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ORIGINAL PAPER

Time-course of ionic responses and proteomic analysis of a Tibetan wild barley at early stage under salt stress

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Abstract Barley (*Hordeum vulgare* L.) is well known for its relatively high salt tolerance among cereal crops. However, the genetic variation of cultivated barley becomes narrower due to continuous artificial selection and breeding processes. Compared with cultivated barley, wild barley contains wider genetic variation and abundant sources for abiotic stress tolerance, considering as an elite resource for mechanism study on salt tolerance. In this study, Tibetan wild barley accession XZ113 identified with high salt tolerance, was used to investigate ionic responses and to identify proteins involved in salt tolerance in roots and shoots at early stage of salt stress, during 48 h. Exposed to salinity, shoot growth is more sensitive than root growth. Conversely, K/Na ratio in the shoots was larger than that in the roots, and both were above 1.0. Steady-state K^+ flux experiment showed XZ113 had a strong K^+-retaining ability under salt stress, maybe contributing to its good performance of the absolute growth rate. Proteomic results suggested that monodehydroascorbate reductase and peroxidases related to reactive oxygen species scavenging in the roots and phosphoglycerate kinase, triosephosphate isomerase and sedoheptulose-1,7-bisphosphatase associated with photosynthesis and metabolisms in the shoots, played important roles in salt tolerance at early stage of salinity in wild barley.

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Keywords Wild barley · Salt tolerance · Ion homeostasis · Proteomics · Time-course

Abbreviations

ATP	Adenosine triphosphate
BPB	Bromophenol blue
DTT	Dithiothreitol
DW	Dried weight
FW	Fresh weight
GR	Growth rate
ICP–OES	Inductively coupled plasma–optical emission spectrometer
IEF	Isoelectric focusing electrophoresis
IPG	Immobilized pH gradient
MALDI-TOF/TOF MS	Matrix-assisted laser desorption/ionization time of flight mass
MIFE	Microelectrode ion flux estimation
PM	Plasma membrane
PIPs	Plasma membrane intrinsic proteins
ROS	Reactive oxygen species
RWC	Relative water content
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SBPase	Sedoheptulose-1,7-bisphosphatase
2-DE	Two-dimensional gel electrophoresis
TCA	Tricarboxylic acid cycle
VDAC	Voltage-dependent anion channel

Introduction

Salt stress is one of major abiotic stresses restricting crop production and yield worldwide. Currently, around 20 % of irrigated land has been affected by salinity, and moreover the soil salinization is expanding rapidly due to unreasonable farming and unavoidable geological factors (Munns and Tester 2008; Rengasamy 2010). To fight against salt stress, development of crop cultivars with high salt tolerance is considered as a most effective method. Plant species differ widely in salt tolerance, and most cereal crops are glycophytes (Glenn et al. 1999; Horie et al. 2012). However, barley (*Hordeum vulgare* L.), ranking the fourth in terms of planting area in the world among cereal crops, is well known for its relatively high salt tolerance. Hence, barley is commonly planted in the saline soil where other cereal crops cannot grow well, and meanwhile it is frequently used in the studies on the mechanisms of salt stress tolerance in plants (Munns and Tester 2008; Munns et al. 2012). But the physiological and molecular mechanisms underlying salt tolerance in barley still remain largely elusive, especially osmotic and ionic stresses at early phases. On the other hand, the genetic background of cultivated barley becomes narrower with development of intensive planting, thus resulting in the loss of elite genes, including those related to salt stress tolerance.

Comparatively, wild barley (*Hordeum spontaneum*) contains a wider source of genetic variation for abiotic stress tolerance (Ellis et al. 2000; Nevo 2007). Wild barley relatives have stronger adaptive ability to various abiotic stresses including cold, drought and salt salinity than cultivated barley. A recent study confirmed that Tibet is one of the centers of domestication of cultivated barley (Dai et al. 2012). Meanwhile, it was found that nearly 70 % of 189 Tibetan wild barley accessions showed higher salt tolerance than a well-known tolerant cultivar, CM72 (Wu et al. 2011; Qiu et al. 2011). Obviously, Tibetan wild barley is highly valuable in breeding for the cultivars of barley as well as other crops with high salt stress tolerance, and it is quite imperative to understand the tolerant mechanisms.

High concentration of soluble sodium (Na) initially causes osmotic and ionic stresses to plants (Zhu 2002; Munns and Tester 2008). Osmotic shock starts immediately when plant roots are exposed to salt solution at a certain threshold level, approximately 40 mM NaCl for most plants (Munns and Tester 2008). Osmotic stress significantly reduces water uptake and tissue growth rates of plants, leading to reduced development and productivity in later phases (Horie et al. 2012; Han et al. 2015). Ionic stress is mainly caused by Na accumulation at a toxic concentration, which causes leaf senescence and cell death (Munns and Tester 2008). Therefore, the effective strategies of fighting against salt stress for plants are to keep low cytosolic Na^+ concentration at the cellular level and low shoot Na^+ concentrations at the whole plant level in response to salt stress at the early phases (Horie et al. 2012). Previous studies also revealed that high cytosolic K^+/Na^+ ratio, especially in shoots is crucial for salt tolerance for glycophyte plants (Gorham et al. 1990; Blumwald 2000; Chen et al. 2005; Shabala et al. 2010). For example, a salt-tolerant rice genotype, PL177 showed significantly less salt damage, higher K^+/Na^+ ratio in shoots and lower Na^+ translocation from roots to shoots than the sensitive genotype, IR64 (Wang et al. 2016). It is also suggested that salt tolerant genotypes are capable of maintaining a higher xylem K^+/Na^+ ratio by HKT transporter localized in plasma membrane and sequestering Na^+ in vacuole by tonoplast-localized Na^+/H^+ exchangers in barley (Shabala et al. 2010). In short, the current studies demonstrate the importance of salt-induced ionic changes in the development of salt stress tolerance in plants.

There are complex signaling and metabolic pathways in plants to cope with salt stress (Zhang et al. 2012a, b). Physiological studies indicated that increased energy metabolism such as glycolysis and tricarboxylic acid cycle (TCA) were necessary to alleviate osmotic and ionic effects caused by salt stress (Kosová et al. 2013a), but little is known on the relationship between signaling and metabolism at the whole plant level (Wang et al. 2016). So, it is imperative to determine the molecular responses to salt stresses in order to understand signaling and metabolic pathways associated with salt tolerance. Obviously, among these molecular components, proteins play direct roles in biological metabolisms and set up the tolerant system in response to salt stresses. Therefore, proteomics analysis is powerful and useful in studies on abiotic tolerance in order to identify the key genes associated closely with stress tolerance (Kosová et al. 2011, 2013b). Zhang et al. (2012a, b) summarized 2,171 salt-responsive proteins in 34 plant species by proteomic studies including those linked with photosynthesis, reactive oxygen species (ROS) scavenging, ion homeostasis, osmotic modulation, signaling transduction and protein synthesis. Those stress-induced proteins may be helpful for obtaining an entire knowledge of molecular networks and beneficial for mining salt-associated genes. However, few attempts have been conducted to investigate the ionic and proteomic responses of wild barley to salt stress, although it shows much higher salt tolerance relative to cultivated barley. Therefore, the current research was done in order to understand ionic responses to salt stress at early stage, and to identify proteins involved in ionic changes and salt tolerance using a Tibetan wild barley accession XZ113, which is high salt tolerant (Qiu et al. 2011).

Materials and methods

Plant materials and hydroponic culture

Seeds of Tibetan wild barley accession XZ113 were surface sterilized with 3 % H_2O_2 solution for 20 min and rinsed thoroughly with distilled water. Seed germination was conducted according to Wu et al. (2014) in a growth condition (22/18 °C, day/night; 14/10 h, light/dark). Seven-days-old seedlings were transplanted into 5 L black plastic containers containing 1/2 Hoagland solution. Aerated hydroponic solution was renewed every 5 days (pH 6.0). Seven days after transplanting, the treatment of 200 mM NaCl was initiated and the solution without NaCl addition was used as the control. For non-invasive MIFE (Microelectrode Ion Flux Estimation) experiments, seeds were placed vertically into floating filter papers in an aerated basic salt medium (BSM) containing 0.1 mM $CaCl_2$ and 0.5 mM KCl, and kept in a growth chamber for 3 days without light until use for measurement.

Root K^+ and H^+ flux measurement

Root net ion fluxes were measured using MIFE technique (Chen et al. 2007). One hour prior to measurement, a root of 3-days-old barley seedling was immobilized to a slide and placed horizontally in a Perspex container with 10 ml BSM. The calibration and operation of K^+ and H^+ ion-selective microelectrodes were followed according to the protocol described by Shabala et al. (2010). The electrode tips were positioned 40 μm above the surface of mature root zone ($\sim$10 mm from the root tip). Steady-state net K^+ and H^+ ion fluxes were measured at each time point of 0, 2, 6 and 24 h after 80 mM NaCl was added into BSM solution according to Chen et al. (2007). Six biological replicates were measured in this experiment. The different significance among treatments was tested by SAS 9.1 software and the difference at $p < 0.05$ and $p < 0.01$ were considered as significant and highly significant.

Growth and element concentration determination

Before sampling, roots were thoroughly rinsed with deionized water for several times. Three biological replicates of roots and shoots were sampled at 0, 2, 6, 24 and 48 h after salt treatment. Fresh weight (FW) of roots and shoots were measured using an electronic balance (Sartorius BSA124S, Germany), then dried at 80 °C for 72 h, and weighed. Absolute growth rate (GR) was calculated according to the formula: GR (mg/h) = ($DW_T - DW_0$)/T, here T is the treatment time. Tissue relative water content (RWC) was calculated by the formula: RWC (%) = (FW − DW)/DW × 100 %. For element extraction, dried samples were digested with 6 ml HNO_3 and 200 μl 30 % H_2O_2 in boiling tubes using a microwave digestion instrument at 200 °C for 1.5 h (Multiwave 3000, Anton Paar GmbH, Australia). Then the contents of Na, K, Ca and Mg in the extracted solution were determined using an ICP–OES spectrometer (Optima 8000 series, PerkinElmer Inc, USA). The different significance was also tested by SAS 9.1 software, as mentioned above.

Protein extraction and purification

Fresh tissue of roots and shoots were sampled at 48 h after salt treatment, frozen immediately in liquid nitrogen, and then stored at −80 °C before use. Total proteins were extracted from 3 g fresh tissue and purified using ReadyPrep™ 2-D cleanup kit (BIO-RAD, Hercules, USA). The Bradford kit (BIO-RAD, Hercules, USA) was used to determine the concentration of proteins.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was conducted according to the method described by Cheng et al. (2009). IEF was carried out on a 24 cm IPG strip (pH 4–7). A total of 460 μl rehydration buffer containing 200 μg (for gel image) or 1200 μg (for MS analysis) protein sample mixed with loading buffer solution (1 % DTT, 1 % IPG Buffer, 1× BPB and rehydration buffer) was loaded in the gels. The gel electrophoresis was set in a total voltage of 52 kVh with variable programs in Ettan IPGphor 3 system (GE Healthcare, Germany) according to Wu et al. (2014). After IEF, the strips were equilibrated in an equilibration buffer for 15 min and placed in the dark for another 15 min. The second dimension of SDS-PAGE was performed on top of 12.5 % running gel, with first voltage of 2 W for 45 min and second 17 W per gel for 5 h. Then the gels were strained with silver staining method and CBB G-250 staining method for further analysis.

2-D gel image analysis

For gel images analysis, 2D gels were scanned by a GS-800 2-DE scanner (BIO-RAD, Hercules, USA) and analyzed by Image Master™ 2D version 5.0 (Amersham Biosciences) according to automated matching and manual editing. Protein spots were analyzed according to Wu et al. (2014).

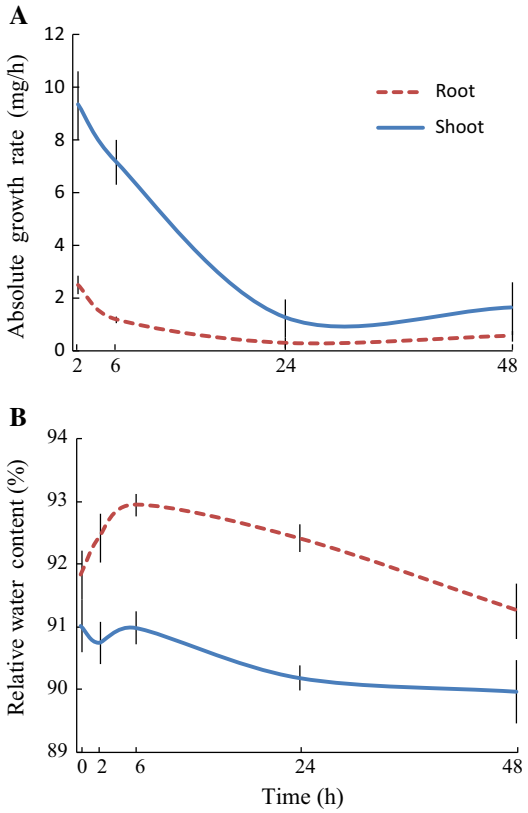

Fig. 1 Effects of salt stress (200 mM NaCl) on time-course of absolute growth rate (**a**) and relative water content (**b**) in roots and shoots of XZ113. For each time point, three replicates were measured independently. Mean ± SE (n = 3)

Three biological and two technical replicates were set in the experiments.

MALDI-TOF/TOF MS analysis

Differentially expressed spots ($p < 0.05$) were excised from the 2-D gels, then washed clearly and digested in trypsin digestion solution according to Cheng et al. (2009). Analysis of peptide mass spectra were operated by an Autoflex speed^{TM} MALDI-TOF–TOF mass spectrometer (Bruker Dalton, Germany) in the mode described by Wu et al. (2014). To analyze data and search for database of MS/MS, MASCOT program was conducted by BioTools (Bruker Dalton, Germany) along with NCBI in the following parameters: peptide mass tolerance of 50 ppm; fragment mass tolerance of ±0.5 Da; peptide charge state of +1; fixed modification of cysteine carbamidamethyl; variable modification of methionine (Met) oxidation. Only target peptides as displayed by Mascot probability analysis ($p < 0.05$) were accepted as candidate significant proteins defined by biological function.

Table 1 Time-course of tissue element contents (mg g^{-1} DW) in XZ113 under 200 mM NaCl

Time (h)	Root (mg g^{-1} DW)					Shoot (mg g^{-1} DW)				
	Na	K	Ca	Mg	K/Na ratio	Na	K	Ca	Mg	K/Na ratio
0	4.21[a] ± 0.22e	39.98 ± 2.05[b]	0.92 ± 0.16a	0.39 ± 0.03ab	9.50	4.47 ± 0.55c	49.69 ± 1.03a	0.17 ± 0.01a	0.19 ± 0.03a	11.12
2	10.90 ± 0.54c	38.91 ± 0.51a	0.89 ± 0.12ab	0.41 ± 0.00a	3.57	4.47 ± 0.20c	49.53 ± 1.71a	0.17 ± 0.01ab	0.18 ± 0.05a	11.08
6	6.02 ± 0.19d	40.46 ± 2.18a	0.56 ± 0.12c	0.33 ± 0.05bc	6.72	4.26 ± 0.59c	49.27 ± 1.86a	0.16 ± 0.01abc	0.17 ± 0.14b	11.58
24	15.30 ± 0.51b	30.65 ± 2.60b	0.62 ± 0.08bc	0.30 ± 0.03c	2.00	9.42 ± 1.13b	48.43 ± 2.95a	0.15 ± 0.02c	0.16 ± 0.14b	5.14
48	20.60 ± 0.62a	21.39 ± 1.12c	0.73 ± 0.26abc	0.31 ± 0.05c	1.04	14.20 ± 1.81a	49.51 ± 0.92a	0.15 ± 0.01bc	0.15 ± 0.12b	3.48

[a] Data represent mean ± SD of three independent experiments

[b] Different letters indicate significant difference at 95 % probability in roots or shoots at different treating times according to the Tukey HSD test

Results

Growth performance of XZ113 in response to salt stress

Tibetan wild barley accession XZ113 is identified as a salt-tolerant genotype in a previous study (Qiu et al. 2011). To understand physiological and molecular responses of XZ113 to salt stress, we firstly determined its growth performance in terms of absolute growth rate (GR) and relative water content (RWC) in a time-course of salt treatment (200 mM NaCl) during 48 h. Obviously, salt treatment caused a significant decrease of GR in roots and shoots, and the decreased extent was more obvious with the treatment time (Fig. 1a). During 24 h treatment, nearly eightfold decrease of GR was found for both roots and shoots, and then kept a low GR after 24 h (Fig. 1a). Compared with the roots, shoots showed larger inhibition of GR in each sampling time, indicating that shoot growth is more sensitive to salt stress than root growth. Meanwhile, salt stress only caused a slight water loss in roots, merely 2 % of RWC decrease after salt treatment. While shoot RWC showed less significant change during 48 h treatment (Fig. 1b). Surprisingly, root RWC increased at 6 h to reach a maximum value and then decreased over the treatment time (Fig. 1b).

Dynamic changes of element concentration in roots and shoots in response to salt stress

The effect of salt treatment on ionic change was significant, and differed between roots and shoots (Table 1). In roots, Na concentration showed significant increase at 2 h after treatment, followed by a slight decrease at 6 h, and then kept rising to 20.6 mg g^{-1} DW at 48 h, nearly twofold higher than that at 2 h. The change of Na concentration in roots was completely opposite to that of root RWC. In contrast, K concentration decreased from nearly 40 mg g^{-1} DW at 0–6 h to 21.39 mg g^{-1} DW at 48 h (Table 1). In shoots, ionic change was slower and less in response to salt stress in comparison with roots. Until 24 h after treatment, Na concentration showed significant increase relative to the control, and then continued increase, reaching to 14.2 mg g^{-1} DW at 48 h, nearly 1.5 fold higher than that at 24 h. While shoot K concentration remained less change during the whole treatment (Table 1). In terms of K/Na

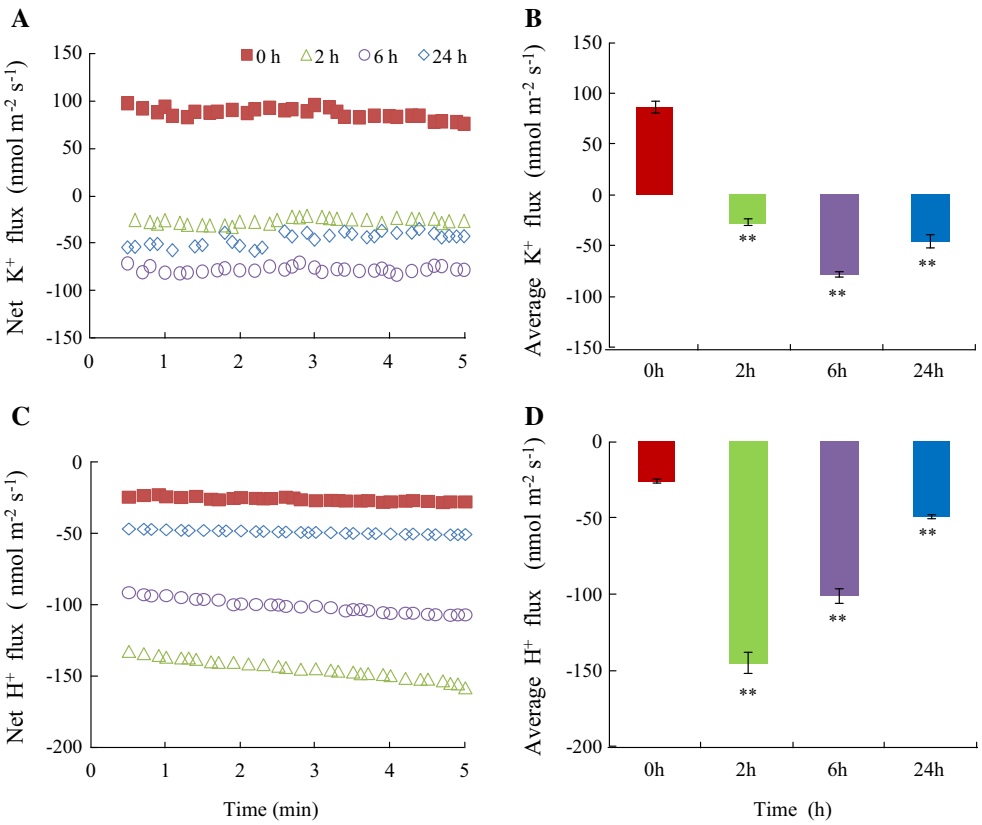

Fig. 2 Steady-state K^+ (**a**) and H^+ (**c**) fluxes measured by MIFE from the mature root zone of 3-day-old seedlings exposed to 80 mM NaCl for 0, 2, 6 and 24 h. The results in **b**, **d** are averaged over 10 min of K^+ and H^+ flux measurement, respectively. Six replications were performed at each time point. **Indicates highly significant between treatment and control at $p < 0.01$ by Tukey HSD

Table 2 The differentially expressed proteins in the root of XZ113 in response to salinity

Spot no.[a]	Accession no.	Protein homologue[b]	Mass (kDa) (Theor./Exper.)[c]	PI (Theor./Exper.)[d]	MASCOT score[e]	No. of peptide	Coverage (%)	CK	T	Fold[f]
A_{D1}	gil326527793	Succinyl-CoA ligase beta-chain	45.12/45.43	6.30/6.30	150	6	10	0.0156	0.0045	0.29
A_{D3}	gil326516792	Predicted protein	28.97/29.24	5.70/5.70	265	6	26	0.0295	0.0077	0.26
A_{D4}	gil326510253	Hydroxyacylglutathione hydrolase	28.61/29.05	5.66/5.66	299	9	35	0.0509	0.0109	0.21
A_{D5}	gil326496021	Dehydroascorbate reductase	23.34/23.44	5.71/5.71	251	8	54	0.0610	0.0000	0.00
A_{U1}	gil326494244	Putative monodehydroascorbate reductase	46.35/46.49	5.50/5.50	352	10	24	0.0000	0.0334	10,000
A_{U2}	gil326521344	Putative class III peroxidase	36.40/36.89	5.67/5.67	197	8	31	0.0271	0.1086	4.00
A_{U4}	gil326521344	Putative class III peroxidase	36.40/36.89	5.67/5.67	262	9	30	0.0000	0.3737	10,000
A_{U5}	gil326506652	Porin family protein	29.47/29.45	9.25/9.25	451	10	44	0.0000	0.0934	10,000

Expression level increase or decrease under salt stress. The fold changes are based on three biologically independent experiments of 2-DE

[a] Spot no.: the number of spot in corresponds to the spots in Figs. 1a and 2. U: increased intensities ratio after 48 h treatment of 200 mM NaCl. D: decreased intensities ratio after 48 h treatment of 200 mM NaCl

[b] Protein Homologue: blast on NCBI database to search the candidate protein of targeted peptides of protein

[c] Mass (Theor./Exper.) (kDa): theoretical/experimental molecular mass of proteins

[d] pI (Theor./Exper.) (kDa): theoretical/experimental pI of proteins

[e] MASCOT score: calculated by MASCOT, and scores above 72 ($p < 0.05$) are considered significant

[f] Fold change: calculated using the mean value of each spot in normalized spot volume comparing salinity and control conditions

ratio, it showed the opposite change as Na concentration did in both roots and shoots. The value of K/Na ratio in roots and shoots was above 1.0 and was larger in shoots than in roots at 48 h after salt treatment (Table 1). In addition, in comparison with control, Ca and Mg concentrations decreased by 39.1 and 26.8 % in roots, and by 11.8 and 21.0 % in shoots, respectively, at 48 h after salt treatment (Table 1).

In order to reveal the effect of salt stress on K^+-retaining ability in XZ113, steady-state K^+ and H^+ flux in the roots exposed to 80 mM NaCl was measured using MIFE technology within 24 h (Fig. 2). The result indicates that K^+ efflux was significantly reduced under salt stress, falling into -20 nmol m^{-2} s^{-1} at 2 h and into -100 nmol m^{-2} s^{-1} at 6 h exposure. However, at 24 h exposure, K^+ efflux was obviously recovered (Fig. 2a, b). Meanwhile, a fast H^+ efflux was observed at 2 h exposure, being sixfold increase compared with that at starting time, and become twofold increase at 24 h (Fig. 2c, d). Apparently, H^+ recovered more quickly and effluxed more than K^+ in the roots of XZ113 when subjected to salt stress. Hence, XZ113 may maintain relatively high K concentration under salt stress, resulting in high K/Na ratio after salt stress, contributing to its salt tolerance.

Identification of salt stress induced proteins in roots

A total of 8 differentially expressed protein spots were successfully identified in roots of XZ113 under salt stress (Table 2; Fig. 3a). Among these protein spots, 4 up-regulated protein spots were putative monodehydroascorbate reductase (A_{U1}), putative class III peroxidase (A_{U2}, A_{U4}) and porin family protein (A_{U5}), respectively. Meanwhile, the spot abundance of succinyl-CoA ligase beta-chain (A_{D1}), predicted protein (A_{D3}), hydroxyacylglutathione hydrolase (A_{D4}) and dehydroascorbate reductase (A_{D5}) were greatly reduced (Table 2). Here, 5 proteins mainly participated in ROS scavenging, were listed as A_{D4}, A_{D5}, A_{U1}, A_{U2} and A_{U4}. Among them, A_{U1} and A_{U4} showed protein abundance specifically under salt stress (Table 2; Fig. 4), indicating highly active ROS activity at early stage of salt stress.

Identification of salt stress induced proteins in shoots

In shoots, there were 8 differentially expressed proteins identified under salt stress when compared with its control (Table 3; Fig. 3b). Three protein spots showing reduction under salt stress were characterized as chloroplastic ATP synthase (B_{D1}, B_{D2}, B_{D3}), belonging to two types of subunit groups, alpha and gamma. On the other hand, 5

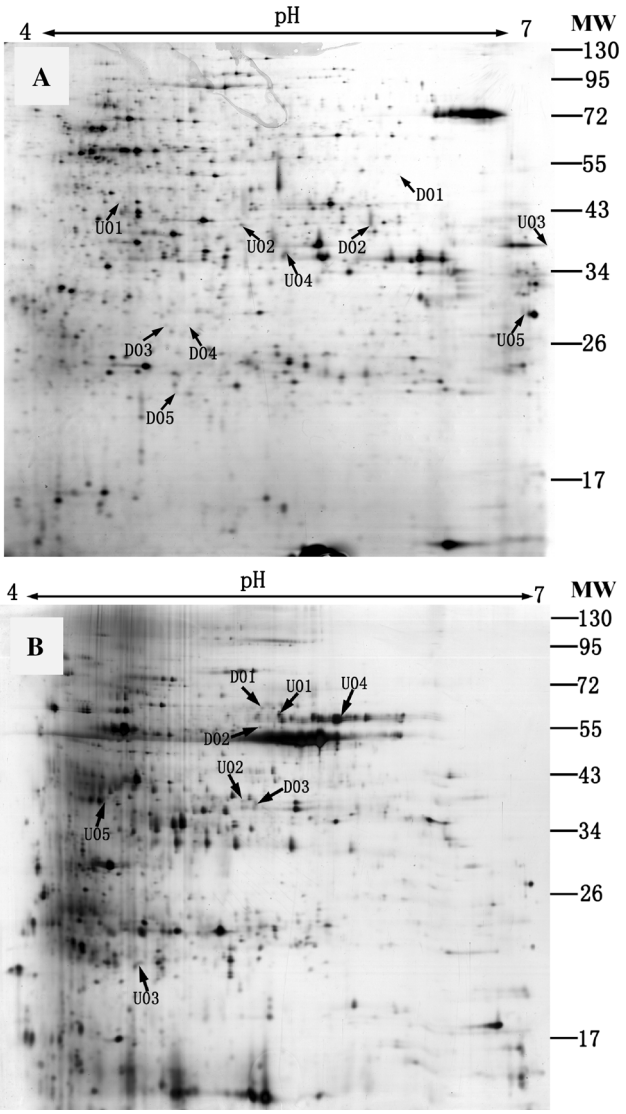

Fig. 3 Two-dimensional gels of differentially expressed protein spots isolated from roots and shoots of XZ113 under 200 mM NaCl treatment or control for 2 day. **a** 2-D gels of root proteome; and **b** 2-D gels of shoot proteome. *Arrows* indicate the location of differentially expressed protein spots. *Numbers* in gels showed different abundance of proteins in roots or shoots after salinity. As D2 and U3 were not identified in MS analysis, they were not discussed in this study

significantly increased proteins were identified as chloroplastic ATP synthase alpha subunit (B_{U1}, B_{U4}), phosphoglycerate kinase (B_{U2}), triosephosphate isomerase (B_{U3}), Sedoheptulose-1,7-bisphosphatase (SBPase, B_{U5}) (Table 3; Fig. 5). Here, 5 ATP synthase subunits (B_{D1}, B_{D2}, B_{D3}, B_{U1} and B_{U4}) were identified, indicating important roles of ATP synthases in response to salt stress in shoots. In addition, SBPase (B_{U5}) showed the maximum change (7.29 folds) as a key enzyme in Calvin cycle of plant photosynthesis, while B_{U2} and B_{U3} are two key enzymes in the pathway of glycolysis, indicating the enhanced activity of

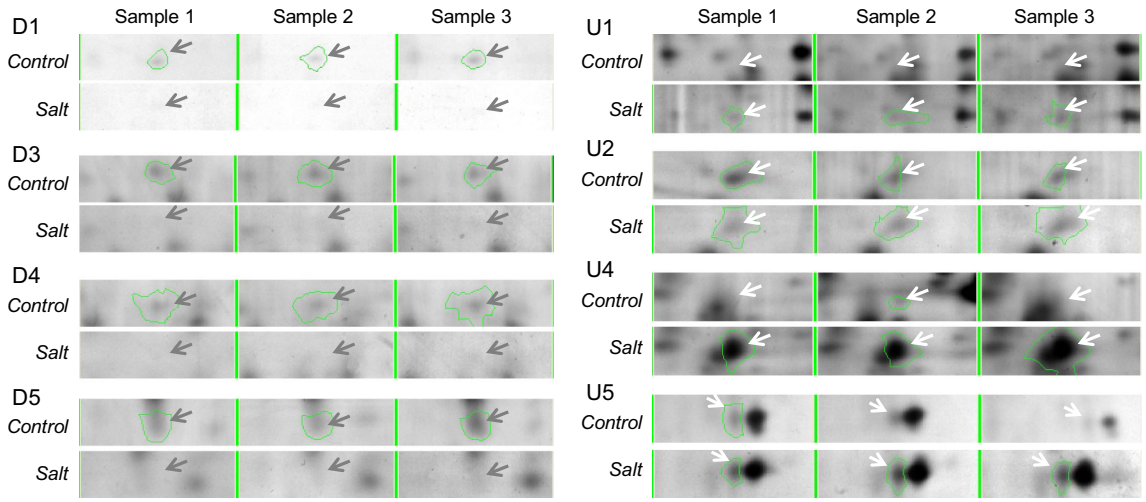

Fig. 4 'Spot view' of the abundance of differentially expressed proteins in roots of XZ113 exposed to salt stress for 2 day. *Green circles* with *black arrows* indicate spots showing decrease (listed as *D*) and *white arrows* indicate spots showing increase (listed as *U*). Protein spot ID corresponds to *spot number* in Table 2 and Fig. 3a. (Color figure online)

Table 3 The differentially expressed proteins in the shoot of XZ113 in response to salinity

Spot no.	Accession no.	Protein homologue	Mass (kDa) (Theor./Exper.)	PI (Theor./ Exper.)	MASCOT score	No. of peptide	Coverage (%)	CK	T	Fold
B_{D1}	gil118430385	ATP synthase (subunit alpha, chloroplastic)	55.29/55.32	6.32/6.32	406	21	36	0.0490	0.0201	0.41
B_{D2}	gil118430385	ATP synthase (subunit alpha, chloroplastic)	55.29/55.32	6.32/6.32	421	21	43	0.0234	0.0171	0.73
B_{D3}	gil285014508	ATP synthase subunit gamma, chloroplastic	39.75/40.01	8.18/8.18	119	7	18	0.0830	0.0327	0.39
B_{U1}	gil118430385	ATP synthase (subunit alpha, chloroplastic)	55.29/55.32	6.32/6.32	373	16	38	0.1167	0.4075	3.49
B_{U2}	gil326522650	Phosphoglycerate kinase	40.19/40.22	5.39/5.39	179	5	14	0.0234	0.0883	3.78
B_{U3}	gil326496613	Triosephosphate isomerase	32.30/32.68	7.04/7.04	306	16	50	0.1182	0.2896	2.45
B_{U4}	gil118430385	ATP synthase (subunit alpha, chloroplastic)	55.29/55.32	6.32/6.32	491	27	47	0.3786	0.8755	2.31
B_{U5}	gil326495216	Sedoheptulose-1,7-bisphosphatase	42.09/45.58	6.17/6.17	189	16	32	0.0191	0.1392	7.29

See more details from Table 2

metabolisms were occurred in shoots of XZ113 under salt stress.

Discussion

Salt tolerance in plants involves complex components of signaling and metabolic regulation networks, and also toughly depends on species, organ, dose and time of salt exposure (Deinlein et al. 2014; Ellouzi et al. 2014). Up to date, most studies on salt resistance are concentrated on a long-term or high salt level, so as to reveal the diverse mechanisms mainly linked to metabolic and ionic adjustment, but few studies were done to understand osmotic and ionic regulation at early stage of salt stress (Munns and Tester 2008; Deinlein et al. 2014). Our previous studies demonstrated that Tibetan wild barley had high salt tolerance (Qiu et al. 2011; Wu et al. 2011, 2013a). In this study, we used a wild barley accession with high salt tolerance, XZ113 as material, to reveal the ionic and proteomic responses to salt stress at early exposure stage. It is interesting to note that the plants could survive well under

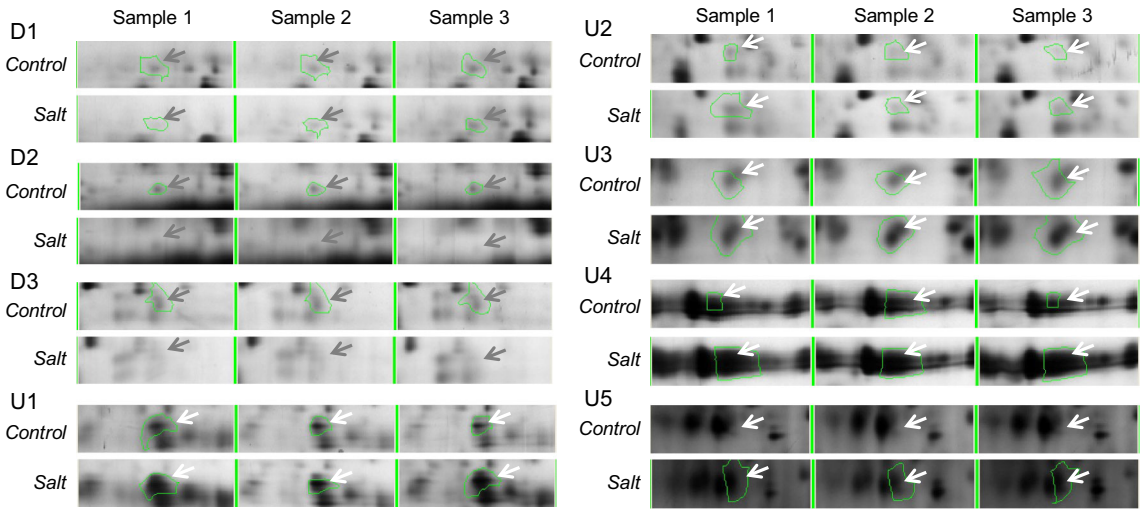

Fig. 5 'Spot view' of the abundance of differentially expressed proteins in shoots of XZ113 exposed to salt stress for 2 day. *Green circles* with *black arrows* indicate spots showing decrease (listed as *D*) and *white arrows* indicate spots showing increase (listed as *U*). Protein spot ID corresponds to *spot number* in Table 3 and Fig. 3b. (Color figure online)

200 mM NaCl, although growth was significantly inhibited relative to control, (Fig. 1a), indicating its extraordinary salt tolerance at seedling stage, and confirming the previous finding (Qiu et al. 2011).

Ionic concentrations were significantly changed under salt stress, especially for Na and K. On the whole, the response to salt stress is characterized by increased Na concentration and decreased K concentration in plant tissues. Maintaining of a high cytosolic K/Na ratio is normally considered as a key feature of salt tolerance in plants (Maathuis and Amtmann 1999; Tester and Davenport 2003; Shabala and Cuin 2008). Moreover, a K/Na ratio of around 1.0 is regarded as a minimum value for keeping ion homeostasis (Maathuis and Amtmann 1999). For example, a salt-sensitive accession of Tibetan wild barley, XZ169 showed K/Na ratios in roots and shoots less than 1.0 even under 150 mM NaCl at germination and seedling stages (Wu et al. 2013b). In the current study, K/Na ratio in roots and shoots of XZ113 was 3.48 and 1.04 at 48 h salt exposure (Table 1), respectively, indicating the capacity of maintaining an optimal K/Na ratio for conferring its salt tolerance. Otherwise, most glycophytes prefer to prevent K loss from cells rather than Na^+ exclusion (Shabala 2000; Chen et al. 2007; Zhang et al. 2012a). Chen et al. (2005) estimated salt tolerance by K^+ flux measurement and suggested the parameter of K^+ efflux is a reliable and heritable indicator of salt tolerance in barley. In this study, we found XZ113 had a strong ability of maintaining K^+, i.e. keeping a low level of efflux under salt stress and even being able to recover slow efflux in the late stage of salt exposure (Fig. 2a, b).

Meanwhile, some salt stress induced proteins were noted in both roots and shoots by 2-DE (Tables 2, 3). In roots, four induced proteins showed extremely expressed changes in comparison with controls, referred to A_{D5}, A_{U1}, A_{U4} and A_{U5}, respectively (Table 2; Fig. 4). Most of them are involved in ROS scavenging. Wu et al. (2014) identified that monodehydroascorbate reductase (A_{U1}) and class III peroxidase (A_{U2} and A_{U4}) showed nearly 2 and 0.2 fold changes of protein level at 48 h after 200 mM salinity in CM72, a well-known salt tolerant cultivar. Here, the above two proteins performed more fold increase of protein level in roots of XZ113 than that in the previous study, after salt treatment. Obviously, these proteins may be attributed to dramatic oxidative activity caused by salt stress and rapid development of antioxidant defenses system in the plant tissues of XZ113. Additionally, A_{D1} is an important component of TCA, and A_{D3} may be helpful for plasma membrane stability. A_{U5} belongs to porin family, which is well known as transmembrane proteins and has the function in cell protection as anion channels (Geiger et al. 1999; Wen et al. 2011). Besides, aquaporins are members of porin family, which transport water and low-molecular weight neutral compounds (Tyerman et al. 2002; Maurel et al. 2008; Horie et al. 2012), enhancing salt tolerance in Arabidopsis (Guo et al. 2006). It may be assumed that ATP synthases act as a pioneer in response to salt stress in shoots of XZ113, and five proteins identified in this study belong to two types of subunits (Table 3; Fig. 5). Here, four subunit α displayed the same molecular mass (55.32 kDa) with different electric charges, identified as B_{D1}, B_{D2}, B_{U1} and B_{U4} (Table 3; Fig. 5). These subunits belong to CF1 complex (Kohzuma et al. 2013). Wu et al. (2014) indicated that H^+-ATPases participated in development of ion homeostasis in salt-tolerant barley genotypes. Interestingly, fast H^+ efflux activity was observed in

the roots of XZ113 (Fig. 2c, d), which may help keeping balance voltage and pH in a proper range. Kosová et al. (2013b) suggested that many CF1 subunits in both halophytes and glycophytes involved in salt tolerance under salt stress. In addition, C3 photosynthesis cycle enzyme, SBPase (Listed as B_{U5}) increased by 7 folds in comparison with control under salt stress (Table 3), hence leading to improved photosynthesis (Rosenthal et al. 2011). Similar result was also found in cultivated barleys, a salt-tolerant genotype Afzal had more up-regulation of SBPase than a sensitive genotype L-527 (Rasoulnia et al. 2011). Therefore the higher SBPase activity should be helpful for enhancing carbon assimilation in leaves of XZ113 under salt stress. Moreover, phosphoglycerate kinase (B_{U2}) and triosephosphate isomerase (B_{U3}) involved in glycolysis pathway were significantly up-regulated after salt stress, indicating that the metabolism level of glycolysis was enhanced by salt stress in shoots of XZ113 as consistent as other findings (Rasoulnia et al. 2011; Wu et al. 2014).

In conclusion, XZ113 showed relatively less growth inhibition in early stage of salt stress exposure, as reflected by the positive GR and relatively high water content. Its high salt stress tolerance may be attributed to the capacity in maintenance of optimal K/Na ratio and high K concentration in plant tissues. Meanwhile, proteomic analysis showed that the wild barley could set up rapidly ROS scavenging enzyme system in roots when meeting an early and transient oxidative burst caused by salt stress. In shoots, ATP synthases were pioneers for tolerant development at early stress exposure stage, and they belong to different subunits, with SBPase contributing to relatively stable photosynthesis and assimilation under salt stress. Further studies are still needed to reveal the exact roles of those proteins whether acting as essential components of salt tolerance in wild barley.

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