950; Mittova *et al*., 2003). Plants have evolved effective reactive oxygen species detoxification mechanism, including the non-enzymatic antioxidant and enzymatic protection system (Scandalios, 2005). Antioxidant protection system including SOD, POD, CAT, glutathione reductase (GR) and others (Caverzan *et al*., 2012; Maruta & Ishikawa, 2017). SOD catalyze the conversion of superoxide anion, such as, singlet oxygen ($^1O2$), superoxide ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$), into less active hydrogen peroxide, and occupy the main position in the enzymes of antioxidant protection system (Zhu *et al*., 2008; Caverzan *et al*., 2012). Currently, the research of *SOD* gene functional investigation is mainly conducted via transferring it into plants for genetic verification. Researchers found that transforming *Cu/Zn-SOD* into tobacco could improve the tobacco resistant to salinity and enhance antioxidant ability (Marček *et al*., 2016). Breusegem *et al*., (1999) found that

tobacco *Mn-SOD* gene transformed into the maize chloroplasts could improve the cold-resistance and oxidative stress of foliar (Breusegem *et al*., 1999). Moreover, overexpression of *Tamarix albiflorum TaMnSOD* increases drought tolerance in transgenic cotton (Zhang *et al*., 2014). It is evident that the research of *SOD* gene has broad prospects and study value. The analysis of *Cu/Zn-SOD* in *C. tagal* under adversity stress of high salt showed that *Cu/Zn-SOD* genes might play important roles in salt stress responses. This not only expounded the reason of high resistance to salinity of *C. tagal* but also clearly showed that the *Cu/Zn-SOD* gene in *C. tagal* was positive correlation with the salt tolerance.

The *C. tagal* grew in coast intertidal zone, as a kind of typical non-secrete salt mangrove plants, formed a set of effective active oxygen removal mechanism under the long-term natural selection. Initially, our laboratory compared the differences of related physiological indicators of *C. tagal* under salt stress about ion balance and anti-oxidation stress. The results showed that the SOD, POD and other enzymes activities were significantly increased. Differential gene screening was performed to isolate the high-expressed gene under salt stress using the transcriptional and digital expression profiling techniques. In this study, a *Cu/Zn-SOD* gene was identified. High concentration of salt stress screening results indicated that the expression of *Cu/Zn-SOD* gene in yeast could significantly improved the salt tolerance of yeast. The effects of *C. tagal* Cu/Zn-SOD in salt resistance of yeast was consistent with the role of cytosolic Cu/Zn superoxide dismutase from *Bruguiera gymnorrhiza* in salt stress response (Takemura *et al*., 2002).

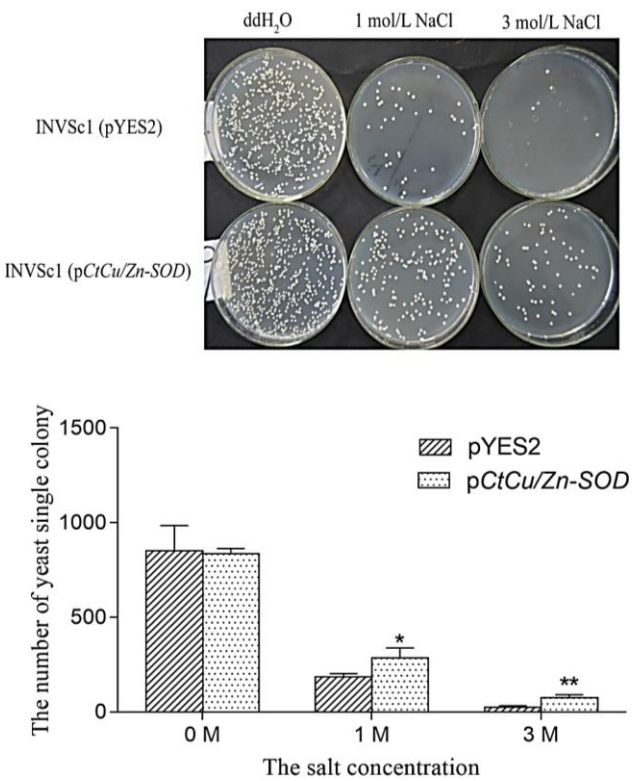

Fig. 5. Phenotype of the transgenic yeast.
A. The growth of INVSc1 (pYES2) and INVSc1 (p*CtCu/Zn-SOD*) treated with different concentration of salt
B. The number of yeast single colony (INVSc1 (pYES2) and INVSc1 (p*CtCu/Zn-SOD*)) treated with different concentration of salt

Conclusions

This was the first study to clone *Cu/Zn-SOD* gene from the root of *C. tagal*, and we revealed the role of it in salt stress resistance in yeast. Furthermore, the characterized *CtCu/Zn-SOD* gene will bring a good foundation to comprehend and further explore its potential functions so that there is a possibility to transmit this gene to crops to change the salt resistance by bioengineering. This study provided the experimental basis about the stress resistance of mangrove plants; however, the underlying physiological and molecular mechanisms are needed to be further studied.

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