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Photosynthetic response and transcriptomic profiling provide insights into the alkali tolerance of clone halophyte *Leymus chinensis*

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Abstract

Alkali stress is one of the important factors in restricting agriculture production. *Leymus chinensis* is constructive halophyte species in alkalized grassland in China. In order to investigate the gene expression response of *L. chinensis* to alkali stress, we used PacBio technology to obtain reference full-length transcript sequences for transcriptomic analysis of alkali stress response. In order to elucidate the alkali tolerance mechanisms of *L. chinensis*, we measured the photosynthetic parameters, concentrations of ions and compatible solutes, chloroplast ultrastructure and anatomy of control and stressed plants. Our results showed that *L. chinensis* shares many alkali-tolerance mechanisms with glycophytes. Higher stability of photosynthetic apparatus under alkali stress may be prominent alkali-tolerance trait of *L. chinensis*. *L. chinensis* may have a strong capacity to decline the toxicity of Na^+ to organelles and cytoplasmic proteins. Enhanced expression of *dehydrin* and *LEA* genes and increased accumulation of carbohydrates may contribute to the development of Na^+-specific stress tolerance of *L. chinensis* under alkali stress.

Additional key words: osmotic adjustment; PacBio sequencing; pigments; RNAseq.

Introduction

Soil salinization and alkalization are important environmental factors restricting agriculture production in the world. In saline soil, harmful salts mainly consist of NaCl, Na_2SO_4, $NaHCO_3$, and Na_2CO_3. About 46% of saline soils contain only the neutral salts NaCl and Na_2SO_4, but the remaining 54% contain both the neutral and alkaline salts (sodic soil) (Tanji 1990). Our previous studies have demonstrated that alkaline salt stress had strong destructive effects to plants compared to the neutral salt stress of the same salinity (Yang *et al*. 2007, 2008b). Alkalization is much more destructive to plants and soil than salinization. For example, in northeast China, about 70% of grasslands was alkalized (Tanji 1990). These alkaline soils contain high concentrations of $NaHCO_3$ and Na_2CO_3, which can not only cause soil compaction and mineral element precipitation but also can destroy the chemical structure of root cell membrane. Only few alkali-tolerant halophytes can live in heavily alkalized grasslands. However, this serious environmental problem has been ignored and always is confused with 'salt-alkaline stress'. In order to reveal the adaptive mechanisms of plant to natural alkalized soils, we must distinguish between salt stress and alkali stress and pay more attention to plant alkali tolerance.

In the past 30 years, salt stress was one of the research hotspots in plant stress biology. A great progress was achieved in ion transport, signal transduction, hormone regulation, and other fields (Flowers and Yeo 1995, Munns and Tester 2008, Flowers *et al*. 2010, Kaashyap *et al*. 2017, Rozentsvet *et al*. 2017, Zhang *et al*. 2018, Zeng *et al*. 2018, Wang *et al*. 2019, Zhu *et al*. 2019). Although soil alkalization has caused serious ecological and agricultural problems in some area of northeast China (Tanji 1990), few studies focus on alkali stress. These alkali stress researches had focused on transgenesis (Wang *et al*. 2016, He *et al*. 2017), organic acid metabolism (Ma *et al.* 2017), physiology and gene expression (Tanji 1990, Zhang *et al*. 2013, Jia *et al*. 2019), metabolome (Guo *et al*. 2016), proteome (Yu *et al*. 2013, Zhang *et al*. 2016, Zhao *et al*. 2019), and microarray analysis (Wang *et al*. 2007a). Most of these descriptive works focused on glycophytes, and only few studies reported alkali tolerance of halophytes (Yang *et al*. 2007, 2008a; Li *et al*. 2010, Yin *et al*. 2019). To date, almost all molecular mechanisms

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Abbreviations: DEG – differentially expressed gene; DM – dry mass; *E* – transpiration rate; FM – fresh mass; GDH – glutamate dehydrogenase; GS – glutamine synthetase; g_s – stomatal conductance; HKT – high-affinity potassium transporter; KEGG – Kyoto Encyclopedia of Genes and Genomes; LEA – late embryogenesis abundant; NCL – sodium/calcium exchanger; NHX – sodium/hydrogen exchanger; NPF – NRT1/PTR FAMILY; NRT – high-affinity nitrate transporter; P_N – net photosynthetic rate.
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of salt tolerance and alkali tolerance were discovered in rice and *Arabidopsis* (Wu *et al*. 2018, Flowers *et al*. 2019, Ganie *et al*. 2019). Most botanists believe that halophytes and glycophytes employ different mechanisms to resist salt stress and alkali stress. In addition, halophytes were distributed in numerous nodes of the phylogenetic tree of higher plants, displaying that halophytes evolved diverse mechanisms against high soil salinity (Flowers *et al*. 2010). This diversity complicates discovery of the salinity or alkali tolerance mechanisms of halophytes. Although research on halophytes is increasing (Flowers and Yeo 1995, Wang *et al*. 2007a,b; Ardie *et al*. 2009, 2010, 2011; Liu *et al*. 2009, Flowers *et al*. 2010, Yu *et al*. 2011, Yu *et al*. 2013, Zhang *et al*. 2013, Wang *et al*. 2017, Zhang *et al*. 2017), genome sequence of extreme halophytes was missing, which greatly restricts the study on salinity tolerance and alkali tolerance of extreme halophytes.

Leymus chinensis is constructive species in alkalized grassland in northern China (Zheng and Li 1999), with high forage value. *L. chinensis* can survive for a long time in alkaline grassland with pH above 10 (Zheng and Li 1999). It is one of the most alkali-tolerant Gramineae halophyte (Liu *et al*. 2015), and it also is close relative of wheat plants. In northern China, *L. chinensis* is an important forage grass that is used to improve alkaline grassland (Zheng and Li 1999). Understanding *L. chinensis* alkali tolerance would improve the current knowledge of alkali tolerance, and even could provide breeders with candidate alkali tolerance genes. Although some studies on physiological response (Liu *et al*. 2015, Wang *et al*. 2015), microRNAs (Zhai *et al*. 2014), and gene expression profiling (microarray chips) (Jin *et al*. 2008) had been carried out in *L. chinensis*, these studies only referred to the genome sequence of other species, which inevitably led to imperfection of the gene expression profiling. In this study, we used PacBio platform to constitute a reference full-length transcript sequences (45,037 high-quality isoforms) for alkali-stressed *L. chinensis*, and we subsequently conducted a RNAseq analysis of alkali stress response by mapping Illumina reads to the generated reference transcript sequences. This approach will improve understanding of gene expression regulation of alkali-stress response in this species. Additionally, we also conducted biochemical and anatomical analysis. We aimed to elucidate the response mechanisms of *L. chinensis* to alkali stress through connecting biochemical analysis and transcriptomic profiling.

Materials and methods

Plant materials: *Leymus chinensis* is a perennial clone grass, sexual propagation (seed reproduction) is rare because of its extreme low seed germination rate, and vegetative reproduction is its dominant reproduction type. To minimize the effects of plant to plant, we used different ramets derived from one clone of *L. chinensis* as experimental materials. *L. chinensis* is not an endangered or protected species, therefore, no specific permissions were required for the plant collection. We transferred the *L. chinensis* individual (named as LC1 plant) from alkalized grassland located in northeast China to a greenhouse. First, we collected partial leaves and roots samples of LC1 plant and stored these samples at –80°C. After this, in order to obtain more rhizomes, we immediately divided the LC1 plant into several ramets, and then these ramets were grown in different plastic pots containing thoroughly washed sand and placed in a greenhouse [24–26°C (day) and 17–19°C (night) temperatures under 16-h light]. The ramets were watered daily with a Hoagland nutrient solution for 60 d. After this, we collected all rhizomes from the ramets, and then the rhizomes were planted in different plastic pots containing thoroughly washed sand; each pot contained five rhizomes. The rhizomes were all generated form the LC1 plant, thus, belonged to a clone. These pots were watered daily with a Hoagland nutrient solution for 30 d. After this, we selected pots with uniform ramets in order to perform further experiments.

Stress treatment for RNAseq, qPCR, physiological experiments, and anatomical analysis: Above mentioned pots (15) were used as control group, and another 15 pots were used as alkali-stress treatment group. Control group was watered with Hoagland nutrient solution, and alkali-stress treatment group was watered with alkaline salt solution that contained the same nutrient composition of Hoagland nutrient solution. Two alkaline salts were mixed in a 9:1 molar ratio ($NaHCO_3$ to Na_2CO_3, pH 8.8) as the alkaline-stress treatment, and total salinity was 200 mM. The stress treatment duration was 2–30 d. All rhizomes used in this work were generated from the same individual through vegetative reproduction without meiosis and fertilization. Therefore, in theory, these ramets were genetically identical, which would improve accuracy of comparative transcriptome analysis. When the seedlings were exposed to alkali-stress treatment for 2 d, we collected the leaf or root samples for biochemical measurements, RNA sequencing experiment, and real-time PCR analysis. When the seedlings were exposed to alkali-stress treatment for 30 d, we prepared the leaf or root samples for biomass, chlorophyll (Chl), photosynthesis, chloroplast ultrastructure, and anatomical analyses. Leaves or roots of five seedlings (ramets) from one pot for each treatment were pooled as a biological replicate, with three biological replicates for each treatment.

Stress treatment for PacBio sequencing: The ramets used in PacBio sequencing experiment and the ramets used in RNA sequencing experiments were both generated from the LC1 plant, and belonged to the clone. First, we exposed the ramets to 300 mM NaCl and 200 mM alkali-stress treatment ($NaHCO_3$ to Na_2CO_3, pH 8.8) for 2 and 30 d through using the method described above. We collected the leaf, root, bud, spike, and flower tissues under three treatment conditions at the tillering, booting, and anthesis stage. Additionally, we also collected partial leaf and root samples of the initial LC1 plant for PacBio sequencing.

Chl and photosynthesis measurements: When the seedlings were exposed to alkali stress treatment for 30 d,

photosynthesis measurements were conducted. Net photosynthetic rate (P_N), stomatal conductance (g_s), and transpiration rate (E) of leaves were determined using a portable open flow gas-exchange system *LI-6400* (*LICOR*, USA) with PAR of 1,200 μmol(photon) m^{-2} s^{-1}, leaf temperature of 28°C, and vapor pressure deficit (VPD) of 2.4–2.8. Chl *a*, Chl *b*, and carotenoids were extracted with 80% acetone, and absorbance of extracted solution was determined at 440, 645, and 663 nm with a spectrophotometer (*T600*, *PERSEE*, China). The calculation used the methods of Zhu (1993).

Biochemical measurement: When the seedlings were exposed to alkali stress treatment for 2 d, leaves and roots were harvested and freeze-dried for biochemical measurement. Roots or leaves of five seedlings (ramets) for each treatment were pooled as a biological replicate, with three biological replicates for each treatment. Concentrations of free amino acids and sugars were determined with the methods of Zhao *et al*. (2017). Briefly, the free amino acids and sugars were isolated from freeze-dried samples using distilled water at 50°C, and were further treated and loaded into a liquid chromatography-tandem mass spectrometry system equipped with a high-performance liquid chromatograph (HPLC) and a triple quadrupole mass spectrometer (*API3200MD*, *AB SCIEX*). Dried samples were digested in 65% HNO_3 at 120°C, and the Na^+ and K^+ contents were measured by an atomic absorption spectrophotometer (*TAS-990super*, *PERSEE*, China).

Anatomical analysis and chloroplast ultrastructure: When the seedlings were exposed to alkali-stress treatment for 30 d, anatomical analysis and chloroplast ultrastructure experiments were conducted. The samples were fixed with FAA solution (10:50:5:35 – formaldehyde:ethanol:acetic acid:water), and then fixed samples were cleaned using 0.1 M phosphate buffer (pH 7.4). The leaf samples for chloroplast ultrastructure were fixed with 2.5% glutaraldehyde at 4°C for 6 h, and were then rinsed with 0.1 M phosphate buffer (pH 7.4) three times. Then fixed samples for both experiments were further treated by 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4). The samples were dehydrated using ethanol and acetone in different concentration gradients. The samples were infiltrated in a solution of 1:1 acetone:embedding agent (*EMBed 812*) for 3 h, in 2:1 acetone:*EMBed 812* overnight, and then pure *EMBed 812* for 8 h, before the samples were kept at 60°C for 48 h. The embedded materials were sliced to 1–2 μm thicknesses with an ultramicrotome (*Leica UC7*, *Leica*), and were dyed with toluidine blue, then photographs were taken by a scanner (*3D HISTECH*, Hungary). The embedded material was also sliced to 70-nm ultrathin sections, and then the sections were stained with uranyl acetate for 15 min, following 15 min staining with lead citrate. The chloroplast ultrastructure was observed under a transmission electron microscope (*HT7700*, *Hitachi*, Japan).

Reference full-length transcript sequence: We used *PacBio Sequel* platform (third generation sequencing technology) to produce reference full-length transcript sequences for reference of mapping analysis of the RNAseq data generated by *Illumina* platform. We mixed all collected RNA samples in equal concentration of RNA, and then this mixed sample was exposed to PacBio sequencing (40 Gb). Finally, we obtained full-length sequences for all expressed transcript. PacBio sequence data were processed using the *SMRTlink* software. Circular consensus sequence (CCS) data was generated from subreads file (default parameters). Then the CCS BAM files were used to produce full-length transcript through using *isoseq3* software (default parameters). Function of the nonredundant transcripts was annotated against following databases: NR (NCBI nonredundant protein sequences), NT (NCBI nonredundant nucleotide sequences), Pfam (Protein family, protein domain), KOG/COG (Clusters of Orthologous Groups of proteins), SwissProt (a manually annotated and reviewed protein sequence database), GO (Gene Ontology), and KO (KEGG Ortholog database). We used the *TransDecoder* software to identify candidate protein-coding regions of transcript on basis of the prediction of an ORF (Open Reading Frame).

RNA sequencing: When the seedlings were exposed to alkali-stress treatment for 2 d, we used conventional method to preform RNA-sequencing experiment. Leaves or roots of five seedlings from one pot for each treatment were pooled as a biological replicate, with three biological replicates for each treatment. Two μg RNA of each sample were used for the RNA input. The libraries were generated using *NEBNext® Ultra™ RNA Library Prep* Kit for *Illumina®* (*#E7530L*, *Neb*, USA). The Libraries were sequenced on *Illumina NovaSeq 6000* and 150 bp paired-end reads were generated. Finally, about 10 Gb clean data for each sample were produced. Clean data were then aligned to reference full-length transcript sequences using *HISAT2 v2.1.0. RSEM* software was used to calculate FPKM (Reads Per Kilobase Millon Mapped Reads) of all nonredundant transcripts. We used *DESeq2* to identify the differentially expressed genes (DEGs) between control and stress treatments (adjusted P value ≤ 0.05 and $|\log2\text{fold change}| \geq 1$). The P-values were corrected by the BH method. DEGs were exposed to KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.kegg.jp/) enrichment by the hypergeometric test, in which P-values were adjusted by multiple comparisons as q-value.

Quantitative real-time PCR analysis: When the seedlings were exposed to alkali-stress treatment for 2 d, we used conventional method to preform real-time PCR analysis. Roots of five seedlings (ramets) for each treatment were pooled as a biological replicate, with three biological replicates for each treatment. The total RNA from each sample was isolated with TRIzol reagent (*Invitrogen*). The RNA was treated with DNaseI (*Invitrogen*), reverse-transcribed using *SuperScriptTM* RNase H-Reverse Transcriptase (*Invitrogen*), and then subjected to real-time PCR analysis. Amplification of the target gene was monitored every cycle by *SYBR Green*. Amplification of the *actin* gene (ID: Gene.42270) was used as an internal quantitative control. The primer sequences of *actin* gene were 5'-TACACGAAGCGACATACAATTCCATCA-3'

(forward sequence) and 5'-AGAACCTCCACTGAGA-ACAACATTACC-3' (reverse sequence). The relative expression of the target genes was calculated using the △△Ct method (Livak and Schmittgen 2001).

Statistical analysis and experimental design: The experimental design was randomized complete block design. Physiological measurements, RNA sequencing experiment, and qRT-PCR experiment all included three biological replicates. Leaves or roots of five seedlings (ramets) from one pot for each treatment were pooled as a biological replicate. The statistical significance of physiological measurements and qRT-PCR were determined by the *t*-test at 0.05 level using *SPSS 16.0* (*IBM*, USA). The gene expression data of RNA sequencing were analysed by *DESeq2 R* package. The *P*-values generated in the RNA sequencing analysis were adjusted by the BH method. DEGs between control and stress treatments were defined as adjusted *P* value ≤ 0.05 and |log2fold change| ≥ 1.

Results

Physiological and anatomical response: Alkali stress markedly limited the photosynthesis and growth of *L. chinensis*. Alkali stress decreased the net photosynthetic rate (P_N), stomatal conductance (g_s), and transpiration rate (E) of *L. chinensis* (Table 1). However, alkali stress did not affect accumulation of photosynthetic pigments (Table 1). Alkali stress decreased the biomass of root and leaf, but only produced small effect on leaf water content (Table 1). Alkali stress increased the Na^+ concentration and decreased K^+ concentration in both roots and leaves (Table 1). Alkali stress affected only marginally the anatomical structure of leaf (Fig. 1). Alkali stress slightly reduced the vessel diameter of the major vein and aerenchyma volume (Fig. 1). Alkali stress increased packing density of thylakoids in chloroplasts, and the chloroplast of control plant (48 grana per chloroplast) had more grana than that of stressed plant (19 grana per chloroplast) (Fig. 2). We detected 19 amino acids in both roots and leaves of *L. chinensis* (Table 2). Alkali stress increased only the concentration of proline in roots and the concentration of asparagine in leaves (Table 2). Accumulation of most carbohydrates was stimulated by alkali stress in leaves, while only concentrations of fructose, glucose, mannose, sucrose, and maltose were enhanced in roots (Table 2). Among four polyols, accumulation of sorbitol/mannitol was stimulated by alkali stress in roots. In stressed leaves, contributions of fructose (9.92%) and sucrose (33.34%) to total molarity were greater than those of other solutes, and they played an important role in osmotic adjustment (Table 2). In

Table 1. Effects of alkali stress on growth, photosynthesis, and ion contents in *Leymus chinensis*. The 30-d-old seedlings were exposed to alkali stress ($NaHCO_3$:Na_2CO_3, 9:1; 200 mM, pH 8.8) for 30 d. Values are means (± SD) of three replicates. Water content = (fresh mass – dry mass) × 100/fresh mass. *Asterisk* in alkali stress treatment column indicates significant difference between control and alkali stress conditions within the same tissue at 0.05 level (*t*-test). NS – no significant difference; P_N – net photosynthetic rate; g_s – stomatal conductance; E – transpiration rate; FM – fresh mass; DM – dry mass.

	Control	Alkali stress
P_N [μmol(CO_2) m^{-2} s^{-1}]	19.56 ± 0.87	10.88 ± 1.09*
g_s [mol(H_2O) m^{-2} s^{-1}]	0.33 ± 0.04	0.09 ± 0.01*
E [mmol(H_2O) m^{-2} s^{-1}]	7.43 ± 0.58	2.49 ± 0.14*
Chlorophyll *a* [mg g^{-1}(FM)]	0.84 ± 0.06	0.80 ± 0.18^{NS}
Chlorophyll *b* [mg g^{-1}(FM)]	0.46 ± 0.02	0.39 ± 0.07^{NS}
Carotenoid [mg g^{-1}(FM)]	0.34 ± 0.02	0.30 ± 0.05^{NS}
Leaf dry mass [g per plant]	2.53 ± 0.11	1.28 ± 0.08*
Root dry mass [g per plant]	0.56 ± 0.06	0.37 ± 0.05*
Leaf water content [%]	72.0 ± 5.3	63.0 ± 0.4^{NS}
Leaf Na^+ [μmol g^{-1}(DM)]	53.8 ± 9.4	321.9 ± 58.6*
Root Na^+ [μmol g^{-1}(DM)]	60.6 ± 28.3	475.4 ± 28.2*
Leaf K^+ [μmol g^{-1}(DM)]	747.5 ± 17.4	509.1 ± 8.9*
Root K^+ [μmol g^{-1}(DM)]	483.7 ± 53.7	140.5 ± 3.1*

Fig. 1. Effects of alkali stress on leaf anatomy of *Leymus chinensis*. (*A*) control leaf and (*B*) stressed leaf. The 30-d-old seedlings were exposed to alkali stress ($NaHCO_3$:Na_2CO_3, 9:1; 200 mM, pH 8.8) for 30 d.

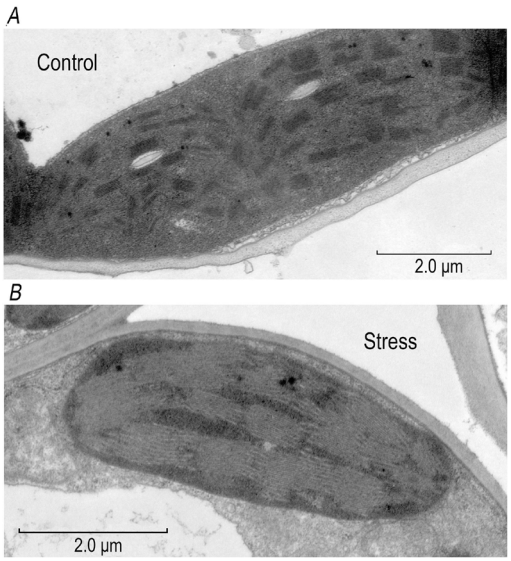

Fig. 2. Effects of alkali stress on chloroplast ultrastructure in *Leymus chinensis*. The 30-d-old seedlings were exposed to alkali stress ($NaHCO_3$:Na_2CO_3, 9:1; 200 mM, pH 8.8) for 30 d.

Table 2. Fold change and percent contribution to total molarity of each compatible solute in *Leymus chinensis*. Fold change is ratio of stress and control. The 30-d-old seedlings were exposed to alkali stress ($NaHCO_3$:Na_2CO_3, 9:1; 200 mM, pH 8.8) for 2 d. Five seedlings (ramets) from one pot for each treatment were pooled as a biological replicate, with three biological replicates for each treatment. CL – control leaf; SL – stressed leaf; CR – control root; SR – stressed root. Percent contribution is calculated with following equation: percent contribution of a given solute = its molarity concentration × 100/total molarity concentration, where total molarity concentration is sum of molarity concentrations of all detected solutes. [a]The solute was not detected in the alkali stressed plants. nd – not detected.

		Percent contribution to total molarity				Leaf		Root	
		CL	SL	CR	SR	Fold change	*P* value	Fold change	*P* value
Free amino acids	Glycine	1.58	2.46	0.85	0.42	3.42	0.490	0.68	0.056
	Alanine	17.84	5.27	7.31	3.74	0.65	0.307	0.71	0.118
	Serine	5.12	5.52	3.43	1.22	2.37	0.498	0.49	0.001
	Proline	2.49	1.54	0.41	1.19	1.36	0.462	3.99	0.000
	Valine	3.16	1.03	2.99	0.70	0.72	0.628	0.33	0.000
	Threonine	3.30	1.77	3.17	0.96	1.18	0.804	0.42	0.001
	Cysteine	1.15	0.69	0.69	0.30	1.32	0.658	0.60	0.058
	Isoleucine	1.60	0.54	1.22	0.30	0.74	0.587	0.33	0.000
	Asparagine	4.46	5.64	29.50	19.08	2.79	0.036	0.89	0.606
	Aspartic acid	6.54	3.83	5.91	6.07	1.29	0.575	1.42	0.005
	Glutamine	0.00	0.46	1.64	0.74	[a]100	0.367	0.62	0.016
	Glutamic acid	2.22	1.72	3.84	4.13	1.70	0.444	1.49	0.006
	Histidine	0.80	1.30	0.77	0.32	3.58	0.462	0.57	0.001
	Phenylalanine	1.54	0.45	0.47	0.14	0.65	0.358	0.42	0.003
	Arginine	2.70	2.27	2.96	2.16	1.85	0.409	1.01	0.878
	Tryptophan	0.34	0.17	0.41	0.23	1.10	0.907	0.79	0.205
	Lysine	4.82	1.87	2.82	0.82	0.85	0.809	0.40	0.001
	Tyrosine	1.95	0.66	0.59	0.19	0.75	0.596	0.44	0.007
	Leucine	3.30	0.95	1.92	0.40	0.63	0.333	0.28	0.000
Free carbohydrates	Erythrose	4.41	3.47	6.52	5.85	1.73	0.063	1.24	0.047
	Fructose	3.48	9.92	1.96	3.98	6.29	0.000	2.81	0.001
	Xylose	0.04	0.01	0.25	0.17	0.88	0.935	0.95	0.851
	Glucose	0.17	0.35	0.11	0.33	4.67	0.048	3.94	0.006
	Galactose	0.02	0.04	0.00	0.00	3.64	0.276	nd	nd
	Mannose	2.29	1.81	1.30	1.47	1.74	0.009	1.56	0.006
	Sucrose	11.63	33.34	9.52	37.20	6.32	0.000	5.40	0.000
	Ribose	0.56	1.12	0.55	0.27	4.39	0.005	0.67	0.239
	Maltose	0.78	0.48	0.43	0.52	1.35	0.008	1.68	0.027
	Trehalose	0.01	0.04	0.01	0.02	11.79	0.035	2.24	0.560
Polyols	Sorbitol/mannitol	0.50	0.15	0.05	0.09	0.68	0.132	2.76	0.015
	Pinitol	9.42	10.50	7.63	6.73	2.46	0.287	1.22	0.789
	Xylitol	0.16	0.08	0.00	0.00	1.11	0.608	nd	nd
	Inositol	1.61	0.56	0.78	0.27	0.77	0.092	0.48	0.009

stressed roots, sucrose (37.2%) and asparagine (19.08%) exhibited much higher contributions to osmotic potential (total molarity concentration) than other solutes (Table 2).

Reference full-length transcript sequence: We used PacBio platform to constitute a reference full-length transcript sequence (Table 1S, *supplement*). Finally, we obtained 45,037 high-quality isoforms (Table 2S, *supplement*).

Transcriptomic profiling: All gene expression data were showed in Tables 3S, 4S (*supplement*). We found that 2,216 genes were differentially expressed under control and stress conditions in the leaves, including 693 upregulated genes and 1,523 downregulated genes. We observed that 1,417 genes were differentially expressed under control and stress conditions in the roots, including 721 upregulated genes and 696 downregulated genes. We exposed all differentially expressed genes (DEGs) to KEGG enrichment. DEGs were significantly enriched in nine pathways in leaves, including antenna proteins, photosynthesis, glycine, serine, and threonine metabolism, glyoxylate and dicarboxylate metabolism, gaffeine metabolism, fatty acid elongation, phenylpropanoid biosynthesis, ribosome biogenesis, and ubiquinone and

other terpenoid-quinone biosynthesis. In the leaves, four pathways (antenna proteins, photosynthesis, glycine, serine and threonine metabolism, and glyoxylate and dicarboxylate metabolism) were significantly restricted by alkali stress, and ribosome biogenesis was significantly promoted by alkali stress (Fig. 3; Table 5S, *supplement*). In KEGG pathway network, photosynthesis pathway is composed of all photosynthetic electron transport protein genes. Key photosynthetic electron transport protein genes (21) and 32 antenna protein genes were downregulated in leaves under alkali stress (Table 5S). In roots, DEGs were significantly enriched in ten pathways, including phenylpropanoid biosynthesis, sesquiterpenoid and triterpenoid biosynthesis, nitrogen metabolism, isoquinoline alkaloid biosynthesis, alanine, aspartate and glutamate metabolism, sulfur metabolism, tropane, piperidine and pyridine alkaloid biosynthesis, amino sugar and nucleotide sugar metabolism, galactose metabolism, and phenylalanine metabolism (Fig. 4). It was obvious that nitrogen metabolism was significantly upregulated in the roots (Fig. 4).

We also identified many differentially expressed salinity-tolerant genes (Table 3). For example, three *NHX* genes were significantly upregulated in roots under alkali stress, and one *NHX* gene was significantly upregulated in leaves (Table 3). Three *late embryogenesis abundant* (*LEA*) genes and one *dehydrin* gene were significantly upregulated in roots under alkali stress, while the four genes all were downregulated in the leaves. One *potassium transporter* gene was significantly upregulated in leaves but not in roots. Both *HKT4* and *HKT8* were downregulated in roots. *V-H$^+$-ATPase* also was downregulated in leaves, but *HKT8* was upregulated in leaves (Table 3). Six *high-affinity nitrate transporter* (*NRT*) genes, one *glutamate dehydrogenase* (*GDH*) gene, and one *glutamine synthetase* (*GS1;3*) gene were upregulated in roots (Table 4). Most of *NRT1/PTR FAMILY* (*NPF*) genes was downregulated in both roots and leaves (Table 4). Many *glutathione S-transferase* and *peroxidase* genes were expressed in stressed leaves or roots, but the most of these expressed genes were downregulated in roots or leaves (Table 6S, *supplement*).

Validation of qPCR: The results of RNAseq were validated by qRT-PCR (Table 7S, *supplement*). Eight out of 11 genes tested showed consistent results between RNAseq and qRT-PCR (Table 7S), indicating the results of RNAseq were reliable.

Discussion

Growth and photosynthesis: In the leaves of *L. chinensis*, many genes involved in photosynthetic light reaction were downregulated by alkali stress (Fig. 3, Table 5S). This might be an adaptive strategy of *L. chinensis* to alkali stress. Alkali stress may promote the shift of the energy from biomass accumulation to stress response by restricting synthesis of proteins involved in building plant body (Munns and Gilliham 2015). Under alkali stress, growth of *L. chinensis* was slowed or even stopped (Table 1), which may be mediated through downregulation of photosynthetic electron transport protein genes. Accordingly, we observed a reduction in P_N, g_s, and E in *L. chinensis* under alkali stress. Surprisingly, alkali stress did not influence photosynthetic pigment content of *L. chinensis*, which was not consistent with finding in wheat plants where alkali stress caused Na^+ excess and destroyed pigments in chloroplasts (Yang *et al*. 2008b). Higher stability of photosynthetic apparatus under alkali stress may be important alkali-tolerance trait of *L. chinensis*. P_N is determined by photosynthetic electron transport and carbon assimilation rate. Under alkali stress, decreased P_N of *L. chinensis* may be due to decreased photosynthetic electron transport rate (Fig. 3, Table 5S) and lower g_s (Table 1). Under alkali stress, reduced aerenchyma volume may also influence the CO_2 influx into mesophyll cells of *L. chinensis* (Fig. 1). In addition, ribosome biogenesis process was significantly promoted by alkali stress in the leaves of *L. chinensis* (Fig. 3). Under alkali stress,

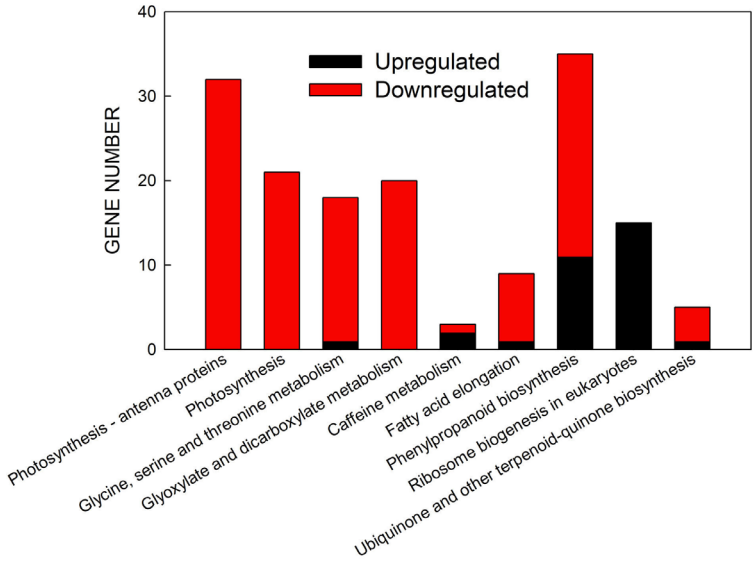

Fig. 3. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of differentially expressed genes in leaves of *Leymus chinensis*. Five seedlings (ramets) from one pot for each treatment were pooled as a biological replicate, with three biological replicates for each treatment. The 30-d-old seedlings were exposed to alkali stress ($NaHCO_3$:Na_2CO_3, 9:1; 200 mM, pH 8.8) for 2 d. KEGG pathways with adjusted P<0.05 are displayed.

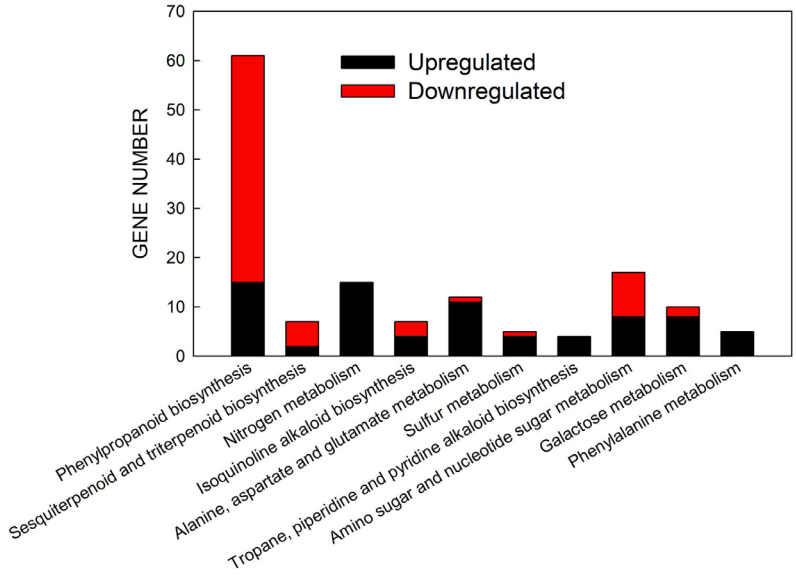

Fig. 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of differentially expressed genes in roots of *Leymus chinensis*. Five seedlings (ramets) from one pot for each treatment were pooled as a biological replicate, with three biological replicates for each treatment. The 30-d-old seedlings were exposed to alkali stress ($NaHCO_3$:Na_2CO_3, 9:1; 200 mM, pH 8.8) for 2 d. KEGG pathways with adjusted $P<0.05$ are displayed.

Table 3. Effects of alkali stress on gene expression involved in osmotic adjustment and ion homeostasis in *Leymus chinensis.* Five seedlings (ramets) from one pot for each treatment were pooled as a biological replicate, with three biological replicates for each treatment. The 30-d-old seedlings were exposed to alkali stress ($NaHCO_3$:Na_2CO_3, 9:1; 200 mM, pH 8.8) for 2 d. NCL – sodium/calcium exchanger; NHX – sodium/hydrogen exchanger; LEA – late embryogenesis abundant protein; HKT – high-affinity potassium transporter.

		Root		Leaf	
Gene ID	Gene name	Fold change	Adjusted *P* value	Fold change	Adjusted *P* value
Gene.15906	*HKT4*	0.49	0.001	0.80	0.358
Gene.20677	*HKT8*	0.24	0.000	25.35	0.167
Gene.16840	*Ca^{2+}/H^+-exchanger*	0.45	0.000	0.99	0.986
Gene.14224	*NCL1*	0.42	0.000	0.63	0.001
Gene.8886:	*Potassium transporter*	0.47	0.007	0.50	0.000
Gene.3756:	*NHX*	1.40	0.039	3.08	0.000
Gene.38145	*NHX*	4.89	0.000	1.66	0.083
Gene.36084	*NHX*	2.14	0.000	1.45	0.079
Gene.19378	*NHX*	3.42	0.001	1.06	0.965
Gene.45017	*V-H+-ATPase*	0.59	0.017	0.17	0.002
Gene.37596	*Potassium transporter*	1.27	0.316	2.75	0.045
Gene.47912	*LEA_4*	2.78	0.037	0.52	0.174
Gene.33695	*Dehydrin*	8.20	0.001	0.02	0.000
Gene.51088	*Dehydrin*	1.22	0.421	0.17	0.000
Gene.33771	*LEA_4*	0.59	0.430	0.38	0.020
Gene.30351	*LEA_2*	2.40	0.000	0.38	0.000
Gene.46909	*LEA_2*	3.42	0.030	0.58	0.482

to support the biosynthesis of stress-response proteins, *L. chinensis* may generate more ribosome through enhanced gene expression involved in ribosome-biogenesis process. Alkali stress increased packing density of thylakoids in *L. chinensis* chloroplasts. Higher-density thylakoids may generate more ATP and NADPH to fuel alkali-stress responses.

Osmotic adjustment and ion homeostasis: High K^+/Na^+ ratios in cytoplasm are salinity-tolerance traits shared by most glycophytes and some halophytes (Wang and Xia 2018, Abdelraheem *et al*. 2019, Ganie *et al*. 2019). Under salinity stress, salinity-tolerant crops or halophytes compartmentalize Na^+ in vacuole in order to reduce its concentrations in cytoplasm. Na^+ compartmentation mechanism is conserved among higher plants, which is mediated by NHX (Flowers *et al*. 2019). To resist the osmotic stress due to high concentration of Na^+ or Cl^- in vacuole, these plants also accumulate compatible solutes and K^+ in cytoplasm. Compatible solutes also function in

Table 4. Effects of alkali stress on gene expression involved in nitrogen metabolism in *Leymus chinensis*. Five seedlings (ramets) from one pot for each treatment were pooled as a biological replicate, with three biological replicates for each treatment. The 30-d-old seedlings were exposed to alkali stress ($NaHCO_3$:Na_2CO_3, 9:1; 200 mM, pH 8.8) for 2 d. GDH – glutamate dehydrogenase; NRT – high-affinity nitrate transporter; NPF – NRT1/PTR FAMILY; GS – glutamine synthetase.

		Root		Leaf	
Gene ID	Gene name	Fold change	Adjusted *P* value	Fold change	Adjusted *P* value
Gene.41379	*GDH2*	2.464	0.000	0.798	0.711
Gene.8701:	*NRT2*	3.043	0.000	0.542	0.329
Gene.40163	*NRT2;1*	25.460	0.000	0.193	0.691
Gene.40014	*NRT2;2*	13.442	0.000		
Gene.40106	*NRT2;2*	12.648	0.000		
Gene.41118	*NRT2;2*	7.759	0.000	2.624	1.000
Gene.49732	*NRT2;1*	2.628	0.000	0.345	0.802
Gene.17652	*NPF2.3*	0.392	0.000	0.569	0.058
Gene.16130	*NPF5.2*	0.271	0.000	0.698	0.003
Gene.39847	*NPF6.2*	0.080	0.158	0.199	0.000
Gene.15644	*NPF6.2*	0.095	1.000	0.471	0.009
Gene.15751	*NPF6.2*	0.187	0.166	0.261	0.000
Gene.12799	*NPF6.3*	0.454	0.000	0.203	0.000
Gene.15464	*NPF2.11*	0.170	0.000		
Gene.39536	*NPF8.3*	0.098	0.008	1.436	0.000
Gene.16031	*NPF8.5*	1.343	1.000	0.246	0.001
Gene.25764	*GS1;3*	2.387	0.003	0.095	0.541

prevention of protein aggregation in cytosol. Our results showed that, in *L. chinensis*, alkali stress enhanced the Na^+ concentration and decreased the K^+ concentration. In *L. chinensis*, accumulation of most carbohydrates was greatly stimulated under alkali stress, but accumulation of few free amino acids and polyols was stimulated (Table 2), indicating that carbohydrate may play more important role in osmotic adjustment and protein aggregation prevention than that of free amino acids and polyols.

Another common mechanism against Na^+ toxicity is the Na^+ exclusion from shoots into roots or from roots into rhizosphere solution. In rice and wheat, *HKT1;5* (*HKT8*) gene mediates this Na^+ exclusion process (Wang and Xia 2018, Abdelraheem *et al*. 2019, Ganie *et al*. 2019). However, we found the *HKT1;5* (*HKT8*) was significantly downregulated in roots of *L. chinensis* but upregulated in leaves (Table 3). We propose that *L. chinensis* may employ different transporter to exclude Na^+ from shoots into roots or from roots into rhizosphere solution. In addition, we observed upregulation of three *NHX* genes in *L. chinensis* roots under alkali stress. This was consistent with finding of Wang *et al*. (2015) in rice plants, indicating that the Na^+ compartmentation mechanism under alkali stress is conserved between *L. chinensis* and glycophyte grass.

Dehydrin and LEA proteins are crucial protective proteins under salinity and osmotic stresses (Rorat *et al*. 2006). As dehydrin and LEA proteins contain tandem hydrophilic amino acids, they play important roles in the preventions of cytosol dehydration and protein aggregation (Rorat *et al*. 2006). LEA and dehydrin proteins also interact with carbohydrates to form intracellular glasses. The intracellular glasses can slow molecular mobility of water and ions (Buitink and Leprince 2008), which restricts the movement of Na^+ and Cl^- in cytoplasm. Combination of enhanced carbohydrate concentration and upregulated *LEA* and *dehydrin* expression would promote accumulation of intracellular glasses in *L. chinensis* leaves under alkali stress, alleviating the ion toxicity. Although we observed that many *glutathione S-transferase* and *peroxidase* genes were expressed in stressed *L. chinensis* plants, few of them were upregulated (Table 6S). This suggested that different members of antioxidant enzyme gene family may play different roles in detoxification of reactive oxygen, with tissue- or stress-type specificity.

Enhanced expression of *nitrate transporter* genes contributed to alkali tolerance of *L. chinensis*: Plant roots absorb nitrate (NO_3^-) by a large family of transporters that include NRT1/PTR FAMILY (NRT1) family and NRT2 family. NO_3^- absorbed by roots is further reduced to nitrite by nitrate reductase and then to NH_4^+ by nitrite reductase (NiR). NH_4^+ generated by nitrate reduction is assimilated by glutamine synthetase (GS) and glutamate synthase (GOGAT) or alternative glutamate dehydrogenase (GDH) pathway (Shi *et al*. 2009). Alkali stress strongly increased the gene expression level involved in nitrogen uptake and assimilation in rice plants, including *GDH* genes, *NRT2* genes, and *GS* genes (Wang *et al*. 2012). Similarly, we also observed that alkali stress mightily upregulated the expression of several *NRT2* genes, *GS1;3* gene, and *GDH2* gene in *L. chinensis*. This displayed that the halophyte grass and glycophyte grass may share the N metabolism response mechanism under alkali stress. NO_3^- uptake is mediated by a H^+/NO_3^- symporter using the transmembrane proton

gradient as driving force (Crawford and Glass 1998). Under alkali stress, the lack of external protons would weaken the NO_3^- uptake (Wang *et al*. 2012). Under alkali stress, *L. chinensis* might enhance the expression of *NRT2* genes in roots to increase the frequency of NO_3^- uptake.

Conclusions: *L. chinensis* shares many alkali tolerance mechanisms with rice or wheat plant. For example, under alkali stress, the *NHX* expression response and nitrogen metabolism response mechanisms of *L. chinensis* were similar to those of rice plants. The apparent difference between *L. chinensis* and glycophyte in alkali-stress response was that photosynthetic pigments of *L. chinensis* were unaffected by long-term strong alkali stress (30 d), but the pigment contents of wheat plants severely decreased under alkali stress. Compared to glycophytes, *L. chinensis* may have strong capacity to lower the toxicity of Na^+ to organelles and cytoplasmic proteins. Enhanced expression of *dehydrin* and *LEA* genes and increased accumulation of carbohydrates may contribute to the development of Na^+-specific stress tolerance of *L. chinensis* under alkali stress.

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