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Ionomic Responses and Correlations Between Elements and Metabolites Under Salt Stress in Wild and Cultivated Barley

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A thorough understanding of ionic detoxification and homeostasis is imperative for improvement of salt tolerance in crops. However, the homeostasis of elements and their relationship to metabolites under salt stress have not been fully elucidated in plants. In this study, Tibetan wild barley accessions, XZ16 and XZ169, differing in salt tolerance, and a salt-tolerant cultivar CM72 were used to investigate ionomic profile changes in tissues in response to 150 and 300 mM NaCl at the germination and seedling stages. At the germination stage, the contents of Ca and Fe significantly decreased in roots, while K and S contents increased, and Ca and Mg contents decreased in shoots, after 10 d of treatment. At the seedling stage, the contents of K, Mg, P and Mn in roots and of K, Ca, Mg and S in shoots decreased significantly after 21 d of treatment. Moreover, Na had a significant negative correlation with metabolites involved in glycolysis, α-ketoglutaric acid, maleic acid and alanine in roots, and metabolites associated with the tricarboxylic acid (TCA) cycle, sucrose, polyols and aspartate in leaves. The salt-tolerant genotypes XZ16 and CM72 showed a lower Na content in tissues, and less reduction in Zn and Cu in roots, of Ca, Mg and S in leaves, and shoot DW than the sensitive genotype XZ169, when exposed to a higher salt level. The results indicated that restriction of Na accumulation and rearrangement of nutrient elements and metabolites in barley tissues are possibly attributable to development of salt tolerance.

Keywords: *Hordeum vulgare* • Ion homeostasis • Ionomics • Metabolite • Salt tolerance.

Abbreviations: ICP-OES, inductively coupled plasma-optical emission spectrometry; PCA, principal component analysis; PC1, first principal component; PC2, second principal component; TCA, tricarboxylic acid..

Introduction

Soil salinity is a global abiotic stress in agricultural systems, and nearly 20% of arable land and half of irrigated land in the world has been affected by salinity, causing a threat to crop production (Yamaguchi and Blumwald 2005, FAO 2009). Excess intracellular sodium (Na) accumulation leads to ion imbalance and toxicity when plants are subjected to salt stress (Zhu 2001, Zhu 2003, Munns 2005). Soil salinity not only inhibits macroelement uptake into tissues, including potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P) and sulfur (S), but also restricts the absorption of microelements, such as copper (Cu), iron (Fe), manganese (Mn), zinc (Zn) and boron (B), thus leading to nutrient deficiency and metabolic disorder in cells (Munns and Tester 2008, Munns 2010, Tavakkoli et al. 2011). Among these elements, N, K, P and S are required in relatively large amounts for plant growth, and the type and amount of metabolites could be greatly altered when these mineral nutrients are deficient in plants (Amtmann and Armengaud 2009). Hence, understanding of mechanisms of ionic detoxification and homeostasis is imperative for improvement of salt tolerance in crops. However, progress in developing salt-tolerant crops is significantly hampered by the physiological and genetic complexity of this trait.

It is well documented that the detoxification of Na and ion homeostasis are crucial for salt tolerance in plants. Plants have developed ion homeostasis through Na and K transport and Na exclusions to adapt to salt stress (Niu et al. 1995, Munns and Tester 2008). However, the responses of other elements and their interactions to salt stress have not been fully elucidated to date. Most available research was only focused on the effects of salinity on one or a few elements (Davenport et al. 1997, Loupassaki et al. 2002, Chen et al. 2005, Pandya et al. 2004, Yousfi et al. 2007, Nazar et al. 2011). The ionome is defined as the mineral nutrient and trace element composition of an organism or tissue, and represents the inorganic component of cellular and organismal systems. Ionomics, the study of the ionome, involves the quantitative and simultaneous measurement of the elemental composition of organisms or tissues and changes in this composition in response to physiological processes, and requires the application of high-throughput elemental analysis technologies and their integration with both bioinformatic and genetic tools (Salt et al. 2008). There

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are four techniques with potential application in ionomics studies, namely inductively coupled plasma-mass spectrometry (ICP-MS), inductively coupled plasma-atom/optical emission spectrometry (ICP-AES/OES), X-ray fluorescence (XRF) and neutron activation analysis (NAA). Several recent studies described high-throughput elemental profiling studies of how the ionome responds to the environment or explores the genetics that control the ionome (Baxter 2009). In Arabidopsis, the leaf ionome was analyzed by ICP-MS, and multivariable ionomic signatures were established to define physiological responses, such as iron (Fe) and phosphorus (P) homeostasis (Baxter et al. 2008). Ionomics has also been applied in understanding the mechanisms of salt tolerance. Sanchez et al. (2011) used ICP-AES for ionomics studies and combined this with metabolite profiling to compare the extremophile *Lotus creticus*, adapted to highly saline coastal regions, and two cultivated glycophytic grassland forage species, *Lotus corniculatus* and *Lotus tenuis*, which demonstrated a differential rearrangement of shoot nutrient levels in the extremophile upon salt exposure. Currently, ionomics are developed and applied in understanding multiple physiological processes in plants, in combination with other platforms such as transcriptomics, proteomics and metabolomics (Salt et al. 2004, Salt et al. 2008). In a previous study, we used a metabolomic method to reveal the physiological and molecular difference in salt tolerance between wild barley (*Hordeum spontaneum*) and cultivated barley (*Hordeum vulgare*) through analyzing the changes in the contents of 82 metabolites in roots and leaves in response to salinity stress (Wu et al. 2013). However, the relationship between ionic responses and metabolite accumulation in barley tissues under salt stress has not been examined.

Barley is the fourth most important cereal crop in the world, characterized by its wide adaptability and high salinity tolerance. Consequently, it is frequently used as a model crop in attempts to understand salinity tolerance in the cereal crops. In particular, wild barley has adapted to a broad range of environmental conditions and formed rich genetic diversities for salt tolerance (Nevo and Chen 2010). For instance, Tibetan wild barley is considered as one of the ancestors of cultivated barley (Xu 1982, Dai et al. 2012), and it is characterized by a wide variation of abiotic stress tolerance. Some accessions with high tolerance to salinity (Qiu et al. 2011, Wu et al. 2011), drought (Zhao et al. 2010) and aluminum toxicity (Dai et al. 2011) have been identified. It is possible for us to explore the elite germplasm in terms of salt tolerance from the wild barley. In our previous study, we have assessed the salt tolerance of 188 accessions of Tibetan annual wild barley and identified some elite salt-tolerant accessions (e.g. XZ16 and XZ26), and the metabolite profiles in wild and cultivated barley in response to salt stress were also determined (Wu et al. 2013). In this study, two accessions from Tibetan wild barley contrasting in salt tolerance, and a well-known salt-tolerant cultivar CM72 (Chen et al. 2005, Chen et al. 2007a), were used to investigate the ionome changes in response to moderate (150 mM NaCl) and high salinity (300 mM NaCl) using ICP-OES at the germination and seedling stages. Moreover, the correlations between ionic and metabolic contents in tissues of wild and cultivated barley were analyzed when plants were exposed to high salinity. The main objectives of this work are to (i) investigate changes in the barley ionome in response to salt stress; (ii) determine the possible difference in tissue ionome responses to salt stress within genotypes and across growth stages; and (iii) provide insights into the physiological mechanisms of ionic homeostasis and metabolic responses to salt stress in barley.

Results

Physiological difference at the germination stage

There was an obvious genotypic difference in germination rate, root and shoot length among the three genotypes after salt treatment (**Fig. 1**). Salt stress reduced the germination rate by 14, 6 and 13% in CM72, XZ16 and XZ169, respectively (**Fig. 1a**). Only the germination rate of XZ16 showed no significant difference between control and treatment (**Fig. 1a**). The lengths of roots and shoots were also significantly affected by salt stress, which was evident by the reduced relative root length at 74, 80 and 62%, and relative shoot length at 79, 78 and 74% for CM72, XZ16 and XZ169, respectively (**Fig. 1b**). However, the physiological parameters were not measured for 300 mM NaCl treatment due to the poor germination (<20%) and tissue formation of all genotypes. According to the germination performance, the genotypes were ranked as XZ16 >CM72 >XZ169 for their salt tolerance at the germination stage.

Tissue ionomic responses to salt stress at the germination stage

In order to reveal the effect of salt stress on the distribution of elements at the germination stage, the contents of Na, K, Ca, Mg, P, S, Mn, Zn, Fe and Cu in roots and shoots were analyzed. A clear separation between samples within treatments and among genotypes was detected according to the principal components analysis (PCA) (**Fig. 2**). The control and treatment samples in both roots and leaves were clearly separated by the first principal component (PC1), representing 34.7% and 44.1% of the total variation, respectively (**Fig. 2a, c**). The major elements which contributed to the PC1 were Na, Mn and Ca in the root ionome, and Na, Mg and Ca in the shoot iomome (**Fig. 2b, d**). The second principal component (PC2) clearly distinguished the samples of XZ169 from those of CM72 and XZ16 in roots and shoots, explaining 23.7% and 31.1% of the variation, respectively (**Fig. 2a, c**). The contribution of elements to the PC2 was dominated by P and Cu in the root ionome, while Zn and Mn were dominant in the shoot ionome (**Fig. 2b, d**).

After 10 d treatment, the Na content in roots was significantly increased from 2.51 to 34.79 mg g^{-1} DW, averaged over the three genotypes. The distribution of Ca, Mn and Fe in roots was significantly reduced by 13.2, 19.4 and 22.9%, respectively, in comparison with the respective control (**Table 1**). In shoots,

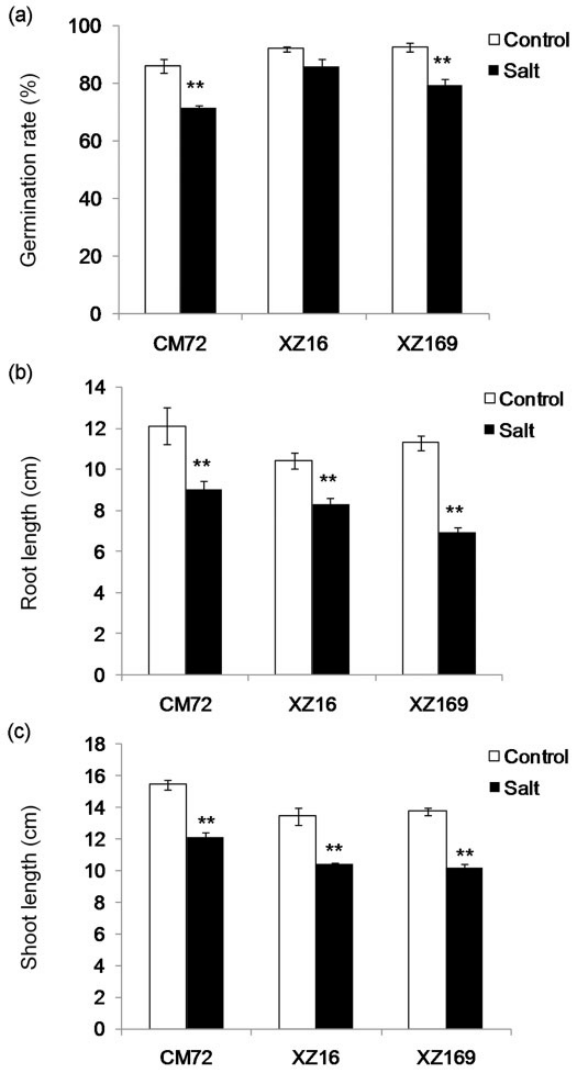

Fig. 1 Growth performances of three barley genotypes under control and salt stress conditions at the germination stage. (a) Germination rates under control and 150 mM NaCl conditions ($n = 4$, bars show the SE); (b) Root length and (c) shoot length after germination for 10 d ($n = 8$, bars show the SE). * and ** indicate significant ($P < 0.05$) and highly significant ($P < 0.01$) differences between controls and treatments, respectively.

the Na content was remarkably increased under salt stress, averaged over the three genotypes, from 2.24 to 25.67 mg g^{-1} DW, whereas the distribution of K and S was significantly enhanced, increasing by 21.4% and 49.9%, respectively, but the opposite was true for Ca and Mg, which were reduced by 48.3% and 52.8%, respectively (**Table 1**).

There was a genotypic difference in the changes of the element content under salt stress. XZ169 had a higher Na content in roots than CM72 and XZ16 (**Table 1**). Notably, the K content was significantly reduced by 32.2% in roots of CM72, and Mn was reduced by 43.6% in roots of XZ16. Meanwhile, the S content was significantly increased by 47.8% in the roots of XZ169. In shoots, XZ169 had lower and higher contents of Na and K in shoots than the other two genotypes, respectively (**Table 1**). S and Cu contents were increased by 40.9% and 41.2% in shoots of XZ169, respectively, but no significant difference was detected between salt stress and control in shoots of CM72 and XZ16. Mn and Fe contents in shoots of XZ16 showed an increase and decrease, respectively, under salt stress (**Table 1**).

Physiological difference at the seedling stage

At the seedling stage, CM72, XZ16 and XZ169 were exposed to 150 and 300 mM NaCl in hydroponics. After 21 d of treatment, significant genotypic differences were detected in plant growth parameters (**Fig. 3**). Under moderate (150 mM) salinity, only the shoot DW of XZ169 showed a significant reduction relative to the control (**Fig. 3a, b**). However, when exposed to 300 mM NaCl, all genotypes showed a significant reduction in shoot DW, with relative shoot DW being reduced by 42, 41 and 69% for CM72, XZ16 and XZ169, respectively (**Fig. 3a, b**). Based on growth inhibition under salt stress at this stage, salt tolerance for the three genotypes could be ranked in the order XZ16 >CM72 >XZ169, being consistent with the rank at the germination stage.

Tissue ionomic responses to salt stress at the seedling stage

According to the PCA, there was a distinct separation of samples between controls and moderate salinity in both roots and leaves (**Fig. 4**), which was distinguished by the PC1, explaining 55.8% and 46.0% of the total variation, respectively (**Fig. 4a, c**). The factors contributing to the PC1 were dominated by Na, Ca, K, Mg and P in the root ionome, and by Na, Mg and S in the leaf ionome (**Fig. 4b, d**). The PC2 basically could separate the samples of the tolerant and sensitive genotypes (**Fig. 4a, c**). Similarly, the PC1 could explain 44.9% and 65.0% of the total variation of the root and leaf ionome, under high salinity, respectively (**Fig. 5**). However, the separation of genotypes was not clearly identified by the PC2 either in the control or under salt stress (**Figs. 4a, c, 5a, c**).

Under moderate salinity, the Na content in tissues was significantly increased, averaged over the three genotypes, being up to 24.41 and 40.69 mg g^{-1} DW in roots and leaves, respectively. However, the contents of all other elements except Cu in roots, and K, Ca, Mg and S in leaves were significantly reduced. Averaged over the three genotypes, the mean reduction of these elements ranged from 27.5% for Ca to 58.3% for K (**Table 2**). Moreover, genotypic difference could be found in the changes in the contents of these element under salt stress. Generally, Tibetan wild barley had much higher contents of Na and K in roots and leaves than CM72 (**Table 2**). XZ169 showed the greatest reduction in Ca, Mg and S contents in leaves, being 64.8, 55.2 and 60.0%, respectively (**Table 2**).

Under high salinity, Na content was dramatically increased in roots and leaves, being up to 36.77 and 40.12 mg g^{-1} DW, averaged over the three genotypes, respectively Meanwhile, the

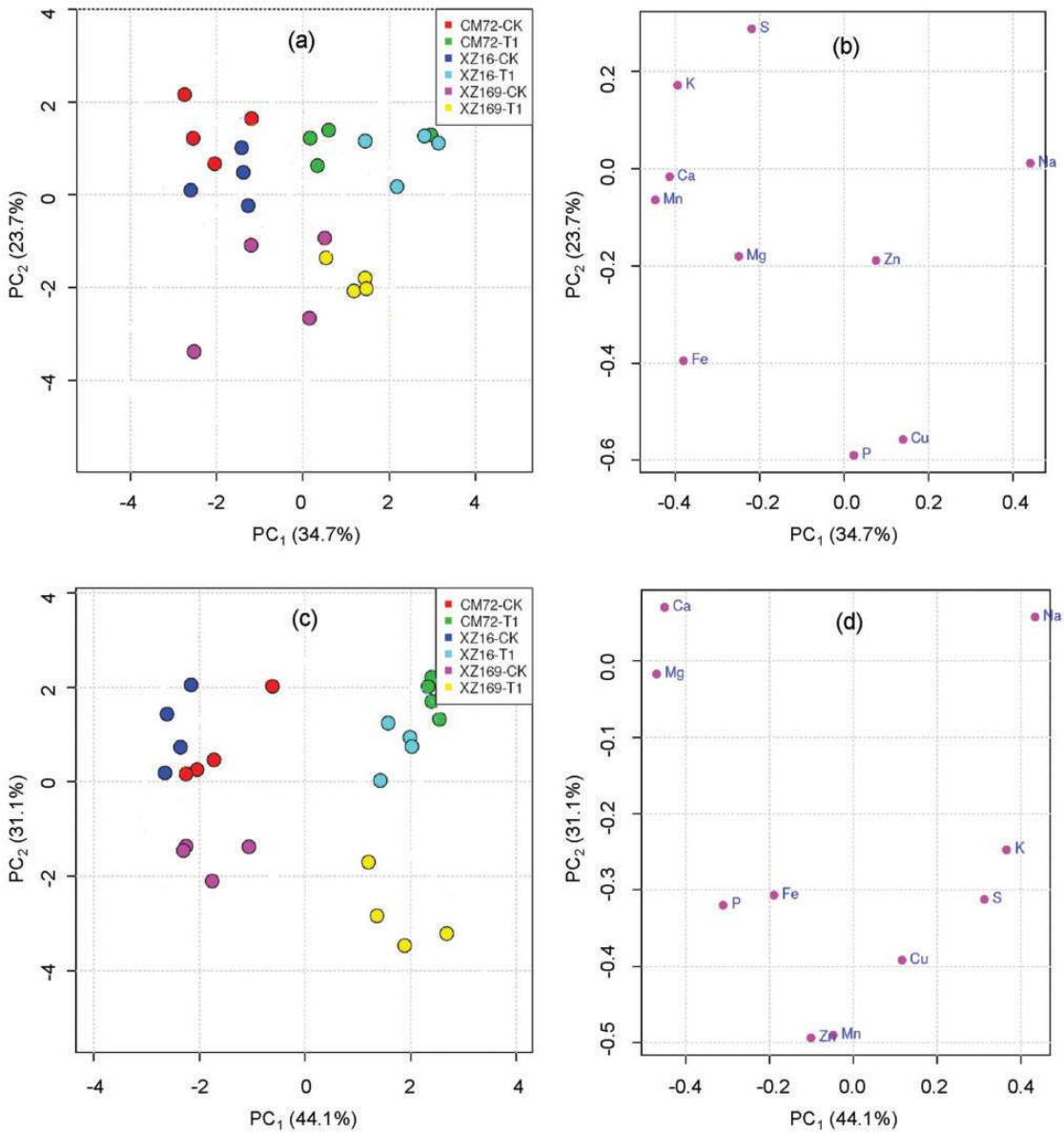

Fig. 2 Tissue ionome variation analysis using PCA at the germination stage and the loadings of elements to the PC1 and PC2. (a) Root ionome variation among samples; (b) the loadings of elements to the PC1 and PC2 in roots; (c) shoot ionome variation among samples; and (d) the loadings of elements to the PC1 and PC2 in shoots. For PCA, the variances in a data set (X) without referring to class labels (Y) are summarized into many fewer variables called scores which are the weighted average of the original variables. CK, controls; T1, 150 mM NaCl; PC1, the first principal component; PC2, the second principal component.

contents of all measured elements, except Zn, Cu and Fe in roots and Fe in leaves, showed a significant reduction, ranging from 17.1% for Ca to 82.1% for Mg (**Table 2**). There was a notable genotypic difference in the response of element content changes to high salinity. XZ169 had the highest Na content among the three genotypes, being 45.55 and 48.35 mg g^{-1} DW in roots and leaves, respectively (**Table 2**). Moreover, XZ169 showed a reduction of 19.3% for Zn content and 14.5% for Cu content in roots under salt stress relative to control, but such a reduction was not detected in roots of CM72 and XZ16 (**Table 2**). For other element contents, significant changes occurred under salt stress, and there was no significant difference among genotypes.

Correlations of elements and metabolites in roots and leaves

The changes in the contents of major metabolites in roots and leaves of CM72 and XZ16 after 21 d salt treatment are shown in **Supplementary Table S1**. Here, the correlations among 10 elements and between these elements and metabolites were determined. In roots, Na had a significant or highly significant negative correlation with K and P, and Mg, respectively.

Table 1 Element contents (mg g^{-1} DW) in roots and shoots of three barley genotypes at the germination stage under control and salt stress conditions ($n = 4$)

Tissue	Treatment	Genotype	Na	K	Ca	Mg	P	S	Zn	Mn	Fe	Cu
Root	Control	CM72	2.305c	6.564a	1.114a,b	1.477a	2.774b	0.989a	0.129a	0.037a,b	0.091a,b	0.010b
		XZ16	1.453c	4.756b	1.155a	1.701a	2.735b	0.675b.c	0.139a	0.039a	0.092a,b	0.010b
		XZ169	3.781c	4.750b	1.159a	1.585a	3.785a	0.508c	0.124a	0.031a,b	0.106a	0.021a
	150 mM	CM72	35.12a,b	4.449b	1.029b,c	1.590a	2.579b	0.770a,b	0.128a	0.030b,c	0.070c	0.012b
		XZ16	32.47b	4.050b	1.007c	1.419a	2.767b	0.555b,c	0.133a	0.022c	0.064c	0.012b
		XZ169	36.77a	4.389b	0.940c	1.596a	4.159a	0.751b	0.149a	0.033ab	0.089b	0.021a
Shoot	Control	CM72	1.459c	13.41c	1.482a	2.310b	6.135ab	0.468bc	0.077bc	0.023bc	0.074ab	0.016b
		XZ16	1.613c	10.89d	1.388a	2.607a	6.210ab	0.340c	0.085b	0.022cd	0.073ab	0.016b
		XZ169	3.648c	12.59c	1.256b	2.468a,b	6.499a	0.636b	0.106a	0.029a	0.076a,b	0.017b
	150 mM	CM72	28.03a	14.72b	0.710c	1.075c	5.305c	0.684a,b	0.066c	0.021d	0.069b,c	0.014b
		XZ16	26.33a	13.61b,c	0.718c	1.167c	5.796b,c	0.583b,c	0.081b	0.025b	0.063c	0.018b
		XZ169	22.64b	16.47a	0.706c	1.240c	6.138a,b	0.896a	0.108a	0.027a	0.077a	0.024a

Different letters in the same column represent a significant difference at 95% probability.

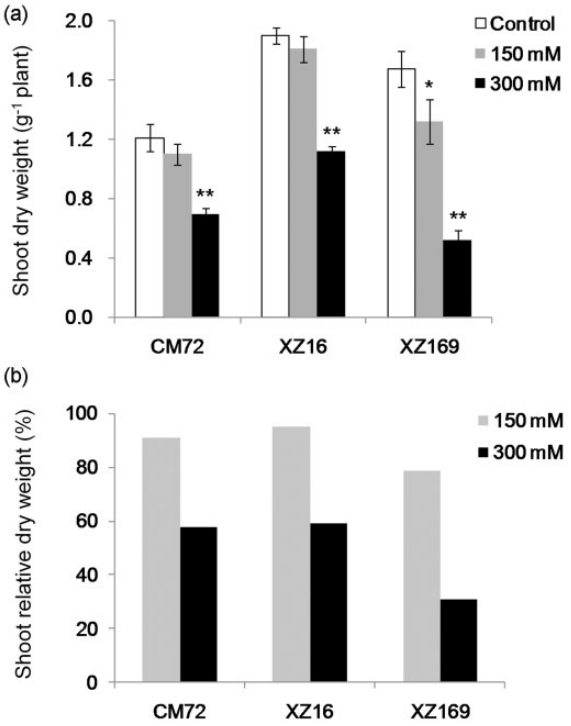

Fig. 3 Shoot growth performances of the three barley genotypes under control and salt stress conditions at the seedling stage. (a) Shoot DW of plants under control and salt conditions ($n = 4$, bars show the SE); * and ** indicate significant ($P < 0.05$) and highly significant ($P < 0.01$) differences between controls and treatments, respectively. (b) Shoot relative DW of plants after 21 d of salt treatment.

In contrast, a significantly positive correlation was found between Na and Cu (**Fig. 6**). In leaves, Na had a highly significant negative correlation with K, Ca, Mg, S and Mn, and a significant negative correlation with P (**Fig. 7**).

A significant correlation was detected between elements and metabolites in both roots and leaves. In roots, Na had a significant negative correlation with the metabolites involved in glycolysis such as fructose-6-phosphate, glucose-6-phosphate and pyruvate, α-ketoglutaric acid, maleic acid and alanine, whereas the opposite correlation between Na and sugars, polyols (i.e. mannitol, inositol and xylitol) and citric acid could be found. On the other hand, K, P and Mg had significantly positive correlations with metabolites involved in glycolysis, the tricarboxylic aid cycle (TCA) cycle and alanine synthesis, and had negative correlations with sugars and polyols (**Fig. 6**). For most microelements, significantly negative correlations could be detected between them and metabolites in roots, including Zn and mannose, fumaric acid, Mn and maltose, Fe and malic acid, Cu and organic acids, and alanine and aspartate, although there was a positive correlation between Cu and polyols. In leaves, Na had significantly negative correlations with metabolites associated with the TCA cycle, polyols and aspartate, but had positive correlations with glyceraldehyde-3-phosphate and raffinose. In addition, the correlations between other elements and metabolites were the opposite to the correlations between Na and metabolites (**Figs. 6**, **7**).

Discussion

In this study, we attempted to determine ionomic responses of barley tissues to salt stress at different growth stages. Salt tolerance at the early growth stage is important because the initial performance of plants has a dramatic effect on subsequent growth and development. Seed germination is a first step in plant development as well as exposure to salt stress (Munns 2005, Zhang et al. 2010). Germination tests have in fact been commonly used to identify genotypic difference in salt tolerance in rice (Hakim et al. 2010), wheat (Almansouri et al. 2001), maize (Carpici et al. 2009) and soybean (Hosseini et al. 2002). A distinct difference was detected among barley genotypes in salt tolerance at this stage (Tajbakhsh et al. 2006, Zhang et al. 2010, Kirmizi and Bell 2012). A barley cultivar, CM72, recognized as a salt-tolerant cultivar (Chen et al. 2005, Chen et al. 2007a), is widely used as a reference genotype for

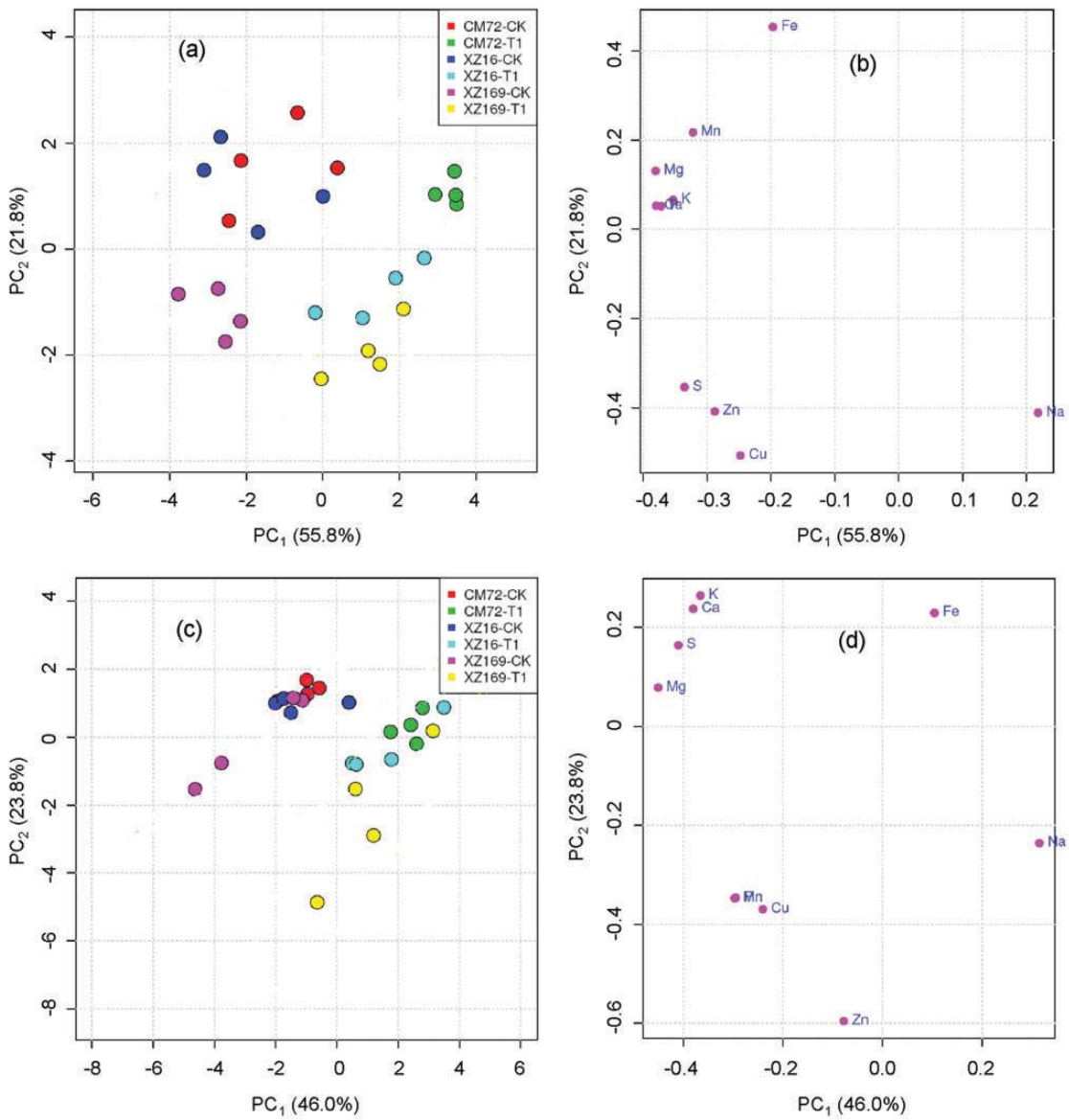

Fig. 4 Tissue ionome variation analysis using PCA at the seedling stage under control and 150 mM salt stress conditions and the loadings of the elements to the PC1 and PC2. (a) Root ionome variation among samples; (b) the loadings of elements to the PC1 and PC2 in roots; (c) leaf ionome variation among samples; and (d) the loadings of elements to the PC1 and PC2 in leaves. CK, controls; T1, 150 mM NaCl; PC1, the first principal component; PC2, the second principal component.

physiological studies on salt tolerance. In previous studies, XZ16 was identified as a salt-tolerant and XZ169 as a salt-sensitive wild barley according to growth parameters at the seedling stage (Wu et al. 2011, Wu et al. 2013). Here, the current results obtained at the germination stage confirmed the previous findings that salt tolerance was in the order XZ16 >CM72 >XZ169.

Na causes ion imbalance and oxidative stress in plants (Zhu 2003, Munns and Tester 2008). Thus, it is imperative to understand the responses of ion and element changes to salt stress in tissues, so as to reveal the mechanisms of ion homeostasis underlying salt tolerance. The effect of salt stress on the germination percentage varies with genotype. For instance, in −1.2 MPa NaCl solutions, CM72 and Numar had >90% germination, while YUQS and ZND3 had only 57% germination (Tajbakhsh et al. 2006). Similar results have also been reported (Zhang et al. 2010, Kirmizi and Bell 2012). In this study, under 150 mM NaCl, XZ16 had 94% germination, much higher than that of CM72 and XZ16 (**Fig. 1**). In addition, root and shoot growth was also affected under salt stress. Excess intracellular Na accumulation causes osmotic and oxidative stresses, thus restraining cell elongation of the roots and shoots (Munns and Tester 2008, Munns 2010). Our results revealed that all three genotypes showed a dramatic increase in Na content in roots

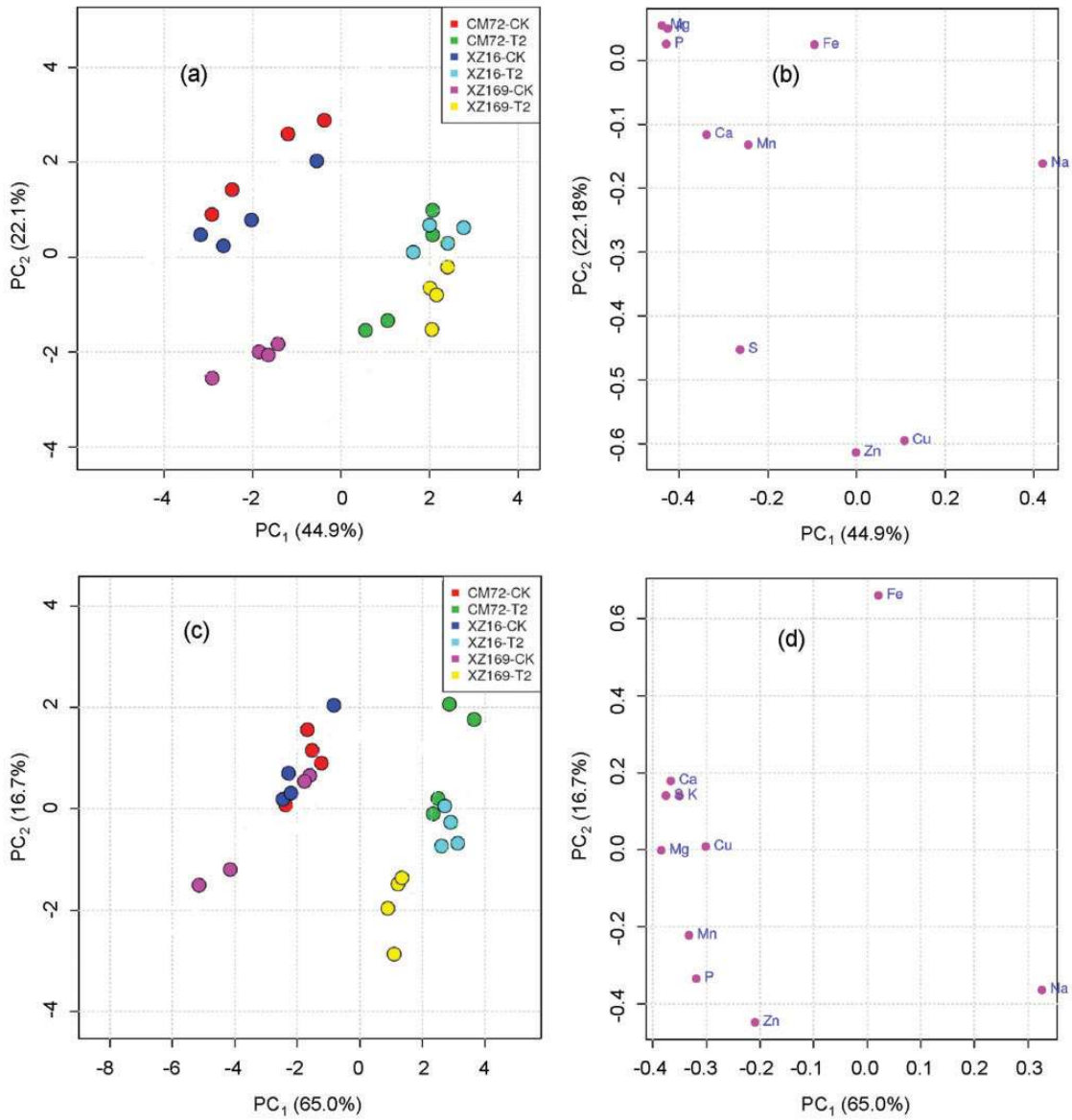

Fig. 5 Tissue ionome variation analysis using the PCA at the seedling stage under control and 300 mM salt stress conditions and the loadings of elements to the PC1 and PC2. (a) Root ionome variation among samples; (b) the loadings of elements to the PC1 and PC2 in roots; (c) leaf ionome variation among samples; and (d) the loadings of elements to the PC1 and PC2 in leaves. CK, controls; T1, 150 mM NaCl; PC1, the first principal component; PC2, the second principal component.

and shoots, a significant reduction in K content in roots and an increase in shoots when exposed to salt stress. Additionally, the salt-sensitive genotype XZ169 had a much higher Na content in tissues than the salt-tolerant genotype XZ16 (**Table 1**). Therefore, lower Na accumulation and a higher K/Na ratio are considered as the mechanism responsible for tolerant genotypes. On the other hand, we found that Ca, Mn and Fe contents significantly decreased in roots, while Ca and S contents increased in shoots under salt stress. It was reported that salt stress had effects on the uptake and distribution of nutrient elements in plant tissues (Shabala et al. 2005, Kirmizi and Bell 2012).

Many growth and physiological parameters at the seedling stage are used to indentify salt tolerance, including root length (Rahnama et al. 2011), leaf cell elongation (Fricke et al. 2006), relative DW (Qiu et al. 2011, Wu et al. 2011), Na and K uptake (Chen et al. 2005, Tajbakhsh et al. 2006, Chen et al. 2007b) and the K/Na ratio (Chen et al. 2007a, Shabala et al. 2010). However, to date, there are no simple or 'standard' physiological traits which can be commonly used in evaluating salt tolerance of barley. Plant DW as well as relative DW should be a more real and reliable trait indicating growth performance under salt stress (Munns 2010). Here, we used DW and relative DW of shoots to assess salt tolerance of the three genotypes. The

Table 2 Element contents (mg g^{-1} DW) in roots and leaves of the three barley genotypes at the seedling stage under control and salt stress conditions ($n = 4$)

Tissue	Treatment	Genotype	Na	K	Ca	Mg	P	S	Zn	Mn	Fe	Cu
Root	Control	CM72	4.369d	13.33a	3.577a	6.112a	6.950a	3.923b	0.741c,d	0.824a,b	1.991a,b	0.023c
		XZ16	3.383d	11.00a	3.493a	7.179a	7.574a	4.817b	1.289b,c	0.970a,b	2.376a,b	0.028c
		XZ169	4.329d	12.51a	3.813a	6.468a	6.054a,b	6.832a	2.482a	1.100a	1.923a,b	0.062a
	150 mM	CM72	14.43c	3.146c	1.948b	0.955c	2.730c	2.188c	0.253d	0.376c	1.444a,b	0.013d
		XZ16	30.78b	7.267b	3.296a,b	2.562b	4.542b,c	3.683b,c	1.022c,d	0.285c	1.083b	0.036c
		XZ169	28.01b	4.933b,c	2.642a,b	2.041b,c	4.551b,c	4.790b	1.846b	0.319c	1.017b	0.049a,b
	300 mM	CM72	31.60b	2.404c	3.706a	1.159c	3.342c	3.463b,c	1.567b	0.771a,b	2.730a	0.044b
		XZ16	32.17b	2.844c	2.672a,b	1.117c	4.031b,c	3.985b	1.665b,c	0.588b,c	1.690b	0.040b
		XZ169	45.55a	2.633c	2.644a,b	1.250c	4.588b,c	4.443b	2.004b	0.816a,b	1.427b	0.053a,b
Shoot	Control	CM72	4.115c	53.38a	7.580a	4.808b	8.327a,b	4.904a,b	0.299a,b	0.091b	0.190a,b	0.022a,b
		XZ16	3.984c	55.26a	7.223a	4.859b	8.258a,b	4.396b	0.278a,b	0.093b	0.183a,b	0.031a
		XZ169	4.028c	50.44a	7.636a	6.401a	10.07a	5.521a	0.360a,b	0.175a	0.165a,b	0.026a,b
	150 mM	CM72	39.37b	21.49b,c	4.076b	2.432c	8.080b,c	2.999c	0.303a,b	0.088b	0.195a	0.017a,b
		XZ16	42.71a,b	23.54b,c	3.358b	3.008c	8.232a,b	3.059c	0.380a,b	0.098b	0.168a,b	0.018a,b
		XZ169	40.00a,b	26.29b	2.691b	2.867c	9.239a,b	2.206c,d	0.507a	0.124b	0.132b	0.024a
	300 mM	CM72	35.56b	18.39c	3.292b	1.671c	5.532c	1.418d,e	0.123b	0.037c	0.203a,b	0.014b
		XZ16	36.45b	21.22b,c	2.468b	1.727c	6.045c	1.015e	0.181b	0.037c	0.152a,b	0.014a,b
		XZ169	48.35a	25.95b	2.828b	2.469c	8.509a,b	1.256d,e	0.454a,b	0.082b,c	0.144a,b	0.021a,b

Different letters in the same column represent a significant difference at 95% probability.

results showed that XZ16 exhibited a smaller change in absolute DW under 150 and 300 mM NaCl in comparison with CM72 and XZ169 (**Fig. 3**), this being consistent with previous findings (Wu et al. 2011, Wu et al. 2013). Hence, XZ16 could be considered as an elite genotype with improved salt tolerance.

Accumulation of many nutrient elements is greatly inhibited in barley tissues at the seedling stage when plants are exposed to salt stress (Yang et al. 2009, Tavakkoli et al. 2010). It is reported that salt stress reduces nutrient element uptake by plants, such as K (Chen et al. 2005, Shabala et al. 2005, Shabala et al. 2010), Ca (Porcelli et al. 1995, Davenport et al. 1997, Kopittke 2012), Mg (Loupassaki et al. 2002, Tavakkoli et al. 2011), P (Martinez et al. 1996, Loupassaki et al. 2002), S (Nazar et al. 2011), Mn (Pandya et al. 2004), Fe (Yousfi et al. 2007) and Zn (Khoshgoftarmanesh et al. 2004). However, most studies were only focused on one or a few elements, and little research was done on ionomic responses to salt stress in barley, especially for wild barley germplasm. Currently, ionomics is an emerging powerful tool for physiological studies in plants. Sanchez et al. (2011) used ICP-AES to analyze the composition of 11 elements in shoots of three *Lotus* species and found that a different rearrangement of shoot nutrient levels occurred under salt stress. In the present study, we analyzed 10 elements in roots and leaves of wild and cultivated barley under 150 and 300 mM salt stress, in order to understand the rearrangement of the elements in response to moderate and high salinity. Among these elements, K, Ca, Mg and S were significantly reduced in tissues under both 150 and 300 mM salt stress, averaged over the three genotypes (**Table 2**). Ca, K, Fe and Mn contents in roots and Ca and Mg contents in leaves were significantly reduced at both stages under 150 mM salt stress. A significant reduction of P, S, Mg and Zn contents in roots, and K and S contents in leaves only occurred at the seedling stage, whereas K and S contents in shoots increased at the germination stage. It was reported that the accumulation of these nutrient elements in some plants would be inhibited by salt stress (Loupassaki et al. 2002, Yousfi et al. 2007, Tavakkoli et al. 2011, Kopittke 2012). The deficiency of any nutrient element will alter plant metabolism, with a subsequent impact on metabolite composition (Amtmann and Armengaud 2009). In our previous study, the results of the metabolomics analysis showed that accumulation of some sugars, including sucrose, trehalose and turanose, was enhanced in roots, but that of fructose-6-phosphate, glucose-6-phosphate and glyceraldehyde-3-phosphate, which are involved in glycolysis, was reduced when plants were exposed to high salinity. In contrast, metabolites associated with glycolysis and amino acid syntheses were increased, while metabolites associated with the TCA cycle were reduced (Wu et al. 2013). Here, we found that there were strong correlations among elements and between elements and metabolites in barley roots and leaves (**Figs. 6**, **7**). It may be suggested that barley develops its adaptation to salt stress through ionic homeostasis and the interaction of ions with metabolite production in tissues.

Barley has the highest tolerance to salinity among cereal crops and can tolerate higher Na content in tissues (Chen et al. 2005, Shabala et al. 2005, Tavakkoli et al. 2011). In this study, the salt-sensitive genotype XZ169 showed more growth reduction and higher Na content than the two tolerant genotypes. Under high salinity, TCA cycle intermediates and sugar accumulation were enhanced, but glycolysis and amino acid synthesis were inhibited in roots. In contrast, metabolites associated with glycolysis and amino acid synthesis appeared to be enhanced in leaves (Wu et al. 2013). We also found that Na has a significantly negative correlation with glycolysis in roots and the TCA cycle in leaves, under high salt stress (**Figs. 6**, **7**). It may be assumed that the salt-sensitive genotype is more affected in terms of metabolism by salt stress. Na exclusion is considered as

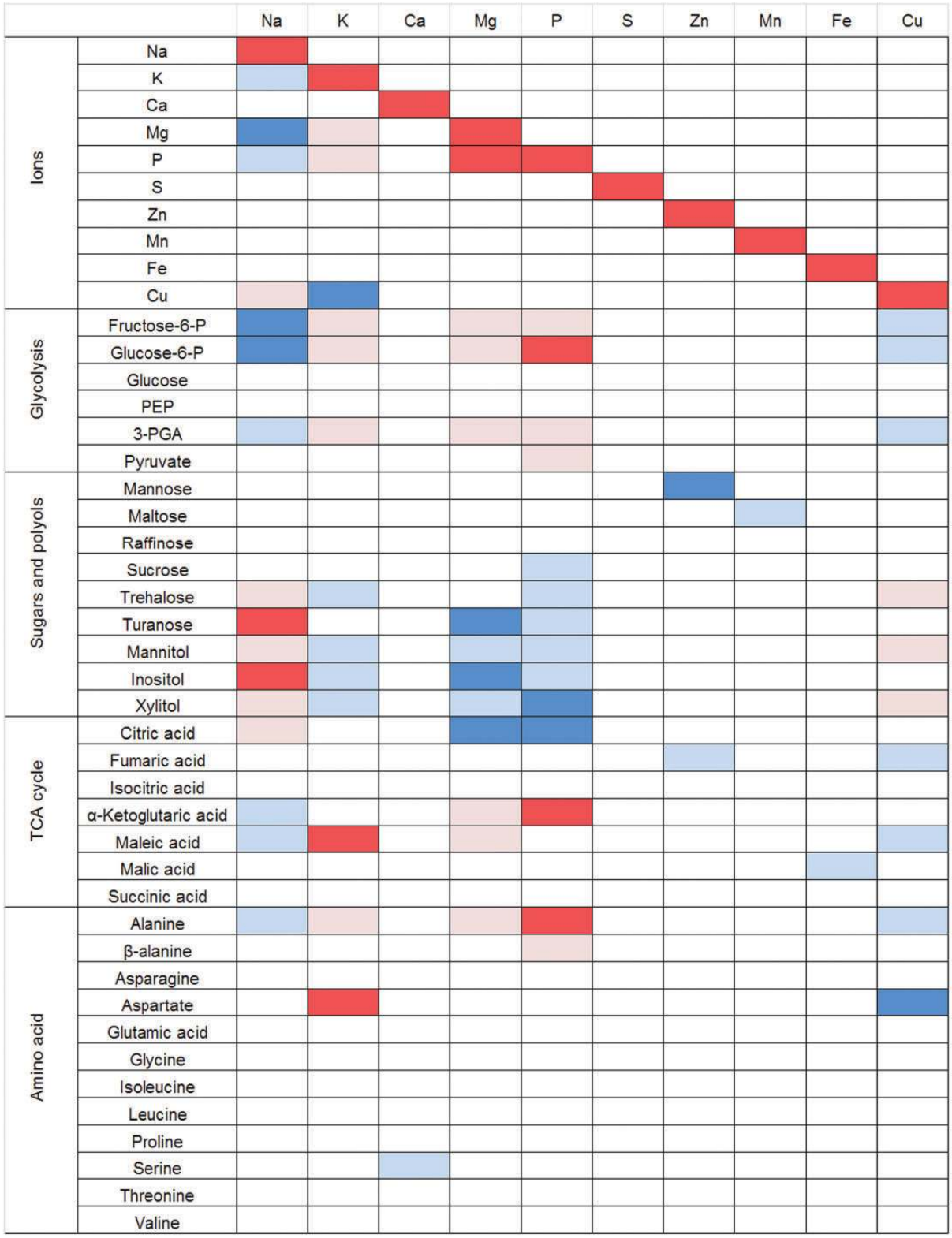

Fig. 6 The correlation among elements and between elements and metabolites in roots of CM72 and XZ16 at the seedling stage under control and 300 mM salt stress conditions. Dark blue and light blue color indicate a significantly negative correlation at the $P < 0.01$ and $P < 0.05$ level, while red and light red color indicate a significantly positive correlation at the $P < 0.01$ and $P < 0.05$ level, respectively.

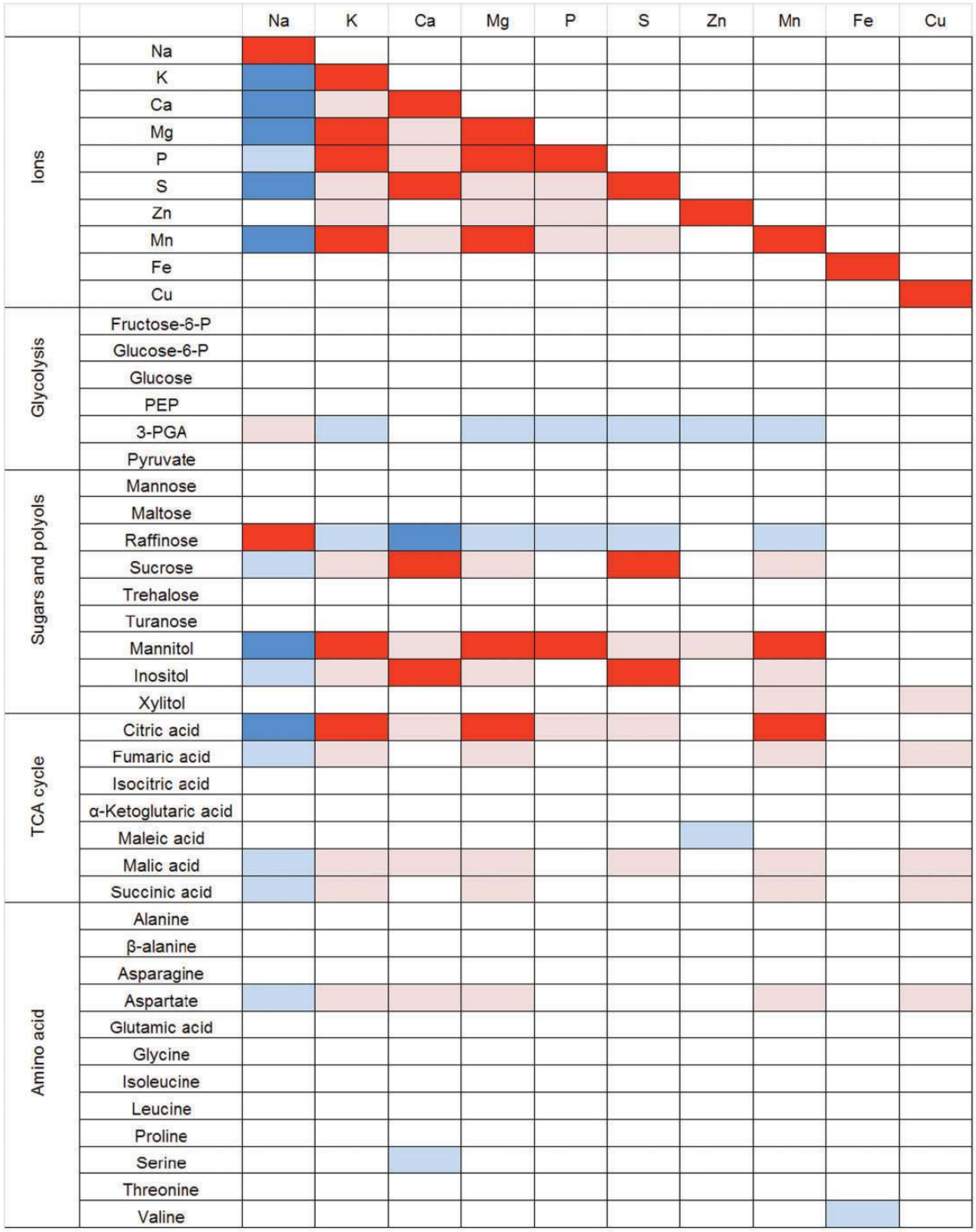

Fig. 7 The correlation among elements and between elements and metabolites in leaves of CM72 and XZ16 at the seedling stage under control and 300 mM salt stress conditions. Dark blue and light blue color indicate a significantly negative correlation at the $P < 0.01$ and $P < 0.05$ level, while red and light red color indicate a significantly positive correlation at the $P < 0.01$ and $P < 0.05$ level, respectively.

an important mechanism for salt tolerance in barley, which is associated with Na transportation from roots to shoots and Na exclusion though ion transporters (Chen et al. 2007b, Shabala and Cuin 2007, Munns and Tester 2008, Shabala et al. 2010). Moreover, XZ169 also showed a dramatic reduction in Ca, Mg and S contents in leaves under 150 mM salt stress, and reduced Zn content by 19.3%, and Cu content by 14.5% in roots under 300 mM salt stress (**Table 2**). Moreover, we found that Mg had significantly positive correlations with metabolites involved in glycolysis, the TCA cycle and alanine synthesis in both barley roots and leaves under salt stress (**Figs. 6**, **7**). Nutrient elements play important roles in metabolic synthesis and physiological processes. K uptake is inhibited by salt stress, and maintenance of a lower Na/K ratio is an important mechanism for salt tolerance in plants (Zhu 2002, Zhu 2003, Munns and Tester 2008, Shabala et al. 2010). Significant positive correlations between K and metabolites involved in glycolysis, the TCA cycle and alanine synthesis were detected in this study. Ca is recognized as a signal in abiotic stress, and it is related to development of salt tolerance (Bressan et al. 1998, Zhu 2001, Kopittke et al. 2012). Transport of Ca from roots to shoots was restricted by salinity (Lynch and Lauchli 1985, Halperin et al. 1997, Davenport et al. 1997). S is a component of many important biological compounds, including amino acids and the tripeptide glutathione (Amtmann and Armengaud 2009). In addition, Cu and Fe are components of some enzymes in plants and play crucial roles in activation of enzymes and redox reactions (Zhu 2001, Yousfi et al. 2007). In order to alleviate the toxicity of salinity, supplemental Ca, K, S and Zn were used in hydroponic and soil studies to reduce the nutrient deficiency (Pandya et al. 2004, Nazar et al. 2011, Genc et al. 2010, Kopittke 2012).

Based on ionomics analysis, the possible mechanisms for tissue ionomic response to salt stress at the germination stage may be assumed to be: (i) restriction of Na accumulation in tissues; (ii) maintenance of Cu, P, S, Mg and Zn contents in roots; and (iii) rearrangement of K and S contents, and preservation of Cu, P, Mn, Zn and Fe contents in leaves. On the other hand, rearrangement of element and metabolite contents in tissues under salinity and restriction of Na accumulation in tissues could be attributed to salt tolerance at the seedling stage. In conclusion, according to ionomic and metabolomic studies on the three barley genotypes differing in salt tolerance, it may be suggested that restriction of Na accumulation and rearrangement of nutrient element levels and metabolite production in tissues under salt stress are attributable to enhanced salt tolerance.

Materials and Methods

Germination assay and salt treatment

Seeds of a salt-tolerant Tibetan wild barley accession XZ16, a salt-tolerant cultivar CM72 and a salt-sensitive accession XZ169 were disinfected with 3% H_2O_2 for 20 min and rinsed with distilled water, then soaked for 12 h in the dark at room temperature. The seeds were transferred onto filter paper moistened with distilled water as controls and with 150 and 300 mM NaCl solutions as salt treatments. Fifty seeds in a germination box were placed in a growth chamber (22/18°C, day/night), as one replicate. Four replicates were set for both control and salt treatments. After 10 d, the germination rate, and the root and shoot length of the three genotypes under control and salt conditions were measured and recorded, and root and shoot tissues were dried at 80°C for use in element measurement. However, these physiological parameters were not measured for 300 mM NaCl treatment due to the poor germination of all genotypes.

Hydroponics and salt treatment at the seedling stage

Seeds of XZ16, XZ169 and CM72 were germinated under normal conditions as described above. Ten-day-old seedlings were transplanted into 48-well plastic containers (35 liters) with aerated hydroponic solution similar to that used by Wu et al. (2011). The pH of the hydroponic solution was adjusted to 6.0 using 1 N HCl and NaOH, as required. Half-strength hydroponic solution was supplied to plants in the first week, and then changed into full-strength solution from the second week and renewed weekly. Plants were grown in a greenhouse with natural light, and a temperature of 20 ±2/15 ±2°C day/night at Zijingang Campus, Zhejiang University, China. Salt treatment was initiated on plants from the third week by adding NaCl at a rate of a 50 mM increment per day, to reach final concentrations of 150 and 300 mM in the solution. The solution without any NaCl addition was used as the control. Four containers were used as replicates for both control and treatments. At 21 d after salt treatment, shoots and roots of the three genotypes under control and treatment conditions were harvested and roots were thoroughly rinsed with tap water before sampling. Then, tissues were dried at 80°C for 72 h before recording the DW. Relative DW was calculated as the ratio of each salt-treated plant to its respective control. Dry root and leaf tissues were used for element measurement. At 21 d after 300 mM salt stress, roots and leaves of XZ16 and CM72 from control and salt-stressed treatments were sampled with six biological replicates, immediately frozen in liquid nitrogen and stored at −80°C until metabolite profiling analysis.

Element and metabolite profiling analysis

Dry roots and shoots (leaves) were ground, and approximately 0.1 g tissue samples were dry-digested in a muffle furnace at 500°C for 6 h, and then 10 ml of HNO_3 : H_2O (1 : 1) was added to extract ions. The contents of Na, K, Ca, Mg, P, S, Cu, Fe, Mn and Zn were determined using an ICP-OES spectrometer (iCAP 6000 series, Thermo Fisher Scientific Inc.), according to the equipment operation manual. For metabolite profiling analysis, metabolites were extracted from barley roots and leaves (100 mg FW) and their contents were determined using a 7890A GC/5975C MS system (Agilent, USA), as described by Wu et al. (2013).

Data analysis

The significance of difference for physiological traits and element content was analyzed by analysis of variance (ANOVA) using SAS software (Version 9.1, SAS Institute Inc.). For ionomic analysis, PCA was carried out to distinguish separation of samples and elements using Metaboanalyst 2.0 (www.metaboanalyst.ca/), according to Xia et al. (2012). The PCA is an unsupervised method with the aim of finding the directions that could best explain the variance in a data set (X) without referring to class labels (Y). The data are summarized into many fewer variables called scores, which are weighted as an average of the original variables. The weighting profiles are called loadings, which in this study refer to elements. Correlation analysis can also be used to identify whether certain features show particular patterns under different conditions. The mean values of element and metabolite contents in roots and leaves of CM72 and XZ16 under control and 300 mM salt stress were used for correlation analysis, which was done using SPSS software (IBM SPSS Statistics Version 19.0). The difference at $P < 0.05$ and 0.01 is considered as significant and highly significant, respectively.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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