

# Development and Optimization of Rabbit Polyclonal Antibodies for Cu/ZnSOD detection in Rice (*Oryza sativa* L.)

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## Original article

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# Abstract

Superoxide dismutase (SOD) activity is an important measure of plant stress tolerance used in cultivar improvement. At present, we are unaware of any widely available immunological reagents for the detection of SOD in *Oryza sativa* (common Asian rice) or other plants. In this study, we used insilico B-cell epitope prediction tools to generate peptides which were immunized into rabbits to yield polyclonal antibodies against Cu/Zn SODs. Immunoblotting demonstrated that the antibody specifically recognized both native and denatured Cu/Zn SODs in rice. In addition, this antibody can confirm the expression tendency of endogenous OsCu/Zn SODs under heat stress by immunoblotting, and has a positive reaction in tomato leaf extracts, as well as human Hela cells. Chloroplast content of Cu/Zn SODs in rice can be identified by ELISA indirect competition method using this antibody. These results suggest that this Cu/Zn SOD rabbit polyclonal antibody may be a useful tool for elucidating the biological functions of Cu/Zn SODs in plants.

## Background

Plant organelles generate reactive oxygen species (ROS) as both secondary messengers and metabolic byproducts (Gill et al. 2010; Noctor et al. 2018; Podgorska et al. 2017). Excessive ROS formation may damage plants, resulting in cell or organism death (Hossain et al. 2016). The enzyme superoxide dismutase (SOD, EC 1.15.1.1) catalyzes the decomposition of superoxide (free radical  $O_2^-$ ) into molecular oxygen ( $O_2$ ) and hydrogen peroxide ( $H_2O_2$ ), which can be degraded by catalase (Foyera et al. 2003). In this respect, SOD activity is a critical component of plant stress resistance. Many studies have observed improved stress tolerance following SOD overexpression in transgenic plants (Badawia et al. 2004; Liu et al. 2015; Guan et al. 2017; Bartels et al. 2005; Tu'rkkan et al. 2005).

At present, SOD levels are typically measured by determination of enzymatic activity and/or quantitative reverse transcriptase PCR. These methods do not directly measure the total amount of SOD in a sample, and widely available antibodies against plant SODs are lacking. Detecting the total Cu/Zn SOD in plants, especially rice, and the protein content encoded by the specific single rice Cu/Zn SOD gene is rare, although there are commercial antibodies against plant SODs or human SODs in papers (Zhao et al. 1995). The development of plant Cu/Zn SOD specific antibodies would facilitate the development of simple immunological assays (e.g.ELISA and immunoblotting) for the quantitation of SOD for use in basic research and plant breeding.

In this study, we compared the Cu/Zn SOD family protein sequences of *Oryza* sub species *japonica* and *indica* in silico. Next we generated recombinant rice chloroplast Cu/Zn SOD recombinant protein and immunized rabbits. We demonstrate specific binding to Cu/Zn SODs from Nipponbare (*Oryza saiva* L.ssp. *japonica*) and 93 – 11 (*Oryza saiva* L.ssp. *indica*), two important rice varieties in Asia, by immunoblot. We also demonstrate cross-reactivity with tomato Cu/Zn SOD, and even human cells. The rice chloroplast Cu/Zn SOD proteins can be detected with an optimized indirect competitive ELISA using this antibody.

## Results

### Sequence Analysis and B-Cell Epitope Prediction of the Cu/Zn SODs in Rice

To guide the design of our immunological assay, a multiple sequence alignment of the amino acid sequences of the SOD gene family members was performed (Fig. 1C) which was then used to generate a phylogenetic tree (Fig. 1A). The amino acid sequence of the SOD with the same LOC was consistent in *O.s. japonica rice* and *O.s. indica rice*. The evolutionary tree revealed that the SOD gene family could be divided into four primary branches based on their coordinated metal cofactors (cations  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ , cations  $\text{Cu}^{2+}$  cation  $\text{Fe}^{3+}$ , and cation  $\text{Mn}^{2+}$ ). It can also be demonstrated that the sequences of Cu/Zn SODs, Fe SODs and Mn SOD have no homology. Cu/Zn SODs are the main components of SOD expression. The expression of *LOC\_Os08g44770* located in chloroplasts was higher than others, as the amino acid sequence homology of Cu/Zn SODs encoded by four genes (Fig. 1C).

Next, we used PlantCare to predict the cis-acting promoter elements of rice Cu/Zn SOD genes. The predicted elements and their numbers are compared and presented in Fig. 1B. The genes of Cu/Zn SODs in *japonica* and *indica* had many conventional cis-acting elements, which can respond to multiple environmental changes. In particular, the genes respond to temperature, light and hormones, and this is well documented in the literature (Chiang et al. 2014; Kimet al. 2010; Gupta et al. 1993; Wu et al. 1999; Wang et al. 2014).

Expected B-cell epitopes derived from amino acids 58–211 AA of *LOC\_Os08g44770* were mapped using the ABCpred server and Lasergene (Fig. 1D). The results revealed that the predicted region of ABCpred measured more than 0.7 points, which was consistent with the positive wave map predicted by Lasergene software. The greater the score, the greater the possibility that the peptide segment will become an antigenic epitope. The green box measuring 0.9 points (Fig. 1D) was consistent with the homologous region of four Cu/Zn SOD proteins (Fig. 1C).

### Characterization of the rabbit polyclonal antibody to OsCu/Zn SODs

The OsSODCP-his recombinant protein, as the antigen, was generated from *E. coli*. The major band of this protein had an apparent molecular weight of 22 kDa on SDS-PAGE, with minor species at 26, 46 and 67 kDa (Fig. 2A). The concentration of OsSODCP recombinant protein was determined to be 0.85 mg/mL by the BCA method. Specific activity of the OsSODCP-his was measured and determined to be 386.5 U/mg (Fig. 2B).

To test for specific binding of the rabbit polyclonal antibody to the recombinant antigen and OsCu/Zn SODs we performed both targets and an unrelated His-tagged protein. Pre-immune rabbit serum was used as a negative control. The blots showed the OsCu/Zn SOD's recombinant proteins bound to the rabbit polyclonal anti-OsSOD and anti-His antibodies, but not to pre-immune serum (Fig. 2C). The

unrelated His-tagged protein was recognized by anti-His tag antibody. The positive reaction to the SODCP-his with immune serum was higher than that of other proteins. The results suggested that the Cu/Zn SOD's homologous region was one of epitopes (Fig. 1C and 1D), which caused the polyclonal antibody have a degree of recognition ability for the 4 types of OsCu/Zn SOD.

## **Rabbit polyclonal antibody binds cell lysates from both in japonica rice and indica rice with SOD activity**

To determine whether anti-OsCu/Zn SOD rabbit polyclonal antibodies bind endogenous Cu/Zn SODs in *japonica rice* and *indica rice*, we prepared protein extracts from lysates obtained from the young leaves of both cultivars. Extracted proteins were electrophoresed, transferred to membranes and immunoblotted with polyclonal antibodies against Cu/Zn SODs. Lysates from both *japonica rice* and *indica rice* (93 – 11) bound anti-Cu/Zn SODs rabbit polyclonal antibodies, but not pre-immune serum (Fig. 3A).

To test the binding of the anti-OsCu/Zn SODs rabbit polyclonal antibodies to the native OsCu/Zn SODs, immunoprecipitation and immunoblotting experiments were performed on lysates of *indica rice* leaves (93 – 11). Native OsCu/Zn SODs were immunoprecipitated by anti-OsCu/Zn SODs rabbit polyclonal antibodies (Fig. 3B) but not by pre-immune serum (Fig. 3B). SOD activity was detected in the anti-OsCu/Zn SOD's immunoprecipitation complex and not in pre-immune serum (Fig. 3C).

To determine whether anti-OsCu/Zn SODs rabbit polyclonal antibodies could measure a dose response, we induced Cu/Zn SODs with high-temperature stress (heat stress). Nipponbare rice seedlings were cultured at 40 °C for 0, 3, 6 and 12 hours. While Cu/Zn SOD's transcript levels increased at each time point, paralleled by an increase in the density of rabbit anti-OsCu/Zn SOD band on immunoblot (Fig. 3E, 3D). In contrast, SOD activity increased at 3 hours and remained at that level for the duration of the experiment (Fig. 3F). These results suggest protein translation was occurring, but that enzyme activity was inhibited. Three other varieties of rice seedlings (93 – 11, STTM398, N22) were also treated in the same way as Nipponbare. The protein extracts of different rice seedlings were immunoblotted with this antibody, and the results are shown in Fig. 3G. At different heat stress times, the protein expression in Cu/Zn SOD seedlings changed significantly, and the response to heat stress was slightly different.

## **Rabbit anti-OsCu/Zn SOD is cross reactive with SOD from tomato and human cells**

Immunoblot experiments were performed on cell lysates obtained from leaves of unrelated plants, tomato and *Arabidopsis thaliana*, to determine if anti-OsCu/Zn SOD was cross reactive with other plant SODs. The blot with anti-OsCu/Zn SOD had banding for rice and tomato, but not *A. thaliana*, suggesting that there is some, but limited, cross reactivity in a species-dependent manner (Fig. 4).

Immunofluorescence experiments were performed on HeLa cells to determine whether anti-OsCu/Zn SOD was cross-reactive with human SODs. The fluorescence signals with anti-OsCu/Zn SOD was the same as

those with anti-human SOD2, the positive control banding for SOD2 in HeLa cells, but not detected in cells with non-immune serum. Fluorescent signals came primarily from the cytoplasm, indicating that the protein bound by the antibody primarily existed in the cytoplasm, which was consistent with SOD cell sublocalization (Fig. 4).

## Quantitative analysis optimization of chloroplast Cu/Zn SOD in rice by indirect competitive ELISA

If the logarithm of the standard concentration was taken as the horizontal coordinate, then the reading value OD405, for the antibody competition sample, with a different dilution degree, was the vertical coordinate, and is shown in Fig. 5. All the groups OD405 reached the detection platform period, which was beyond the detection range (Fig. 5A). The quantitative standard curve of competitive antibody with different dilutions was obtained under the concentrations from 60 to 60000 ng/mL for linear fitting (Fig. 5A). The optimal antibody dilution for quantitative detection of chloroplast Cu/Zn SOD by indirect competitive ELISA was 1:16000, and the fitting degree was the highest with the standard curve  $R^2 = 0.9942$ . The homologous proteins OsSODC1-his, OsSODC2-his, OsSODCS-his, and OsSODCP-his, were tested with the antibody at the same time as the standard. Non-related proteins were negative controls. The results showed that OD405 were not dependent with the concentrations of the standard protein except OsSODCP-his (Fig. 5B). The ELISA system can only quantify chloroplast Cu/Zn SOD in rice, and this may be related to the presence of antibodies in this polyclonal antibody that only specifically recognize chloroplast Cu/Zn SOD.

The standard curve linear equation and group with OsSODCP-his as the standard protein, the linear equation of the standard curve and the  $R^2$  results are shown in Fig. 5C. In this scheme, the content of chloroplast Cu/Zn SOD in rice was quantitatively analyzed in the range of 0.06 ng/mL to 60000 ng/mL.

## Discussion

As a primary catalyst of oxygen free radicals, superoxide dismutase plays an important role in the stress tolerance of rice. Many studies have revealed that increased expression of SOD improves stress tolerance in plants (Liu et al. 2015; Tu'rkkan et al. 2005; Bartels et al. 2005; Badawia et al. 2004; Guan et al. 2017; Chiang et al. 2014; Kim et al. 2010; Gupta et al. 1993; Wu et al. 1999; Wang et al. 2014). The regulation of gene expression in organisms is both a transcriptional and posttranscriptional regulation process. In eukaryotic organisms, miRNAs inhibit the expression of target genes by mRNA degradation and translation inhibition, which belongs to posttranscriptional expression regulation (Baumberger et al. 2005; Llave et al. 2002). The specific recognition of antibodies and antigens can be used to detect protein levels. This method is widely used in animal protein detection, but is rare against plant proteins.

In *Arabidopsis thaliana*, miRNA can inhibit translation in endoplasmic reticulum in the presence of AMP1, and does not produce the enzyme hydrolysis activity of mRNA (Shengben et al. 2013). MiR398 not only inhibits the expression of Cu/Zn SOD by the mRNA degradation response to heat stress, but also

inhibiting the translation of Cu/Zn SOD response to Cu stress in *Arabidopsis thaliana* (Guan et al. 2013; Sunkar et al. 2006; Dugas et al. 2008). MiR398 exhibits strong homology between *Arabidopsis thaliana* and rice. Many studies have shown that Cu/Zn SOD in rice is also regulated by miR398 (Balyan et al. 2017; Yao et al. 2014; Li et al. 2019) and there are multi-level regulation among Natural antisense transcripts of MIR398, miRNA and SOD in *Arabidopsis thaliana* (Li et al. 2020), however the protein level expression of SODs are not reported in rice. In our study, a rabbit polyclonal antibody against OsCu/Zn SOD was used to test its expression under high-temperature. These results suggested that enzyme activity was not consistency with the transcriptional and protein level of SOD after heat treatment 3 h, that may be related to the protein modification and degradation of SOD. Rice cv. N22 have strong resistance to heat stress, many articles have been reported (Jagadish et al. 2011; Poli et al. 2013). STTM398 transgenic lines is the rice mutant obtained after silencing miR398 from Nipponbare using STTM technology. It showed some of the phenotypes such as decreased plant height, panicle length, and grain number per panicle, compared with Nipponbare (Zhang et al. 2017). As the heat stress time increased, the protein content of Cu/Zn SODs in rice leaves continuously changed. The heat response speed and Cu/Zn SODs expression changes are not completely consistent in different rice. This may be related to the inconsistent ability of different rice to resist heat stress. Other stress treatments may also affect the expression of SOD in plants, and the SOD antibody could be used to unfold the basis between expression regulation of SOD and those stress-related ROS scavenging signaling pathways.

In this study, rabbit polyclonal antibodies against OsCu/Zn SOD were obtained using rice chloroplast Cu/Zn SOD recombinant protein as an antigen, which can specifically identify the Cu/Zn SOD in *japonica* rice, *indica* rice, tomato and even in humans. The quantitative analysis of chloroplast Cu/Zn SOD in rice by the indirect competitive ELISA method using this antibody, is another optimization scheme in plant SOD enzyme activity detection. For the first time, the changes of chloroplast Cu-Zn SOD in rice were identified using this method. This study can provide a research tool for the detection of Cu/Zn SOD expression levels in rice and tomato. This will help to further explore the mechanisms of Cu/Zn SOD gene expression regulation.

## Materials And Methods

### Animals

Adult male New Zealand white rabbits (9weeks, 2.5kg, n = 2) were purchased from Taiping Biotechnology (Yiyang, China) and housed at a density of 1 per cage at Foreverttech Biotechnology. The rabbits were fed enough food and free access to water, with a 10-h light/14-h dark cycle and a 22 °C room temperature. Any procedures that caused potential pain or stress for the animals were conducted under isoflurane anesthesia. Adequate depth of anesthesia was determined by response to a paw pinch and by monitoring respiration. The animal experiment scheme was approved by the Animal Ethics Committee of the ProMab Biotechnology Inc. (Changsha, China & CA, USA) in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council 2011).

# Identification of SODs in rice genome

A keyword search of “superoxide dismutase” was performed against the eRice Epigenetic & Epigenomic Database (Version 1.0) (Zhang et al. 2018). SOD genes were mapped onto chromosomes by identifying their positions as given in the eRice database. RNA-seq data were expressed as fragments per thousand reads mapped (FPKM).

Phylogenetic and promoter element sequence analysis of SODs in Japonica group cultivar Nipponbare (Nip) and Indica group cultivar 93-11

Sequence alignment was carried out using Clustal W (Version 2.0.8) (Saitou et al. 1987). Phylogenetic trees were constructed using the neighbor joining method in MEGA (Version 7.0).

Two thousand base pairs upstream of Cu/Zn SOD genes were submitted to the PlantCare database (Kumar et al. 2016) for prediction and analysis of promoter sequence elements.

## Sequence Analysis and B-Cell Epitope Prediction of the Cu/Zn SODs in Rice

The amino acid sequences of Cu/Zn SOD were aligned in DNASTAR's Lasergene sequence analysis software (Version 7.1.0) (Burland et al. 2000). B-cell epitope prediction was performed in Lasergene and with the ABCpred server (Saha et al. 2006). The final identification of amino acid sequences can be used for immunization. A BLAST search was performed with the resulting sequence on NCBI to identify homologs.

## Expression Plasmid Constructs and the Recombinant protein purification

The candidate Cu/Zn SOD sequences were codon optimized for expression in *E. coli* codon and the synthesized genes transferred into the prokaryotic expression vector pET21a. Plasmid sequences were verified by Sanger sequencing (Hongxun Biotechnology Co. Ltd., Suzhou, China) and are described in Table 1.



Table 1

Gene name	Insert sequence (AA)	Recombinant plasmid	Molecular weight of recombinant protein (KD)	Molecular weight of Epigenetic protein (KD)
<i>LOC_Os08g44770</i>	58–211	OsSODCP-his	18.6	15.7
<i>LOC_Os03g22810</i>	1-152	OsSODC1-his	18.1	15.3
<i>LOC_Os07g46990</i>	1-152	OsSODC2-his	18	15.1
<i>LOC_Os03g11960</i>	1-159	OsSODS-his	19.5	16.5

OsSODCP-his recombinant protein was expressed in *E. coli* strain BL21 (DE3) (Forevertch Biotechnology Co. Ltd., Changsha, China). His-tagged protein was purified using a Ni-NTA agarose column (Qiagen, Germany). Protein concentration was determined using a BCA Assay Kit (Biosharp, AnHui, China). Apparent molecular weight was visualized by 12% polyacrylamide Bis-Tris gel followed by commassie stain.

## Rabbit immunization and Polyclonal Antibody Production

Two animals were immunized with 0.5 mg immunogen in PBS mixed with an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO, USA). A secondary immunization was repeated at two weeks. Two subsequent immunizations were performed using 0.25mg of the immunogen mixed with an equal volume of Freund's incomplete adjuvant (Sigma, USA) at biweekly intervals. One week after the third immunization, venous blood was collected from the ear for titer determination, and the fourth immunization was carried out as in the previous dose. Seven days after booster immunization, rabbits were euthanized and the anti-serum was harvested. Polyclonal antibodies were affinity purified from the anti-serum using a Protein G Sepharose (GE, Boston, MA, USA) and stored at -80 °C. Collection of rabbit serum the day before the first immunization was used as a negative control.

## Germination, Cultivation and High temperature treatment of Rice Seedlings (*Oryza sativa* L.)

Rice seeds were soaked in water in clean petri dishes and placed in a constant temperature incubator at 28 °C for two days in the dark. On the third day, dishes were transferred to a light incubator (light intensity 3000 lux and 16 h photoperiod, relative humidity 80% with 28 °C). When the buds grew to 1 cm, the germinated rice seeds were transferred and immersed in aseptic 1/2 MS liquid medium, and cultured at 25 °C in the light in a culture room. After 20 d, plants were transferred to the light incubator at 40 °C. Time-course samples were collected at 0, 3, 6 and 12 h for subsequent analysis.

# RNA and protein isolation, cDNA preparation and quantitative real-time PCR

Total RNA and protein from rice leaf tissues were isolated from Trizol (Invitrogen, Carlsbad, CA) using the modified protocol described by Xiao et al (Xiao et al. 2010). Total protein was isolated from organic and interphases followed by isopropanol precipitation.

cDNA synthesis was performed using ReverTraAce qPCR RT Kit (Toyobo, Osaka, Japan). cDNA reaction mix was diluted 20-fold for use as qPCR template. SYBR green based qPCR was performed with NovoStart SYBR qPCR SuperMix Plus Kit (Novoprotein, Shanghai, China) using the Step-One Plus thermocycler (ABI, Carlsbad, CA, USA). Each reaction contained 10µL 2x master-mix, 0.5 µL 10 mM forward and reverse primer, 1 µL cDNA and H<sub>2</sub>O to 20µL. All primer pairs used in this study are listed in Table 2. Cu/Zn SOD transcript levels were normalized using the  $2^{(-\Delta\Delta T)}$  method. Mean and standard errors presented are average of triplicates of biological replicates.

Table 2

Target	Length(bp)	Forward primer	Reverse primer
<i>LOC_Os03g22810</i>	231	GATCTTGGAAAGGGTGGGCA	CACCAAAAACCATGCGCAGA
<i>LOC_Os07g46990</i>	199	GTTGCTTGCGGAATCATCGG	AACGCATGCACTCAAGTCAA
<i>LOC_Os08g44770</i>	230	ACTTGCATGCGGTGTTGTTG	GGAGAACAGGGGATGTGACG
<i>LOC_Os03g11960</i>	195	GGGCAGTTGTTGTTTCATGCT	GGAGCGCCAATGATTTCCAT
<i>OsActin</i>	97	CCTCTTCCAGCCTTCCTTCAT	TCTCCTTGCTCATCCTGTCAG

## Immunoprecipitation

Young leaf tissues (100 mg) of rice strain 93 – 11 were ground with a mortar and pestle in liquid nitrogen. Total soluble proteins were extracted using 1 mL IP Lysis Buffer (25 mM Tris-HCl pH7.4, 150 mM NaCl, 1mM EDTA, 1% NP-40, 5% glycerol) as per the manufacturers protocol (Thermo, Waltham, MA, USA), and supplemented with PMSF and a phosphatase inhibitor cocktail (CST, Danvers, MA, USA). Total protein was quantified using a BCA Assay Kit (Biosharp, AnHui, China) prior to the determination of SOD activity with the SOD Enzyme Activity Detection kit (Jiancheng, NanJing, China). Samples were then split and 1 mg total protein incubated with 4µg anti-OsCu/Zn SOD rabbit polyclonal antibody, or 4µg pre-immune rabbit serum in 1ml IP Lysis Buffer overnight at 4 °C. Antibody complexes were captured with 20 µL Protein Agarose (GE, Boston, MA, USA) with gentle shaking for 1 hour at 4 °C. Proteins were eluted with 80 µL elution buffer (100mM glycine-HCl pH2.5) followed by pH neutralization with 20 µL 1M Tris (pH 9.5). Immunoprecipitates were then separated by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

## Immunoblotting

Immunoblot was performed on recombinant Cu/Zn SOD, leaf tissue lysate and immunoprecipitated leaf tissue lysate. Proteins were separated on 15% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose (Thermo, Waltham, MA, USA) using a semidry transfer apparatus (Biorad, Hercules, MA, USA). Following transfer, membranes were blocked with 5% non-fat dry milk/20 mM tris buffered saline, pH7.6 and 0.05% Tween 20 at 4 °C overnight. Incubation with primary antibodies was performed at room temperature for 2 hours followed by 5x wash over for 5 minutes. Secondary HRP-labeled antibodies (Millipore, MA, USA) were incubated at room temperature for 2 hours. Blots were visualized using enhanced chemiluminescence immunoblotting detection kit (Biorad, Hercules, MA, USA).

## **Determinations of SOD Enzyme Activity**

SOD activity was measured using a colorimetric SOD Enzyme Activity Detection kit (Jiancheng, NanJing, China). Samples were prepared and analyzed in triplicate according to manufacturer's protocol. Absorbance values were measured using an Epoch™ 2 Microplate Spectrophotometer (Biotek, Winooski, VT, USA) at 25 °C.

## **Indirect immunofluorescence assay**

For the immunofluorescence assays, Hela cells were fixed with 4% paraformaldehyde. Then after 30 mins, the cells were incubated with the rabbit polyclonal antibody diluted to 1:400, anti-Cu/Zn SOD Mab (ProMab) diluted to 1:200 as a positive control, and non-immuned serum diluted to 1:400 as a negative control, respectively. This was followed by incubation with the FITC-labeled anti-mouse IgG antibody 1:100 (Sigma-Aldrich) or anti-rabbit IgG antibody 1:200 (Sigma-Aldrich) corresponding to the primary antibody. The cells were examined using a laser confocal microscope.

## **Indirect competitive ELISA**

The titer of the antibody was tested by an indirect competitive ELISA method as described below. The microplates were coated with the coating antigen, OsSODCP-his recombinant protein at 10ng/well, and incubated at 37°C for 2 h. Plates were washed three times, blocked with 250 µL/well of blocking buffer, and incubated at 4°C overnight. Plates were subsequently washed three times.

The sensitivity of antibodies was determined by the same method as described above, except that different concentrations (0, 0.06, 0.6, 6, 60, 600, 6000, 60000 ng/mL) of antigen (50µL/well) were mixed with the antibodies (dilution 1:4000, 1:8000, 1:16000, 1:32000, 1:64000, 50 µL/well) that was then added to the plates coated with the coating antigen at 10ng/well concentration. These were incubated for 1 h at room temperature. After washing, goat anti-rabbit IgG-AP (1:3000, 100 µL/well) was added and incubated for 30 min at room temperature. Plates were washed three times and PNPP substrate solution was added. After that, the plates were incubated for 15 min at room temperature. The color development was inhibited by adding stop solution (100 µL/well) and the absorbance at 405 nm was measured. Absorbance values were corrected by a blank reading. The antibody titer was defined as the reciprocal of the dilution that resulted in an absorbance value twice that of the blank value. According to the results, the optimal dilution of competitive antibody is inferred.

The specificity of the antibody in optimal dilution was evaluated by measuring inhibition curves using four functionally or structurally similar analogues as competitors, including OsSODC1-his, OsSODC2-his, OsSODCS-his (coding from other 3 Cu/Zn SOD genes in rice), and a non-related-hisprotein as a negative control. The specificity was expressed as the cross-reactivity of each compound.

Data analysis was performed by GraphPad Prism v.7 (GraphPad Software Inc.). The results of the ELISA are indicated as the mean  $\pm$  SD of three replicates. The concentration standard curve of OsSODCP-his protein was calculated by the linear regression analysis.

## Statistical Analyses

Statistical analyses were performed using Prism 7.0 (GraphPad, San Diego, CA, USA).

## Abbreviations

AA: amino acid

AMP1: amplitude of circadian rhythm 1

AP: alkaline phosphatase

ATG: autophagy-related

BCA: bicinchoninic acid

d: days

E.coli.: Escherichia coli

EDTA: Ethylene Diamine Tetraacetic Acid

ELISA: enzyme linked immunosorbent assay

FPKM: fragments per thousand reads mapped

h: hours

HRP: Horseradish Peroxidase

IB: Immunoblotting

ic-ELISA: indirect competitive enzyme-linked immunosorbant assay

IF: Immunofluorescence

IgG: immunoglobulin G

IP: Immunoprecipitation

kDa: Kilodaltons

Kg: kilogram

mg: milligram

mins: minutes

miRNA: micro-Ribonucleic Acid

mL: milliliter

mM: millimolar per 1 liter

ng: nanogram

N22: Nagina 22

OD: Optical Density

O.s.: Oryza sativa

Pab: polyclonal antibody

PMSF: Phenylmethanesulfonyl fluoride

PNPP: Disodium 4-nitrophenylphosphate

R<sup>2</sup>: Determination coefficient

RNA: Ribonucleic Acid

ROS: reactive oxygen species

SD: Standard Deviation

SDS-PAGE: sodium dodecylsulphate polyacrylamide gel electrophoresis

SOD: superoxide dismutase

STTM: short tandem target mimic

Tris: tris(hydroxymethyl)aminomethane

μL: microliter

WB: Western Blot

WT: wild type

## **Declarations**

## **Availability of Data and Materials**

The datasets supporting the conclusions of this article are included with in the article and its additional files.

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## **Authors' Contributions**

D Xiong, and QM Wang designed the experiment and wrote the manuscript. D Xiong, F Wang, and YH Luo conducted the experiments and performed data analysis. B Luo, CW Liu and T Zhou participated in material development, sample preparation and data analysis. LQ Rao, C Wu, WC Li, and W Hartley corrected the manuscript. All authors read and approved the final manuscript.

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## **Availability of Date and Materials**

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

## **Ethics Approval and Consent to Participate**

The animal experiment scheme was approved by the Animal Ethics Committee of the ProMab Biotechnology Inc. (Changsha, China & CA, USA) in accordance with the “Guide for the Care and Use of Laboratory Animals” (National Research Council 2011).

# Consent for Publication

Not applicable.

## Competing Interests

The authors declare that they have no competing interests.

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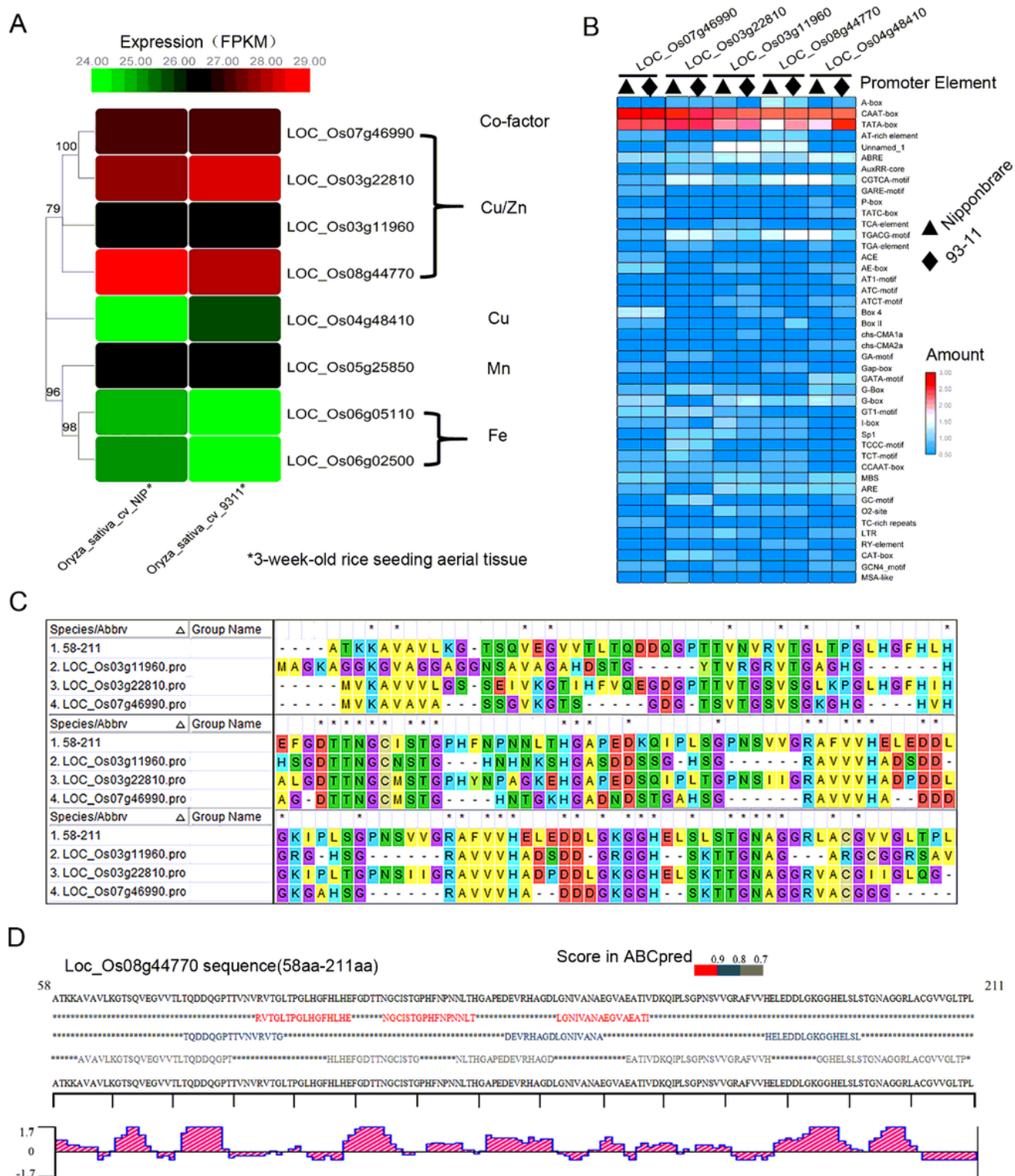
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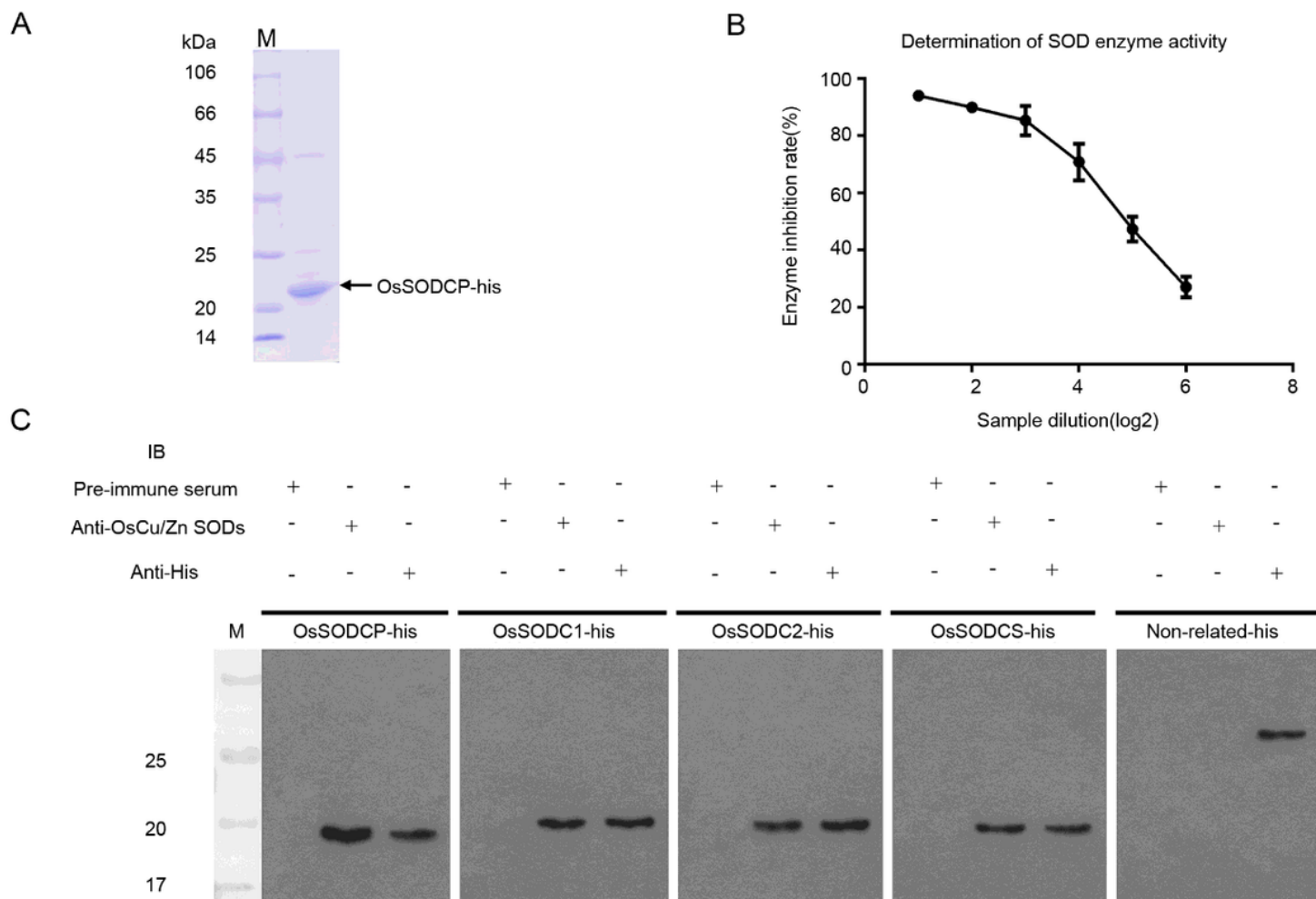
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## Figures



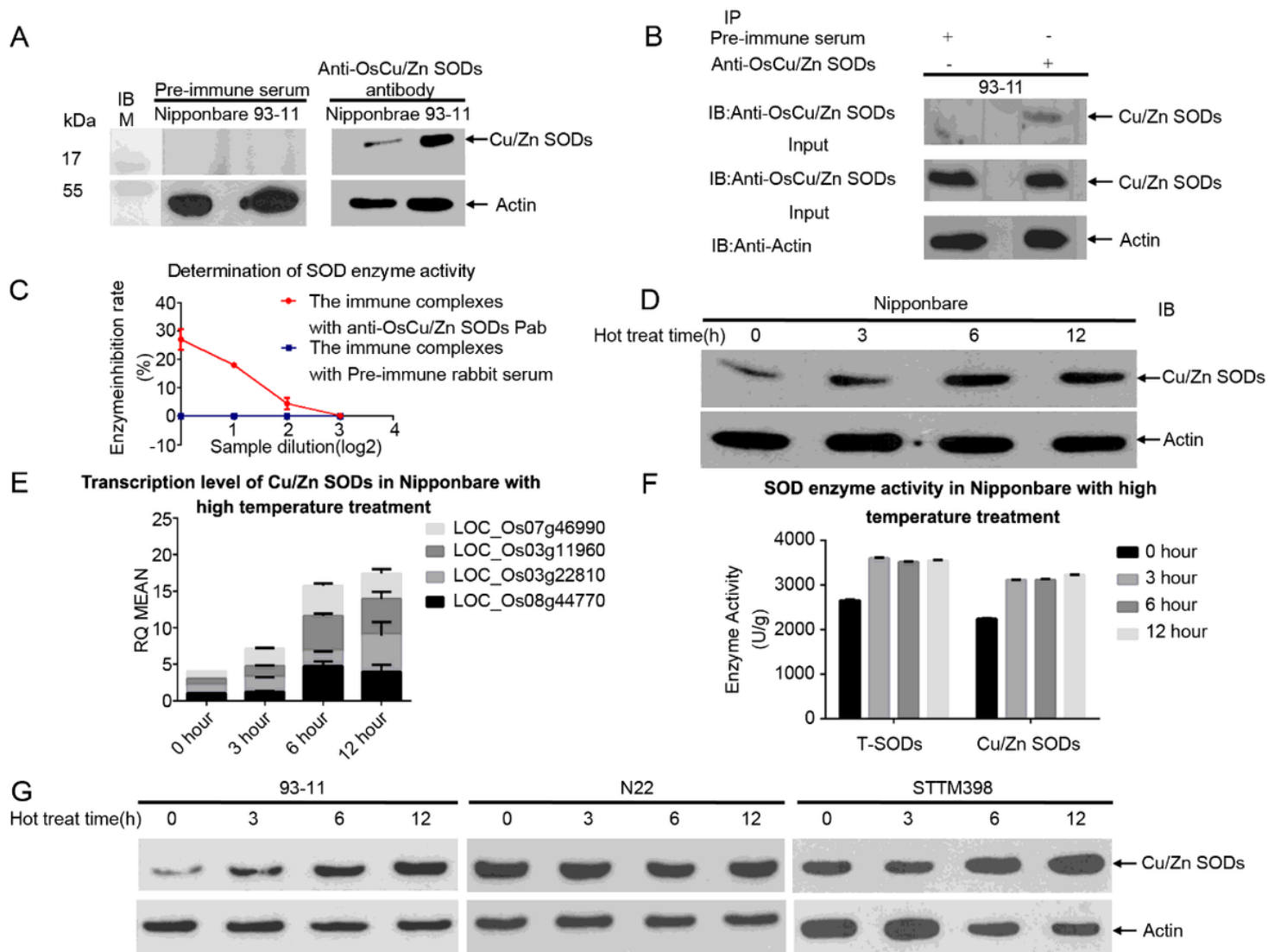
**Figure 1**

Sequence Analysis and B-Cell Epitope Prediction of the Cu/Zn SODs in Rice



**Figure 2**

Characterization of the rabbit polyclonal antibody to OsCu/Zn SODs



**Figure 3**

Rabbit polyclonal antibody binds cell lysates from both in japonica rice and indica rice with SOD activity

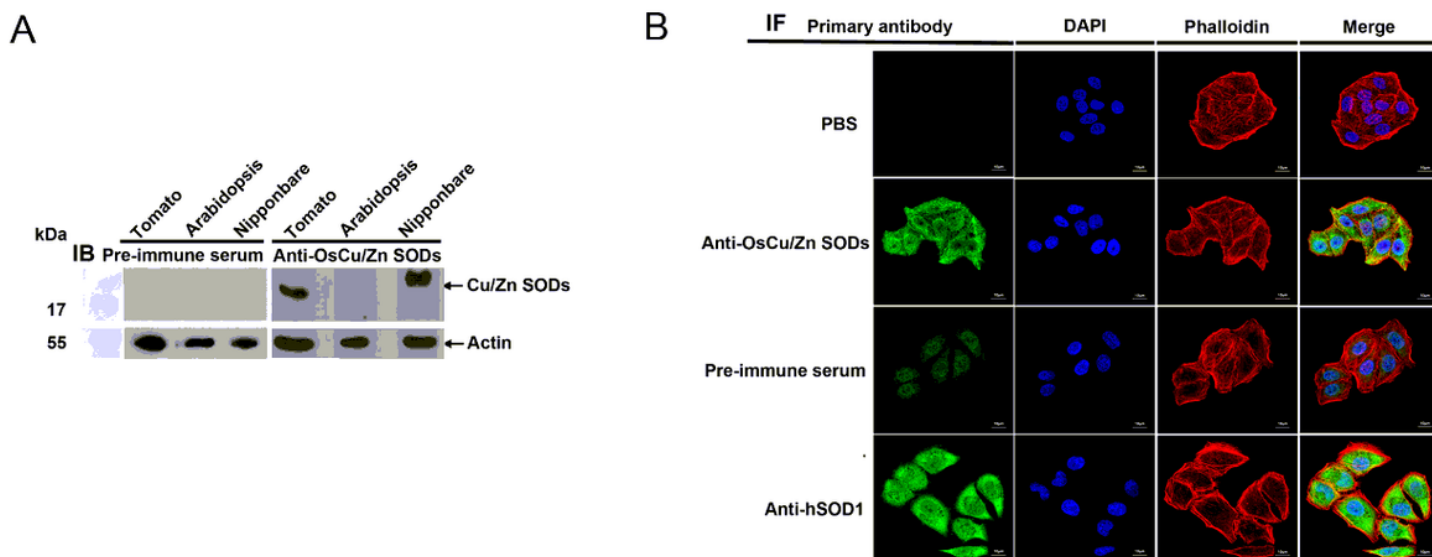


Figure 4

Rabbit anti-OsCu/Zn SOD is cross reactive with SOD from tomato and human cells

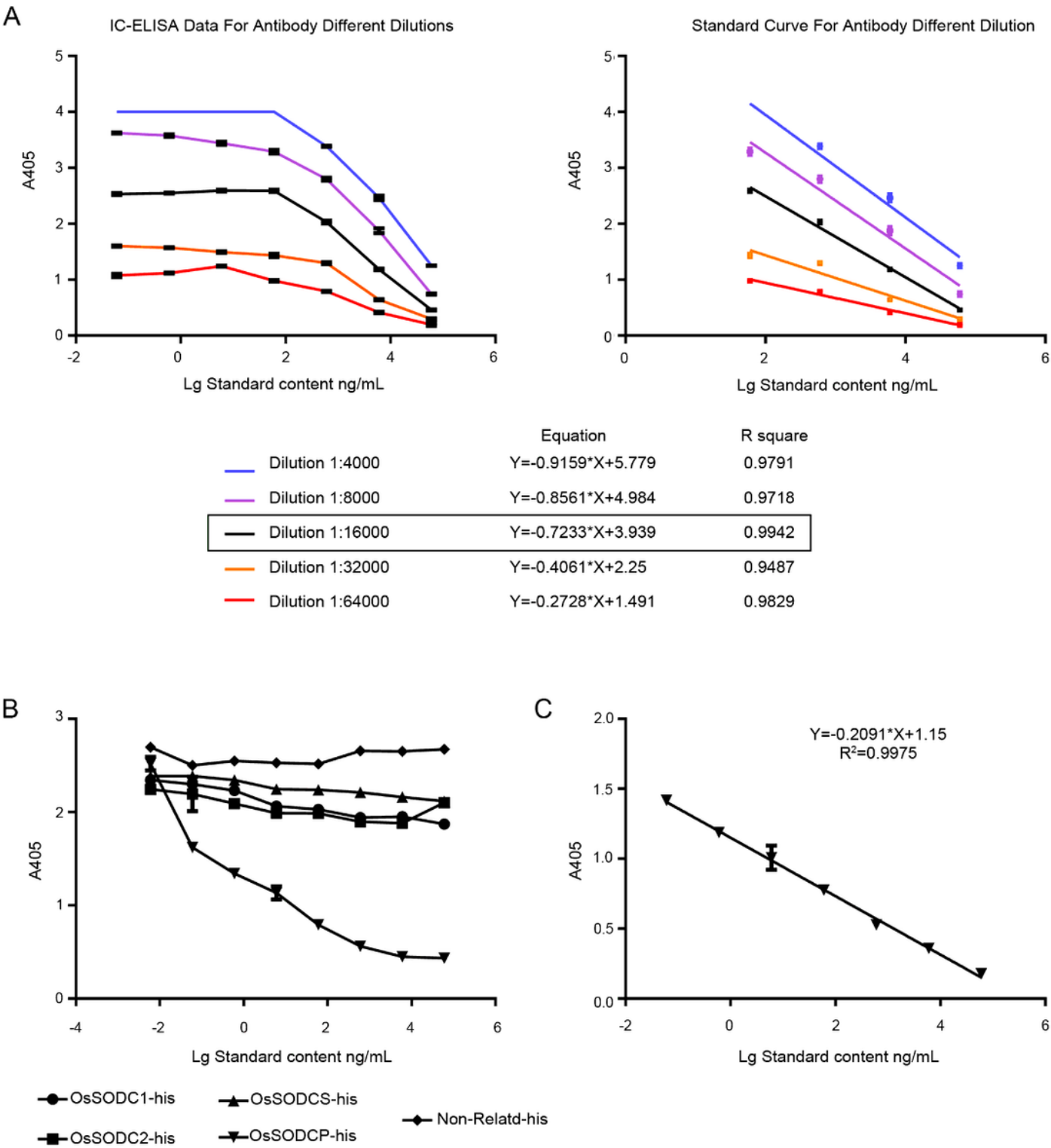


Figure 5

Quantitative analysis optimization of chloroplast Cu/Zn SOD in rice by indirect competitive ELISA