DOI: 10.1007/s10535-014-0442-5 BIOLOGIA PLANTARUM 58 (4): 751-757, 2014

Zinc improves salt tolerance by increasing reactive oxygen species scavenging and reducing Na^+ accumulation in wheat seedlings

L.H. XU[1,2], W.Y. WANG[1]*, J.J. GUO[2], J. QIN[3], D.Q. SHI[2,4], Y.L. LI[5], and J. XU[1,2]*

College of Life Science and Geography, Qinghai Normal University, Xining 810008, P.R. China[1]
Key Laboratory of Tropical Plant Resource and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun, Mengla, Yunnan 666303, P.R. China[2]
Cereal and Oil Crop Institute, Hebei Academy of Agricultural and Forestry Sciences, Shijiazhuang 050031, P.R. China[3]
College of Life Science, Henan University, Kaifeng 475000, P.R. China[4]
Center for Agricultural Resources Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Huaizhong RD 286, Shijiazhuang 050021, P.R. China[5]

Abstract

Salt decreases the uptake of Zn and other minerals and causes nutritional disorders in plants. Zn is an essential micronutrient for all organisms and it is reasonable to hypothesize that Zn status is essential for maintaining salt tolerance in plants. In this study, the physiological and molecular mechanisms of Zn-based alleviation of salt stress in wheat seedlings were investigated. Our results indicate that sufficient Zn nutrition maintained antioxidative enzyme activities and decreased a reactive oxygen species over-accumulation in wheat seedlings. Our data also reveal that sufficient Zn nutrition improved the expression of Na^+/H^+ antiporter genes, *TaSOS1* and *TaNHX1*, thereby decreasing the Na^+ accumulation and subsequently improving salt tolerance in wheat seedlings.

Additional key words: antioxidative enzyme, ascorbate peroxidase, Na^+/H^+ antiporter, peroxidase, superoxide dismutase, *Triticum aestivum*.

Introduction

Salinity limits the growth and distribution of plant species and is responsible for significant reductions in the yield of many important crops (Ghoulam *et al.* 2002, Guo *et al.* 2009). Increased salinization will result in a 50 % reduction in arable land by the middle of the 21st century (Wang *et al.* 2003, Mahajan and Tuteja 2005, Chen *et al.* 2011). With the goal of increasing the productivity of marginal soils, the development and cultivation of salt-tolerant crops has received more attention in recent years.

There are four factors that constrain plant growth under salt stress: osmotic stress, ionic toxicity, oxidative stress, and nutrient imbalance (Grattan and Grieve 1999, Chen *et al.* 2011, Mansour 2013). Plants use different strategies to avoid salt injury, from molecular and cellular mechanisms to those that operate at the morphological level. Plants maintain the low content of intracellular Na^+ by regulating ion uptake. Intracellular Na^+ can be transported back into the cell wall, or it can be transported

Submitted 31 July 2013, *last revision* 15 January 2014, *accepted* 16 January 2014.
Abbreviations: APX - ascorbate peroxidase; CAT - catalase; CM-H2DCFDA - 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetylester; DAB - 3-diaminobenzidine; DCFH-DA - 2,7-dichlorofluorescein diacetate; DAPI - 4,6-diamidino-2-phenylindole; NBT - nitroblue tetrazolium; PCD - programmed cell death; PI - propidium iodide; PM - plasma membrane; POD - peroxidase; RT-qPCR - reverse transcriptase quantitative polymerase chain reaction; ROS - reactive oxygen species; SOD - superoxide dismutase.
Acknowledgments: This work was supported by the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-EW-Q-25), the China National Natural Sciences Foundation (31170228, 31272239), the Key Project of State Key Laboratory of Desert and Oasis Ecology, Xinjiang Institute of Ecology and Geography of Chinese Academy of Sciences, and the Hebei Province National Natural Sciences Foundation for Distinguished Young Scientists (C2013503042). L.H. Xu, W.Y. Wang, and J.J. Guo contributed equally to this work.
* Corresponding authors; fax: (+86) 311 85871745, e-mails: xujin@xtbg.ac.cn, wangwy0106@aliyun.com

into the vacuole. Efflux occurs through Na^+/H^+ antiporters on the plasma membrane, such as SOS1 (Zhu 2003). Compartmentalization occurs through vacuolar Na^+/H^+ antiporters, such as NHX1 (Blumwald *et al.* 2000, Upadhyay *et al.* 2012).

Salt stress induces the over-accumulation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radical, resulting in metabolic disturbance, lipid peroxidation, chlorophyll breakdown, and other negative effects (Dong *et al.* 2001, Tsai *et al.* 2004). To avoid the deleterious effects of ROS, plant cells possess efficient antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX). These enzymes have been observed to specifically counteract oxidative damage in plants subjected to salinity (Tsai *et al.* 2004, Chen *et al.* 2011). SOD is the primary scavenger involved in the detoxification of ROS in plants, and its function is to convert superoxide to H_2O_2 (Asada and Kiso 1973). According to a metal that binds in the active site, SODs can be separated into Cu/Zn-SOD, Mn-SOD, or Fe-SOD isoforms. CAT, POD, and APX play important roles in detoxifying H_2O_2 by catalyzing the reduction of H_2O_2 to H_2O (Foyer 1996).

Salt decreases water uptake and mineral uptake, causing nutritional disorders. Furthermore, a high pH in saline soil reduces mineral solubility in most cases. Therefore, the maintenance of a stable content of intracellular mineral ions is critical for plant adaptation to salt stress (Xu *et al.* 2010, Chen *et al.* 2011). Zn is an essential micronutrient for all organisms and serves as cofactor for more than 300 enzymes (Gonzalez-Guerrero *et al.* 2005). Therefore, Zn is closely involved in a wide range of cellular processes, such as free radical defence, electron transport, protein and auxin biosynthesis, cell proliferation, and reproduction. Zn deficiency is one of the most widespread limiting factors in crop production, especially in saline soil. Zinc deficiency reduces the activity of Cu/Zn SOD, thereby increasing oxidative damage in plants. Therefore, it is reasonable to hypothesize that Zn status is essential for maintaining salt tolerance in plants. However, a detailed description of the role of Zn in salt tolerance in plants is lacking.

To elucidate the physiological and molecular mechanisms of the effect of Zn on salt tolerance in wheat, we chose a Zn-accumulating winter wheat cultivar Jingdong8 which has been cultivated in the Hebei province of China for over ten years and we chose the winter wheat cultivar Shi4185 as control. We wanted to support the model in which Zn contributes to ROS scavenging in response to salt stress and to underline the role of Zn in Na^+ accumulation in plants.

Materials and methods

Plants and treatments: Seeds of wheat (*Triticum aestivum* L.) cvs. *Shi4185* and *Jingdong8* were treated with 70 % (v/v) ethanol for 30 s, rinsed five times with sterile distilled water, and subsequently germinated on double-layer filter paper wetted with double distilled H_2O. Cultures were maintained at a temperature of 22 - 25 °C, a 16-h photoperiod, an irradiance of 800 μmol m^{-2} s^{-1}, and a relative humidity of 50 %. Seven-day-old seedlings were transferred to 5-dm^3 plastic vessels (16 plantlets per vessel) with a half-strength Hoagland's solution (containing 2 μM $ZnSO_4$, pH 6.5, and Fe supplied as Fe-EDTA) (Hoagland and Arnon 1950) supplemented with 50 mM NaCl and grown under the above mentioned conditions for 20 d. To examine the effects of Zn on salt tolerance of wheat seedlings, 2 μM $ZnSO_4$ was added to a half-strength Hoagland's solution with 50 mM NaCl. The culture solution was replaced every 6 d. After a stress treatment, pictures of the seedlings were digitized using a scanner (*Epson Perfection 1670*, *Seiko Epson*, Suwa, Nagano, Japan). The growth of the seedlings was measured using the *ImageJ* software, *v. 1.38* (http://rsbweb. nih.gov/ij/download.html).

Determination of ROS: After 10 d of the treatments, roots were specifically stained for H_2O_2 and O_2^-. A fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA), 3-diaminobenzidine (DAB), and nitroblue tetrazolium (NBT) have been successfully employed for H_2O_2 and O_2^- detections in plants (Ramel *et al.* 2009, Xu *et al.* 2010). For the H_2O_2 detection, the roots were washed and stained with 25 μM 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetylester (CM-H2DCFDA; *Molecular Probes*, Invitrogen, USA) for 30 min (Xu *et al.* 2009). The roots were then washed thoroughly and viewed under a *Leica* (Germany) laser scanning confocal microscope. All images were scanned under excitation at 488 nm and emission at 525 nm. For localizing the H_2O_2 produced, the treated roots were immersed in a solution of 1 mg cm^{-3} 3-diaminobenzidine (DAB)-HCl (pH 3.8) for 5 h and cleared by boiling in alcohol (95 %, v/v) for 5 min. The content of O_2^- was monitored by staining in a solution of 2 mM nitroblue tetrazolium (NBT) in a 20 mM phosphate buffer (pH 6.1) for 20 min. The reaction was stopped by transferring the seedlings into distilled water. Images were obtained using the *Carl Zeiss* (Thornwood, USA) imaging system.

Detection of cell death: To assess cell death in root tips under the salt stress, the roots were immersed in a water solution of 3 μg cm^{-3} propidium iodide (PI) for 7 min. After washing, the samples were examined using an excitation wavelength of 546 nm. For each treatment, at least 10 roots were analyzed using a compound microscope (*Zeiss Axioskop*). For 4,6-diamidino-2-

phenylindole (DAPI) staining, the roots were incubated with 1 μg cm^{-3} DAPI (*Beyotime*, Jioangsu China) in a 1×PBS buffer with 1 % (v/v) *Triton X-100* for 25 min, followed by three washes in a PBS buffer. The samples were viewed microscopically with a UV filter.

Antioxidative enzyme activities: Roots and leaves (0.5 g) of 10 or 20 d treated seedlings were ground in liquid nitrogen to release total protein. The obtained powder was suspended in 3 cm^3 of an homogenization medium containing 50 mM sodium phosphate buffer (pH 7.5), 1 % (m/v) polyvinylpyrrolidone (PVP), and 0.1 mM EDTA. To determine an APX (EC 1.11.1.11) activity, the root powder was suspended in 50 mM Hepes buffer (pH 7.5) containing 1 % (m/v) PVP and 0.5 mM ascorbate. After centrifugation (12 000 *g*, 30 min), the supernatant was used and the activities of SOD (EC 1.15.1.1), POD (EC 1.11.1.7) and APX were analyzed using the methods described by Liu *et al.* (2007) and Xu *et al*. (2010). For the assay of APX activity, a reaction mixture containing 100 mM phosphate buffer (pH 7.5), 0.5 mM ascorbate, and 0.2 mM H_2O_2, equivalent amount of protein from the enzyme source was added and absorbance was recorded at 290 nm (Xu *et al.* 2010). The activity of APX was estimated using a coefficient of absorbance of 2.8 mM^{-1} cm^{-1}. One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction of NBT by 50 %. The POD activity was determined by measuring the increase in the absorbance at 470 nm. The activity of POD was estimated using the coefficient of absorbance of 26.6 mM^{-1} cm^{-1}. One unit of POD activity was defined as the amount of the enzyme required for formation of 1 μmol of tetraguaiacol per minute at room temperature.

Determination of Na^+ content: After 20 d of the stress treatments, the seedlings were harvested and thoroughly rinsed with deionized water and subsequently oven-dried for 72 h. Dried plant tissues were ground to fine powder and allowed to stand overnight in digestion tubes with concentrated nitric acid at room temperature prior to digestion at 80 °C for 1 h and at 120 - 130 °C for 20 h. The cooled digested solutions were diluted with *Millipore*-filtered, deionized water and Na^+ concentration was analyzed by atomic absorption spectrometry (*Shimadzu AA-6300*, Kyoto, Japan).

Reverse transcription quantitative PCR analysis of gene expression: Total RNA was extracted from leaves and roots of the wheat seedlings using a *TRIzol* reagent (*Invitrogen*, CarlsbadUSA). The RNA quality and integrity were checked before the cDNA was synthesized using a *Bio-Rad* (Hercules, CA, USA) *StdSens* analysis kit. Poly(A)$^+$ mRNA was purified from pooled RNA from all tissues using a *PolyA* kit (*Promega*, Madison, WI, USA) according to the manufacturer's instructions. For RT-qPCR, we firstly accurately quantified the RNA concentration using spectrophotometry. Next, cDNA was synthesized from the DNase-treated total RNA (1 μg) using a reverse transcription system kit (*Promega*) and oligo(dT) primers. The cDNA produced was diluted 1:15 with ddH_2O, and 3 mm^3 was used in each RT-qPCR reaction, which was run in a *7500 Real-Time* system (*Applied Biosystems*, USA) using *Platinum® SYBR® Green* qPCR *SuperMix-UDG* (*Invitrogen*, Life Technologies, Carlsbad, CA, USA). The RT-qPCR of each gene was performed with three biological replicates. The relative transcription was determined for each sample and averaged over the six replicates. We performed control reactions using *TaACT* primers (5'-CGTGTT GGATTCTGGTGATGGT-3' and 5'-TCTTCATCA AACAGTCAGTTAGGTCG-3'). We designed the following specific primers: *TaSOS1*: 5'-GTTTTG TATCCACTGTTGCGTCAC-3' and 5'-TAGCACGTT TAACGGACAAAGAGA-3'; *TaNHX1*: 5'-TCTGAG TGGCATTCTAACCGTG-3' and 5'-CCATCCCGACAT AGAGAAAAAGA-3'. All the primer pairs produced only one peak in the DNA melting curves indicating their high specificity.

Statistical analysis: For each treatment, six seedlings were analyzed; all the experiments were repeated at least three times. The results are presented as means ± SD. The statistical analysis was performed using the Duncan's multiple range test ($\alpha = 0.05$).

Results and discussion

In a previous study, we found that salt stress decreases Zn accumulation in wheat seedlings. However, an increased antioxidative capacity increases a Zn content in wheat seedlings under salt stress indicating that the increased accumulation of Zn under salt stress is, at least partially, related to the decreased content of ROS (Chen *et al.* 2011). To further elucidate the correlation between Zn accumulation and salt tolerance in wheat seedlings, we measured wheat seedling growth under salt stress with different Zn supply. The salt stress markedly inhibited the growth of the wheat seedlings. After 20 d exposure to 50 mM NaCl, the root growth and the plantlet height of the wheat cultivar *Shi4185* decreased by 32 and 14 %, respectively, relative to the untreated control. Addition of Zn increased the root growth and the plantlet height relative to the salt treatment alone (Table 1). To further support the positive role of Zn in salt tolerance, we compared Zn-accumulating cv. *Jingdong8* (Tan *et al.* 2013) and normal cv. *Shi4185*. After the salt treatment, the root growth and the plantlet height of the wheat

Table 1. The effects of Zn (2 μM) on plant growth relative to the control, on activities of SOD [U g^{-1}(f.m.)], APX [nmol(ascorbate oxidized) g^{-1}(f.m.) min^{-1}], and POD [U g^{-1}(f.m.)], on Na^+ content [μg g^{-1}(d.m.)], and on relative transcription of *NHX1* and *SOS1* genes in salt-treated (50 mM NaCl) wheat seedlings for 10 or 20 d (control - untreated seedlings). Means ± SD, n = 3. Different letters indicate significant differences at the 5 % level using the Duncan's multiple range test.

Parameters	Organs	Time [d]	Shi4185 control	NaCl	NaCl+Zn	Jingdong8 control	NaCl	NaCl+Zn
Height	shoot	20	1a	0.86±0.05b	0.92±0.04c	1a	0.98±0.03a	0.99±0.03a
Length	root	20	1a	0.68±0.05b	0.80±0.06c	1a	0.89±0.03b	0.97±0.05a
SOD	leaf	10	128.77±5.7a	171.11±6.1b	185.13±4.6c	139.02±6.7a	178.60±31.7b	202.82±9.2c
		20	181.74±8.2a	157.77±6.1b	183.24±7.9a	166.84±0.7a	178.67±4.2b	196.35±4.1c
	root	10	187.12±46.4a	166.86±11.9a	294.22±16.4b	91.80±12.3a	229.19±18.9b	220.44±23.3b
		20	126.21±5.9a	122.26±8.9a	127.25±4.2a	174.72±2.3a	125.66±3.8b	150.24±8.8c
APX	leaf	10	159.17±57.3a	202.50±17.6a	137.97±41.3a	280.83±20.3ab	336.25±67.1b	243.80±41.6a
		20	146.90±12.8a	418.75±12.3b	247.08±104.3c	537.12±171. 4a	525.00±100.1a	158.68±25.9b
	root	10	486.67±34.3a	999.17±35.1b	1450.8±101.9c	211.54±19.5a	1000.8±19.4b	2633.7±5.3c
		20	1136.6±218.5a	1118.3±23. 9a	1431.7±287.3a	1439.2±463.8a	1620.2±856.2a	1860.8±288.8a
POD	leaf	10	14986 ±926.4a	17485 ±357.3b	16321 ±698.2c	21088 ±319.4a	28365 ±220.8b	22446 ±1266.5a
		20	18936 ±1904a	25750 ±3780b	21007 ±1983a	26147 ±810.3a	36648 ±3990b	32115 ±4659b
	root	10	52534 ±91043a	60987 ±2489a	49700 ±5768a	34120 ±756.7a	61695 ±2292b	57971 ±10994b
		20	632178±2883a	72540 ±1037.8b	59695 ±5185a	72230 ±2356a	64715 ±4708ab	61157 ±3024b
Na^+	leaf	20	98.0 ±27a	451.7 ±36b	303.0 ±46.8c	109.0 ±29.65a	267.4 ±31.3b	187.8 ±45.4c
	root	20	165.0 ±45.1a	740.1 ±32.3b	588.1 ±103c	231.0 ±34a	1045 ±45.1b	347.2 ±35.3c
NHX1	leaf	10	1 ±0.1a	1.40±0.4b	2.00±0.4c	1 ±0.1a	0.70±0.3a	0.80±0.1a
		20	1 ±0.2a	0.14±0.01b	0.40±0.03c	1 ±0.2a	0.30±0.02b	2.00±0.2c
	root	10	1 ±0.2a	1.50±0.3b	1.10±0.3a	1 ±0.1a	0.80±0.1b	1.10±0.1a
		20	1 ±0.2a	2.80±0.02b	2.70±0.5b	1 ±0.3a	0.75±0.2b	0.70±0.1b
SOS1	leaf	10	1 ±0.1a	0.65±0.1b	5.20±0.3c	1 ±0.2a	0.20±0.02b	0.70±0.15c
		20	1 ±0.2a	0.80±0.2b	0.90±0.2b	1 ±0.3a	0.60±0.2b	1.70±0.2c
	root	10	1 ±0.4a	1.30±0.4a	1.80±0.2b	1 ±0.3a	0.52±0.2b	1.40±0.3a
		20	1 ±0.1a	6.00±0.9b	6.10±2b	1 ±0.4a	0.80±0.2b	1.30±0.2a

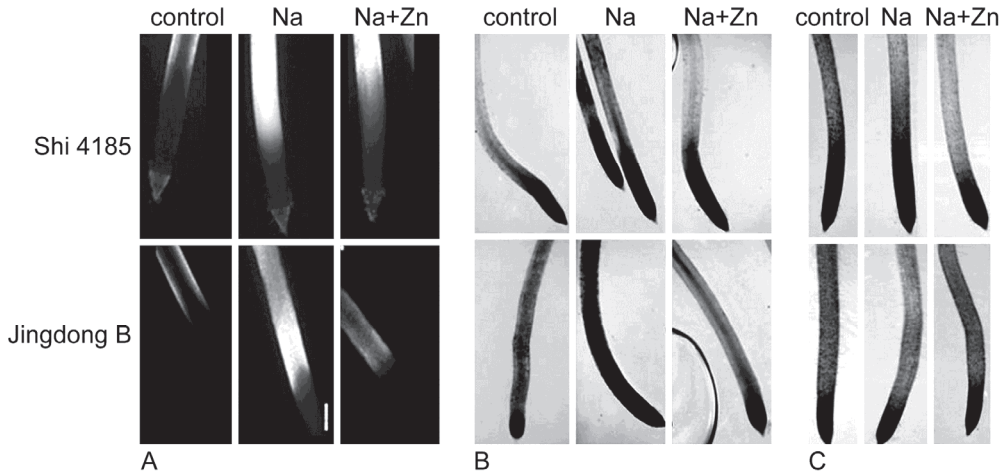

Fig. 1. Visualization of H_2O_2 detected with a DCFH-DA fluorescent probe (*A*), DAB staining (*B*), and visualization of O_2^- by NBT staining (*C*) in roots of wheat seedlings treated with 50 mM NaCl alone or with additional Zn supply for 10 d (control - untreated plats, Na - 50 mM NaCl, Na+Zn - 50 mM NaCl + 2 μM $ZnSO_4$).

cultivar *Jingdong8* decreased by 11 and 2 %, respectively, relative to the untreated control indicating that *Jingdong8* is more tolerant to the salt stress than *Shi4185* (Table 1). Addition of Zn markedly increased the root growth relative to the salt treatment alone also in this cultivar. These results indicate that Zn alleviated the salt stress in

the wheat seedlings.

Several studies have demonstrated the effects of Zn on the activities of antioxidative enzymes (SOD, APX, and CAT) in plants (Wojcik *et al.* 2006, Tewari *et al.* 2008, Wang *et al.* 2009, Daneshbakhsh *et al*. 2013). To determine whether Zn affects the activity of antioxidative enzymes in the salt-treated wheat seedlings, we measured the activities of SOD, APX, CAT, and POD, and examined changes in these activities under Zn supplementation (Table 1). The SOD activity increased in the leaves of *Shi4185* and *Jingdong8* as well as in the roots of *Jingdong8* when seedlings were treated with 50 mM NaCl for 10 d and in the leaves of *Jingdong8* treated for 20 d. However, the SOD activity decreased in the leaves of *Shi4185* and in the roots of *Jingdong8* after 20 d of the treatment. Addition of Zn increased the SOD activity in the leaves of both the cultivars, and in the roots of *Shi4185* treated with NaCl for 10 d and in the roots of *Jingdong8* treated for 20 d. The APX activity increased in the leaves of *Shi4185* after 20 d of the salt treatment as well as in the roots of *Shi4185* and *Jingdong8* after 10 d of the salt treatment. Addition of Zn decreased the APX activity in the leaves of *Jingdong8* after 10 d of the salt treatment and *Shi4185* after 20 d of the salt treatment, whereas Zn increased the APX activity in the roots of both the cultivars after 10 d of the salt treatment. The CAT activity decreased in the NaCl treated seedlings, and addition of Zn did not significantly affect it (data not shown). The salt treatment increased the POD activity in the leaves of the two wheat cultivars as well as in the roots of *Jingdong8* after 10 d of the salt treatment and of *Shi4185* after 20 d of the salt treatment. However, addition of Zn decreased the POD activity in the leaves of both the cultivars after 10 d of the salt treatment and in the leaves and roots of *Shi4185* after 20 d of the salt treatment. Manchandia *et al.* (1999) suggested that the up-regulation of antioxidant activity provides a basic defence against cellular damage from oxidative burst. Activities of APX, CAT, and SOD significantly decrease by Zn deficiency (Cakmak and Marschner 1993), and activities of APX and CAT are also inhibited in Fe-deficient plants (Takahiro *et al.* 2003). NaCl reduces the uptake of Zn and other minerals and causes nutritional disorders in plants. Further, Zn deficiency decreases antioxidative enzyme activities and so increases ROS accumulation and subsequent oxidative damage. Daneshbakhsh *et al*. (2013) found that Zn application increases the root activities of CAT and SOD in wheat cvs. Rushan and Kavir, and a differential tolerance to salt stress between these wheat cultivars is related to their tolerance to Zn-deficiency and root activities of CAT and SOD.

Therefore, we further investigated the productions of H_2O_2 and O_2^- in roots, and NaCl-induced accumulations of H_2O_2 (Fig. 1*A*,*B*) and O_2^- (Fig. 1*C*) were detected. Addition of Zn inhibited the productions of H_2O_2 and O_2^- compared with the salt treatment alone. These data indicate that Zn decreased the H_2O_2 and O_2^- content

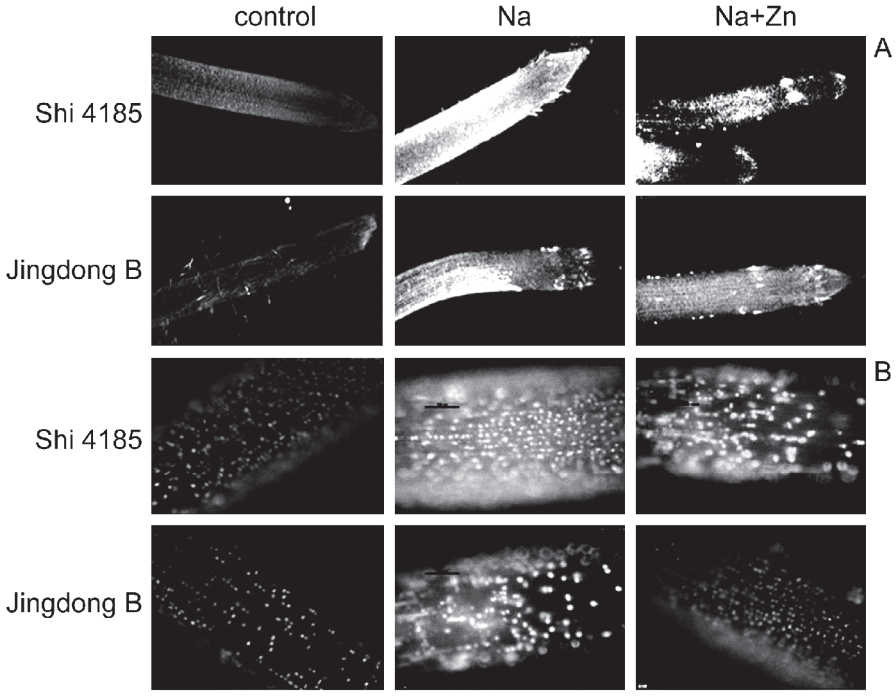

Fig. 2. Plasma membrane integrity (*A*) and programmed cell death in root tip cells (*B*). Wheat seedlings treated with 50 mM NaCl alone or with additional Zn supply for 10 d. Roots were excised and subsequently stained with PI and DAPI dyes as described in Materials and methods (control - untreated plats, Na - 50 mM NaCl, Na+Zn - 50 mM NaCl + 2 μM $ZnSO_4$).

which is consistent with the increased activities of some antioxidative enzymes.

To determine whether Zn alleviates membrane injury and cell death of root tips, we examined the plasma membrane (PM) integrity in root tips using propidium iodide (PI) staining. PI is a membrane-impermeable dye that binds to nucleotides; it is generally excluded from living cells. A PI-positive nucleus is a strong indication of loss of membrane integrity (De Cnodder *et al.* 2005). PI staining indicated that the salt stress induced cell death of the wheat root tips after 10 d of the salt treatment, but less in *Jingdong8* compared with *Shi4185* (Fig. 2*A*). Salt-induced programmed cell death (PCD) in root tips has been reported (Huh *et al.* 2002). To determine whether Zn affects the process, we investigated the chromatin condensation by DAPI staining. DAPI is a DNA fluorochrome that binds stoichiometrically to AT-rich regions of DNA. Addition of Zn decreased the DAPI signals in the roots of the wheat seedlings after 10 d of the salt treatment (Fig. 2*B*) indicating that addition of Zn effectively reduced salt-induced PCD in the root tip cells. The modification of root system architecture by PCD of root tip cells is an adaptive mechanism that may be essential for survival of plants under salt stress. The elucidation of the exact molecular mechanisms should be addressed in future research.

Further, we measured the Na^+ content by atomic absorption spectrometry in the wheat seedlings exposed to 50 mM NaCl for 20 d. The Na^+ content in the leaves and roots of the plants was increased due to the salt treatment, but less after addition of Zn. *Jingdong8* showed a lower Na^+ accumulation in the leaves, however, more Na^+ accumulated in its roots compared with *Shi4185*. We also examined the expression of two genes coding Na^+/H^+ exchangers TaSOS1 and TaNHX1, contributing to plasma membrane and tonoplast membrane Na^+ transports, respectively (Xu *et al.* 2008a,b). Addition of Zn further increased the expression of *TaSOS1* and *TaNHX1* especially in the roots after 10 d of the NaCl treatment (Table 1). The elevated expression of *TaNHX1* increased storage of Na^+ in root cell vacuoles, thereby decreasing its transport from the root to the stem.

The data presented here provide new insights regarding salt tolerance mechanisms in wheat. Our results demonstrate that sufficient Zn nutrition maintainedthe antioxidative enzyme activities and decreased ROS over-accumulation in the wheat seedlings. Our data also reveal that sufficient Zn nutrition improved the expression of the Na^+/H^+ antiporter genes, *TaSOS1* and *TaNHX1*, thereby decreasing Na^+ accumulation in the wheat shoots.

References

Asada, K., Kiso, K.: Initiation of aerobic oxidation of sulfite by illuminated spinach chloroplasts. - Eur. J. Biochem. **33**: 253-257, 1973.

Blumwald, E., Aharon, G.S., Apse, M.P.: Sodium transport in plants. - Biochim. biophys. Acta **1465**: 140-151, 2000.

Cakmak, I., Marschner, H.: Effect of zinc nutritional status on activities of superoxide radical and hydrogen peroxide scavenging enzymes in bean leaves. - Plant Soil **155/156**: 127-130, 1993.

Chen, L.N., Yin, H.X., Xu, J., Liu X.J.: Enhanced antioxidative responses of a salt-resistant wheat cultivar facilitate its adaptation to salt stress. - Afr. J. Biotechnol. **10**: 16887-16896, 2011.

De-Cnodder, T.. Vissenberg, K, Van-Der-Straeten, D., Verbelen, J.P.: Regulation of cell length in the *Arabidopsis thaliana* root by the ethylene precursor aminocyclopropane-1-carboxylic acid: a matter of apoplastic reactions. - New Phytol. **168**: 541-550, 2005.

Daneshbakhsh, B., Khoshgoftarmanesh, A.H., Shariatmadari, H., Cakmak, I.: Effect of zinc nutrition on salinity-induced oxidative damages in wheat genotypes differing in zinc deficiency tolerance. - Acta Physiol. Plant. **35**: 881-889, 2013.

Dong, H.L., Young, S.K., Chin, B.L.: The inductive responses of the antioxidant enzymes by salt stress in the rice (*Oryza sativa* L.) - J. Plant Physiol. **158**: 737-745, 2001.

Foyer, C.H.: Free radical processes in plants. - Biochem. Soc. Trans. **24**: 427-434, 1996.

Ghoulam, C., Foursy, A., Fares, K.: Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. - Environ. exp. Bot. **47**: 39-50, 2002.

Gonzalez-Guerrero, M., Azcon-Aguilar, C., Mooney, M., Valderas, A., MacDiarmid, C.W., Eide, D.J., Ferrol, N.: Characterization of a *Glomus intraradices* gene encoding a putative Zn transporter of the cation diffusion facilitator family. - Fungal Genet. Biol. **42**: 130-140, 2005.

Grattan, S.R., Grieve, C.M.: Salinity-mineral nutrient relations in horticultural crops. - Sci. Hort. **78**: 127-157, 1999.

Guo, Y.H., Yu, Y.P., Wang, D., Wu, C.A., Yang, G.D.: GhZFP1, a novel CCCH-type zinc finger protein from cotton, enhances salt stress tolerance and fungal disease resistance in transgenic tobacco by interacting with GZIRD21A and GZIPR5. - New Phytol. **183**: 62-75, 2009.

Huh, G.H., Damsz, B., Matsumoto, T.K., Reddy, M.P., Rus, A.M., Iveas, J.I., Narasimhan, M.L., Bressan, R.A., Hasegawa, P.M.: Salt causes ion disequilibrium- induced programmed cell death in yeast and plants. - Plant J. **29**: 649-659, 2002.

Liu, K., Xu, S., Xuan, W., Ling, T., Cao, Z., Huang, B., Sun, Y., Fang, L., Liu, Z., Zhao, N., Shen, W.: Carbon monoxide counteracts the inhibition of seed germination and alleviates oxidative damage caused by salt stress in *Oryza sativa.* - Plant Sci. **172**: 544-555, 2007.

Mahajan, S., Tuteja, N.: Cold, salinity and drought stresses: an overview. - Arch. Biochem. Biophys. **444**: 139-158, 2005.

Manchandia, A.M., Banks, S.W., Gossett, D.R., Bellaire, B.A., Lucas, M.C., Millhollon, E.P.: The influence of amanitin on the NaCl-induced upregulation of antioxidant enzyme activity in cotton callus tissue. - Free Radical Res. **30**: 429-

438, 1999.

Mansour, M.M.F.: Plasma membrane permeability as an indicator of salt tolerance in plants. - Biol. Plant. **57**: 1-10, 2013.

Ramel, F., Sulmon, C., Bogard, M., Couée, I., Gouesbet, G.: Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in *Arabidopsis thaliana* plantlets. - Plant Biol. **9**: 28, 2009.

Takahiro, I., Rapolu, M., Shigeru, S.: Effect of iron on the expression of ascorbate peroxidase in *Euglena gracilis*. - Plant Sci. **165**: 1363-1367, 2003.

Tan, J.J., Wang, J.W., Chai, T.Y., Zhang, Y.X., Feng, S.S., Li, Y., Zhao, H.J., Liu, H.M., Chai,X.P.: Functional analyses of *TaHMA2*, a P1B-type ATPase in wheat. - Plant Biotechnol. J. **11**: 420-431, 2013.

Tewari, R.K., Kim, S.Y., Hahn, E.J., Paek, K.Y.: Involvement of nitric oxide-induced NADPH oxidase in adventitious root growth and antioxidant defense in *Panax ginseng*. - Plant Biotechnol. Rep. **2**: 113-122, 2008.

Tsai, Y.C., Hong, C.Y., Liu, L.F., Kao, C.H.: Relative importance of Na^+ and Cl^- in NaCl-induced antioxidant systems in roots of rice seedlings. - Physiol. Plant. **122**: 86-94, 2004.

Upadhyay, A., Upadhyay, A.K., Bhirangi, R.A.: Expression of Na^+/H^+ antiporter gene in response to water and salinity stress in grapevine rootstocks. - Biol. Plant. **56**: 762-766, 2012.

Wang, C., Zhang, S.H., Wang, P.F., Hou, J., Zhang, W.J., Li, W., Lin, Z.P.: The effect of excess Zn on mineral nutrition and antioxidative response in rapeseed seedlings. - Chemosphere **75**: 1468-1476, 2009.

Wang, W., Vinocur, B., Altman, A.: Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. - Planta **18**: 1-14, 2003.

Wójcik, M., Skórzynska-Poli, E., Tukiendorf , A.: Organic acids accumulation and antioxidant enzyme activities in *Thlaspi caerulescens* under Zn and Cd stress. - Plant Growth Regul. **48**: 145-155, 2006.

Xu, H., Jiang, X., Zhan, K., Cheng, X., Chen, X., Pardo, J. M., Cui, D.: Functional characterization of a wheat plasma membrane Na^+/H^+ antiporter in yeast. - Arch. Biochem. Biophys. **473**: 8-15, 2008a.

Xu, H.X.: Bioinformatics analysis of antiporter gene *TaNHX1* on the tonoplast membrane in wheat. - J. Anhui agr. Sci. **13**: 5354-5356, 2008b.

Xu, J., Yin, H.X., Li, X.: Protective effects of proline against cadmium toxicity in micropropagated hyperaccumulator, *Solanum nigrum* L. - Plant Cell Rep. **28**: 325-333, 2009.

Xu, J., Yin, H.X., Li, Y.L., Liu, X.J.: Nitric oxide is associated with long-term zinc tolerance in *Solanum nigrum*. - Plant Physiol. **154**: 1319-1334, 2010.

Zhu, J.K.: Regulation of ion homeostasis under salt stress. - Curr. Opin. Plant Biol. **6**: 441-445, 2003.