Bioinformatics and Activity Analysis of Antioxidant Enzymes in Wheat Seedlings under Salt Stress and Their Malondialdehyde Content Changes

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Abstract [**Objectives**] This study was conducted to improve crop yield and select excellent wheat varieties. [**Methods**] Wheat seedlings were treated with different concentrations of NaCl solution, and the activities of superoxide dismutase (SOD) and peroxidase (POD) and the content changes of malondialdehyde (MDA) in the leaves of seedlings were determined. A control group (distilled water) and three treatment groups (NaCl concentrations of 1, 2 and 3 mmol/L) were set up. When the wheat seedlings grew to two leaves and one heart, they should be treated with different concentrations of NaCl solution (the wheat seedlings grew uniformly, and 20 ml of each NaCl concentration was used for treatment of wheat). When the wheat seedlings grew to four leaves and one heart under stress, samples were taken separately, once every 2 d, for three times, with 5 g of leaves each time. The SOD and POD activities and MDA content of seedlings in the control group and treatment groups were determined, and related protein sequences were analyzed by bioinformatics, including signal peptide prediction, transmembrane domain prediction, phosphorylation prediction and protein structure prediction. [**Results**] Under NaCl stress, the growth rates of seedling length and root length of wheat decreased obviously, and SOD and POD in leaves decreased, while the MDA content in leaves after treatment increased compared with the control group. SOD had no signal peptide, while POD had signal peptides and a transmembrane region. SOD and POD were different in terms of secondary and tertiary structures and the number of phosphorylation sites. [**Conclusions**] These results lays a solid theoretical foundation and application prospect for the study on salt tolerance mechanism of wheat seedlings in the later stage.

Key words Wheat; Salt stress; SOD activity; POD activity; MDA content; bioinformation

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Wheat is an important food crop, that feeds more than one third of the world's population, and its yield is directly related to world food security[1]. Soil salinization is an important external factor affecting the decline of wheat yield, and China is the country with the largest saline-alkali land area in the world[2-3]. High salt concentration will increase the accumulation of reactive oxygen species (ROS) in wheat cells, damage the protein in leaves and decrease metabolic activity, and it is thus one of the important factors preventing the growth of seedlings. In order to avoid excessive accumulation of reactive oxygen species under salt stress, plants have formed a set of defense mechanisms to remove, neutralize and capture reactive oxygen species. Such defense mechanism consists of antioxidant enzymes and non-enzymatic antioxidants. The main antioxidant enzymes for removing reactive oxygen species include superoxide dismutase (SOD) and peroxidase (POD)[4-5]. Therefore, the important way to solve the problem of land salinization is to improve the ability of crops to resist adversity. Therefore, in the early stage of this study, seedlings of Zhoumai 28 were treated with different concentrations of NaCl solution to observe the seedling length and root length. The activities of superoxide dismutase (SOD) and peroxidase (POD) and malondialdehyde (MDA) content in the leaves of seedlings were measured in the middle stage, and signal peptide prediction, transmembrane domain prediction, phosphorylation prediction and protein structure prediction of protein sequences related to SOD and POD were analyzed in the later stage by bioinformatics. These results provide experimental evidence for improving crop yield and selecting excellent wheat varieties.

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Materials and Methods

Culture of experimental materials

The seeds of wheat variety Zhoumai 28 with uniform size were carefully selected and disinfected for 5 min using a $HgCl_2$ solution with a mass concentration of 1.0 g/L. The treated seeds were then washed with running water for many times. Next, the wheat seeds were soaked in distilled water at 25 ℃ for 12 h. After the seeds showed white buds, they were placed in a Petri dish having a diameter of 18 cm with two layers of filter paper at the bottom, and cultured to seedlings with two leaves and one heart for experimental treatment.

Experimental methods

Salt stress Group setting: Three experiment groups corresponding to three NaCl concentration gradients (1, 2, 3 mmol/L) were set up, and distilled water was used as a control group. Each group were set with three replicates, and the culture solution was replaced once every 5 h to ensure that the salt concentration was unchanged. Samples were taken on days 2, 4 and 6, respectively.

Determination methods

Statistics of leaf length and root length After salt stress

treatment, wheat seedlings were washed clean, and the seedling length and longest root length were measured for three times repeatedly .

SOD determination Test tubes of the same size were taken, and 20 μl of enzyme solution (which needed to be diluted by 10 times) was transferred to each tube, which was then added with 3 ml of SOD reaction solution and illuminated at 4 000 Lux for 30 min (the light conditions were the same). Meanwhile, seven test tubes were taken, including four as determination samples, one as blank (no enzyme solution, using buffer solution instead), and one added with enzyme solution but free of illumination treatment. The four sample tubes and one blank control were illuminated at 4 000 Lux for 30 min, and then preserved in dark place. After zero setting, absorbance was determined at a wavelength of 560 nm. The control tube free of illumination treatment was used as reference, and three replicates were set.

$SOD_{activity}$ (absorbance/g Fw) = $(Ack - Ae) \times (W \times 0.5 \times Ack)$

Unit: Photoreduction of 50% of NBT was taken as the unit.

POD determination POD activity was determined according to the method of Hernandez *et al.* [6], with slight modifications. In specific, 20 μl of enzyme solution and 3 ml of reaction solution were added in a cuvette (the reaction was fast, so the solutions should be added next to the spectrophotometer), and determined at 470 nm for three times, once every one minute. The enzyme activity was the change of absorbance per minute, and three replicates were set.

$$POD_{activity} = \triangle A_{470} \times V/Va/W = \triangle A_{470} \times 5/0.02/0.5 = \triangle A_{470} \times 500$$

MDA determination According to the method of Yu *et al.* [7], 1 ml of enzyme solution and 2 ml of 0.6% TBA mixed solution were transferred into a centrifuge tube, which was sealed and heated in boiling water for 15 min. Next, the tube was quickly cooled and centrifuged, and the supernatant was collected and determined at three wavelengths of 600, 532 and 450 nm.

$$MDA_{content} = (6.45 \times (D_{532} - D_{600}) - 0.56D_{450}) \times 0.03/W$$

Analysis of SOD and POD bioinformation

After entering the Protein database of NCBI, SOD and POD of wheat were input to get the sequences, and sequence analysis were then performed. The database and software information are shown in Table 1.

Table 1 Database and software information

Item	Function	Site
Uniprot	Searching protein sequence	https://www.uniprot.org/
SignalP	Predicting signal peptides	https://services.healthtech.dtu.dk/services/SignalP-6.0/
TMHMM	Predicting transmembrane structure in protein	https://services.healthtech.dtu.dk/services/TMHMM-2.0/
SOPMA	Predicting secondary structures	https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl? page=/NPSA/npsa_sopma.html
Swiss-model	Predicting tertiary structures	https://swissmodel.expasy.org/
NetPhos3.1	Predicting phosphorylation sites in protein	https://services.healthtech.dtu.dk/services/NetPhos-3.1/

Data processing

Wheat enzyme activity data were recorded by Excel, and each treatment was repeated for three times. SPSS 26.0 and Design Expert 8.0.6 were employed to describe the test data and check the significance of difference.

Results and Analysis

Effects of NaCl stress on leaf length and root length of wheat seedlings

With the concentration of NaCl increasing, the growth rates of seedling length and root length of wheat showed an obvious downward trend with significant differences ($P < 0.05$). In the CK group, the leaves of wheat were fresh and slender, and with the increase of NaCl concentration, the leaves of wheat became wide and yellow (Fig. 3). As shown in Fig. 1, the seedling length in the CK group increased by 5.96% from 2 to 4 d and by 8.61% from 4 to 6 d. In the 1 mol/L group, it increased by 9.09% from 2 to 4 d, and by 7.35% from 4 to 6 d. In the 2 mol/L group, it increased by 13.08% from 2 to 4 d and by 8.16% from 4 to 6 d. In the 3 mol/L group, it increased by 4.96% from 2 to 4 d and by 0.79% from 4 to 6 d. As shown in Fig. 2, the root length in the CK group increased by 15.94% from 2 to 4 d, and by 27.5% from 4 to 6 d. In the 1 mol/L group, it increased by 2.86% from 2 to 4 d, and by 1.59% from 4 to 6 d. In the 2 mol/L group, it increased by 1.31% from 2 to 4 d, and by 2.59% from 4 to 6 d. In the 3 mol/L group, it increased by 5.47% from 2 to 4 d and by 5.66% from 4 to 6 d. Therefore, the growth rates of seedling length and root length of wheat under NaCl stress showed an obvious downward trend.

Effects of NaCl stress on SOD and POD activity in leaves of wheat seedlings

Different concentrations of NaCl significantly reduced the SOD activity of wheat seedlings, and the SOD activity decreased by 13.98%, 27.41% and 38.57% respectively in 2 d compared with the control. It decreased by 28.72%, 35.15% and 47.86% respectively in 4 d compared with the control. Further, it decreased by 35.45%, 42.95% and 53.77% respectively in 6 d compared with the control. Therefore, with the concentration of salt increasing, the SOD activity of wheat decreased, which was because the balance of produced normal and scavenged superoxide radicals in wheat cells under salt stress was damaged, and the SOD activity decreased, which caused harm to the growth of the body[8]. The POD activity showed the same trend with SOD activity of wheat seedlings. In specificity, the POD activity decreased by 10.96%, 19.28% and 32.14% respectively compared with the control in 2 d. It decreased by 14.16%, 22.61% and 35.73%

respectively in 4 d. To day 6, The POD activity decreased by 10.72%, 21.58% and 35.99% respectively.

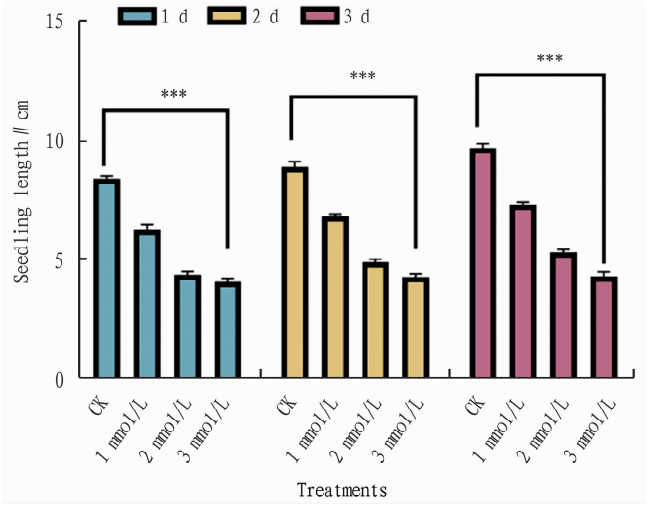


Fig. 1 Seedling length of wheat seedlings under salt stress

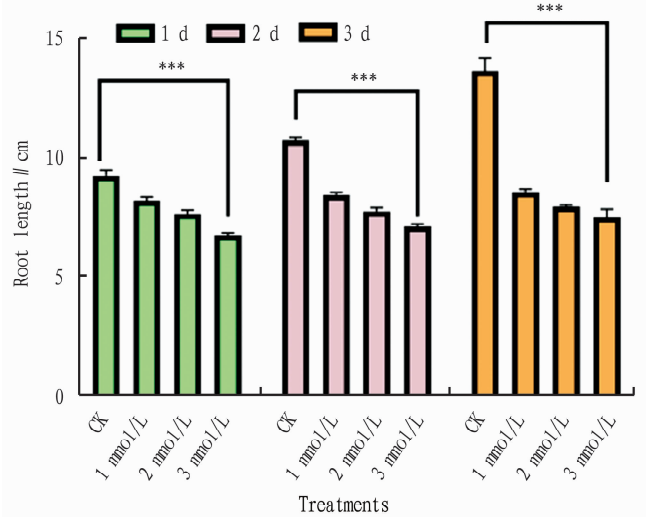


Fig. 2 Longest root length of wheat under salt stress

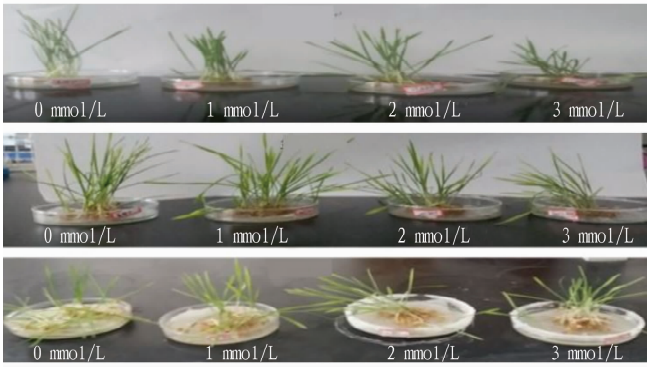


Fig. 3 Wheat growth under salt stress

Effects of NaCl stress on MDA content in leaves of wheat seedlings

When plants are in adversity or organ senescence, changes in the degree of membrane lipid peroxidation and the strength of plant response to adversity conditions often occur[10]. It can be seen from Fig. 6 that with the concentration of NaCl increasing, the degree of membrane lipid peroxidation in young leaves of wheat deepened, showing an upward trend of malondialdehyde content. The content of MDA increased by 62.2%, 103.41% and 174.28% respectively in 2 d. It increased by 58.54%, 69.79% and 130.42% respectively in 4 d. The content of MDA decreased by 74.81%, 72.88% and 125.19% respectively in 6 d.

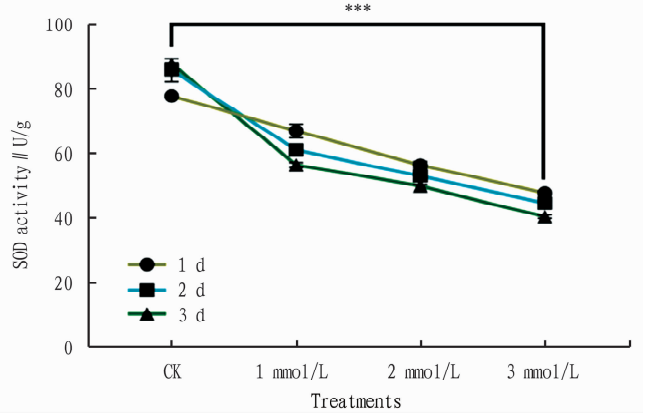


Fig. 4 Changes of SOD activity in wheat seedlings under different NaCl concentrations

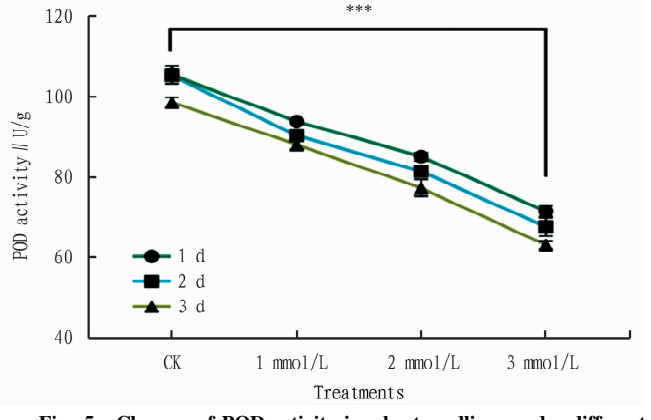


Fig. 5 Changes of POD activity in wheat seedlings under different NaCl concentrations

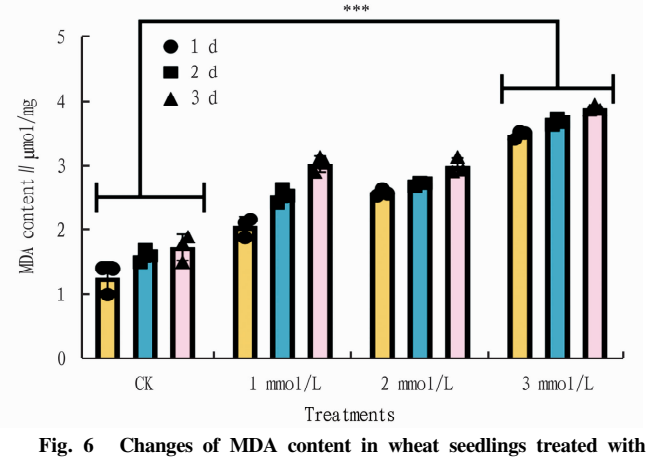
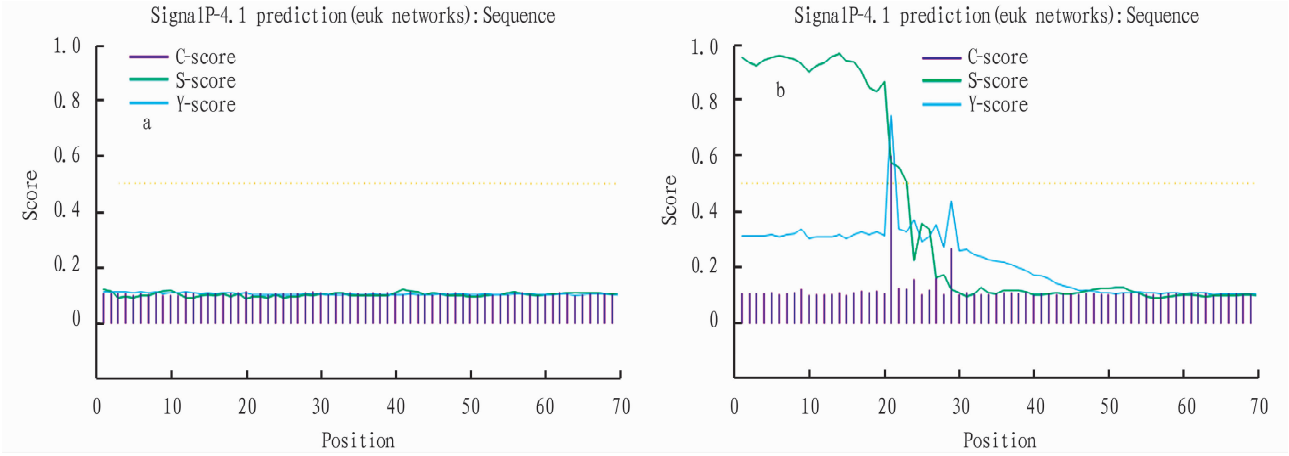


Fig. 6 Changes of MDA content in wheat seedlings treated with different concentrations of NaCl

Prediction on signal peptides and transmembrane domains of SOD and POD in wheat

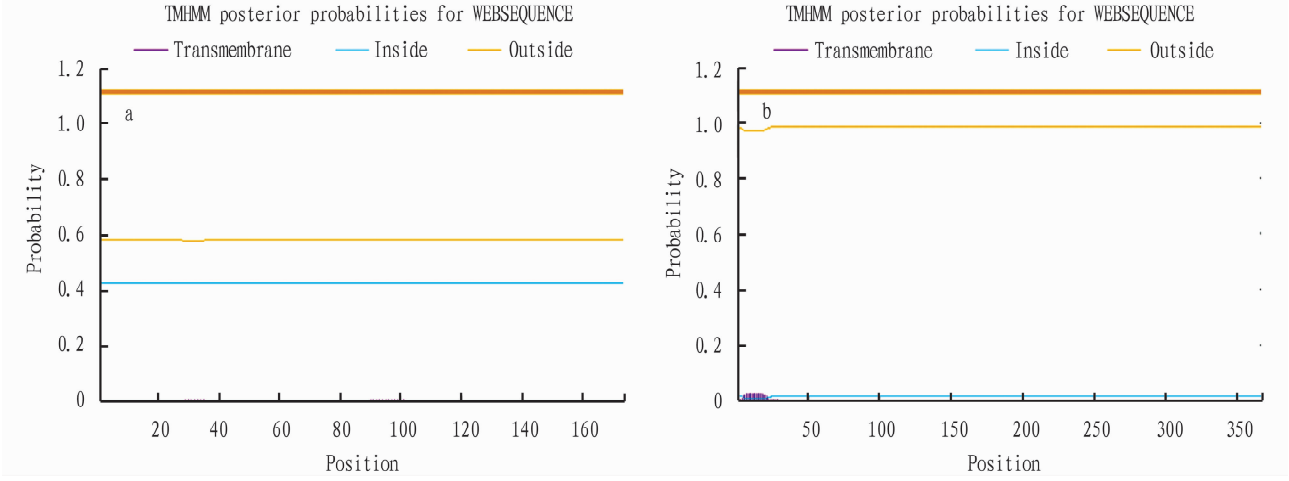
The amino acid sequence information of SOD (ABN50913.1) and POD (XP_044344719.1) was obtained from NCBI database, and signal peptide prediction, protein phosphorylation analysis, transmembrane domain structure prediction and hydrophobicity analysis were carried out. Signal peptide analysis with the help of SIGNAP software showed that the C value of BN50913.1 protein in Fig. 7a was low, and BN50913.1 protein had no nuclear output signal. In Fig. 7b, the C value of XP_044344719.1 protein was higher than 0.5 at 20, and XP_044344719.1 protein had nuclear output signals.

The transmembrane domain analysis of SOD and POD in wheat was carried out by using TMHMM. As shown in Fig. 8a, the related gene had no transmembrane domain, so there was no transmembrane domain, indicating that this SOD protein was not a membrane receptor protein related to cell signal transduction. Fig. 8b shows that the related protein XP_044344719.1 had a semi-transmembrane domain, so there was a semi-transmembrane domain, indicating that the POD protein was a membrane receptor protein related to cell signal transduction.



a. Signal peptide cleavage site analysis for ABN50913.1 protein; b. signal peptide cleavage site analysis for XP_044344719.1 protein.

Fig. 7 Signal peptide cleavage site analysis for proteins



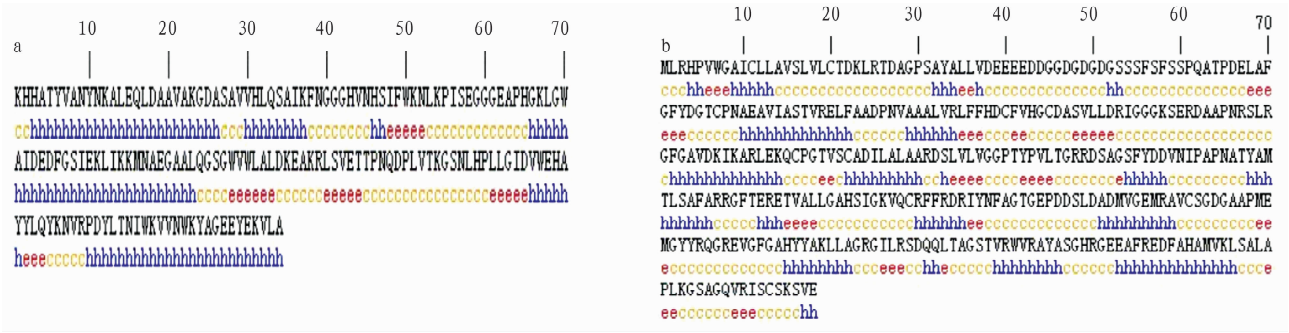
a. Transmembrane domain of ABN50913.1; b. transmembrane domain of XP_044344719.1.

Fig. 8 Prediction results of transmembrane domains

Prediction and analysis on secondary structures and tertiary structures of SOD and POD in wheat

As can be seen from Fig. 9, SOD (ABN50913.1) is composed of β turns (0%), extended strands (13.79%), α helixes (53.45%) and random coils (32.76%). POD (XP_044344719.1) consists of β turns (0%), extended strands (13.86%), α helixes (32.88%) and random coils (53.26%). The results showed that most amino acids of these two enzymes formed α helixes and random coils, which was beneficial to the formation of spherical protein and enzyme active center.

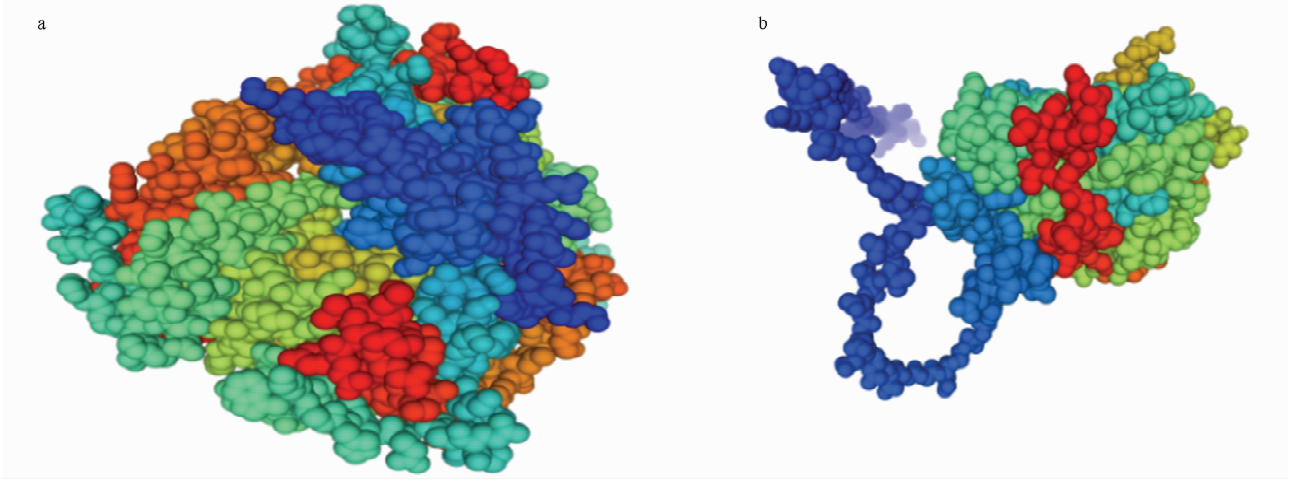
The tertiary structures of SOD (ABN50913.1) and POD (XP_044344719.1) were predicted using Swiss model. As shown in Fig. 10, the total sum of various residues was estimated to be 0.93 and 0.84 for SOD (ABN50913.1) and POD (XP_044344719.1), respectively, based on the standardized weight of the target sequence length. Therefore, the Cα dihedral angles formed by all amino acid residues were in a reasonable acceptable state, which showed that these amino acid residues were all in acceptable regions, and the prediction models were accurate, reliable and of high quality.



h. α helix; e. extended strand; t. β turn; and c. random coil.

a. Secondary structures of ABN50913.1; b. secondary structures of XP_044344719.1.

Fig. 9 Prediction results of secondary structures

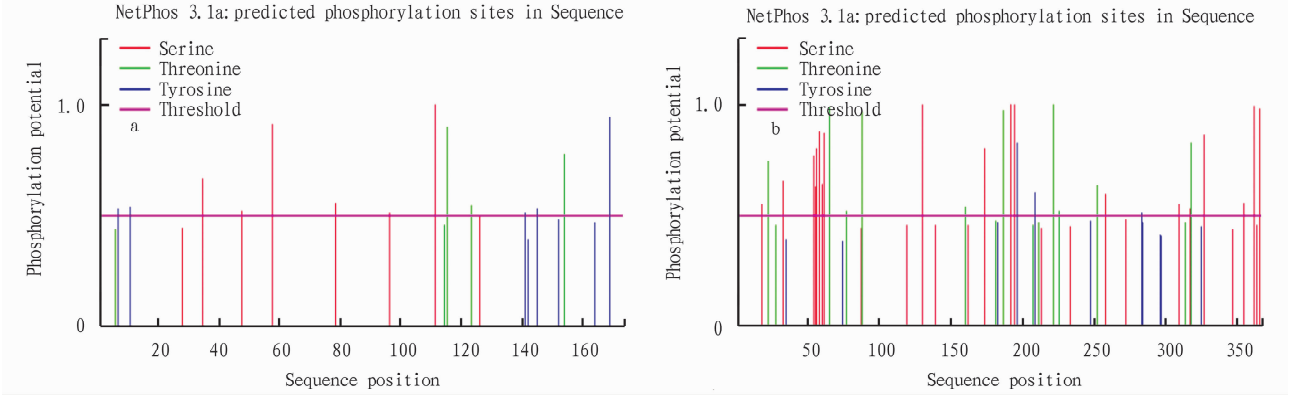


(a) Tertiary structures of ABN50913.1; (b) tertiary structures of XP_044344719.1.

Fig. 10 Prediction results of tertiary structures

Prediction and analysis on phosphorylation modification sites of SOD and POD enzymes in wheat

According to Fig. 11, SOD (ABN50913.1) had 21 potential phosphorylation sites, including eight serine (ser), five threonine (thr) and eight tyrosine (Tyr). It is helpful to further exploration on the phosphorylation mechanism of wheat protease.



(a) Phosphorylation sites of ABN50913.1; (b) phosphorylation sites of ABN50913.1.

Fig. 11 Prediction results of phosphorylation sites

Conclusions and Discussion

Reactive oxygen species (ROS) in normal state have no toxic effect on wheat. Once plants are affected by salt stress, the cell membrane of plants will increase their permeability, metabolic disorder and free radical accumulation in the body[11]. There are corresponding free radical scavenging protection systems and non-enzymatic protection systems in plant cells[12]. The important

(Continued on page 8)

each other. Because asparagus is dioecious, the tissue culture and propagation of parents is also the key technique of hybrid seed production which ensures the breeding of hybrids. In a word, asparagus industry needs new all-male varieties with high quality, high yield and good disease resistance, and systematic cross breeding is expected to realize this ideal.

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components of enzyme protection system are SOD and POD. The coordination of SOD and POD can keep active oxygen free radicals in plants in a stable state, so that wheat can carry out normal circular metabolism. On this basis, in the early stage of this study, the longest root length and seedling length of Zhoumai 28 seedlings were used as survey indicators for measuring the effects of salt stress on the characters of Zhoumai 28. The activities of SOD and POD in Zhoumai 28 were determined in the middle stage, and basic physical and chemical properties of POD and SOD in wheat were studied by bioinformatics analysis in the later stage. The results showed that the SOD activity of Zhoumai 28 decreased with the concentration of NaCl increasing, indicating that the balance of produced normal and scavenged superoxide radicals in wheat cells was damaged under salt stress, so the SOD activity decreased, and the superoxide radicals that could not be effectively eliminated caused harm to the growth of the body[13]. The activity of POD was consistent with SOD. The MDA content increased significantly, which led to a significant impact on seedling growth, which was reflected in a significant decline in root length and seedling height, resulting in salt damage, and finally a decline in yield[14]. It shows that membrane lipid peroxidation often occurs when plants are in adversity or organ aging[15]. There were differences between SOD (ABN50913.1) and POD (XP_044344719.1) of Zhoumain in terms of signal peptide, structure and transmembrane domain.

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