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LncRNAs PARTICIPATE IN SALT TOLERANCE RELATED PATHWAYS BY REGULATING TARGET GENES IN WHEAT

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Abstract

Long non-coding RNAs (lncRNAs) are key regulatory elements that play important roles in plant development as well as stress responses in plants. A genome-wide analysis of lncRNA expression in salt tolerant (Xiaoyan60) and salt sensitive (Lumai21) wheat cultivars under salt stress conditions was performed. We identified a total of 746 differentially expressed lncRNAs under salt stress that 675 were expressed in Xiaoyan60 and 592 in Lumai21. Gene ontology enrichment analysis indicated that differentially expressed genes were enriched in biological process, cellular component and molecular function. KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis revealed that the distinct lncRNAs were involved in salt tolerance-related pathways, including plant hormone signal transduction and 27 other pathways. We identified lncRNAs that were significantly upregulated (i.e., lnc_521, lnc_593 and lnc_743) in Xiaoyan60 when compared with Lumai21 after salt stress. These results indicated that lncRNAs were involved in salt tolerance, and our findings provided an important insight regarding wheat adaptation to salt stress.

Key words: Wheat; Salt stress; lncRNA; Target genes; Up-regulation; Down-regulation.

Introduction

Salt stress is a major abiotic factor that affects photosynthesis, growth and development in plants; and high soil salinity is a major global problem (Ma *et al.,* 2016; Sun *et al.,* 2016). Salinity is both an osmotic and ionic stressor. Plant survival in a saline environment depends upon protective adaptations including osmolyte synthesis, increased expression of key response genes and ion compartmentation (Yeo *et al.,* 2017; Fu *et al.,* 2013; Munns & Tester, 2008; Hirayama & Shinozaki, 2010; Rajendran *et al.,* 2009). Plant response to salinity is mediated by multiple approaches, including calcium signaling and other metabolic pathways (Alsahli *et al.,* 2019).

Long noncoding RNAs (lncRNA) are a class of >200nt endogenously expressed non-coding RNAs that are transcribed primarily by RNA polymerase II. Two other lncRNA classes are transcribed by RNA polymeraseIV and RNA polymeraseV, respectively. They are required for regulation of gene expression by gene silencing and epigenetic controls (Wierzbicki *et al.,* 2008; Zhang & Chen, 2013). LncRNAs are transcribed from multiple locations in the genome including introns, exons and intergenic regions (Shumayla *et al.,* 2017). They are key regulators of gene expression and function at both transcriptional and post-transcriptional levels (Heo *et al.,* 2013; Zhang *et al.,* 2014). The regulatory mechanisms that employ lncRNAs are complex, and they act both directly and indirectly (Wilusz *et al.,* 2009). LncRNA functions in plants are largely unknown but available evidence associates them with plant development and stress responses (Xin *et al.,* 2011； Wang *et al.,* 2017).

A previous study of lncRNAs in *Arabidopsis* identified 1832 of these molecules that regulate plants responses to drought, cold, high-salt, abscisic acid (ABA) and bacterial elongation factor Ef-Tu (Liu *et al.,* 2012). In addition, 13 salt-responsive lncRNAs in *Arabidopsis* were identified and validated by qRT-PCR (Di *et al.,* 2014). The over expression of npc536 in *Arabidopsis* showed a heightened root growth in salt stress (Amor *et al.,* 2009). In *Medicagotruncatula,* 2477 lncRNAs were found up-regulated during salt stress (Wang *et al.,* 2015). TE-LincRNA11195, alncRNA associated with transposable elements was reportedly involved in abiotic stress responses including salt treatments in plants (Wang *et al.,* 2017). However, lncRNAs in wheat have not been fully cataloged, although some associated with wheat stripe rust and powdery mildew have been identified (Zhang *et al.,* 2013; Zhang *et al.,* 2016). In addition, 125 putative wheat stress-responsive long non-protein coding RNAs responsible to powdery mildew infection and heat stress have been identified (Xin *et al.,* 2011). An analysis of 52 RNA-Seq data sets in *Triticumaestivum* indicated that lncRNAs were regulated during tissue development and under abiotic stress (Shumayla *et al.,* 2017). The co-expression of lncRNA with other regulatory mRNAs indicated that lncRNAs are involved in numerous biological processes such as ABA biosynthesis and some acted as target mimics of known miRNAs (Shumayla *et al.,* 2017).

Further studies on lncRNAs and their molecular mechanisms in response to salt stress in wheat are needed. In the current work, we studied expression patterns of the salt stress-responsive lncRNAs of the salt-tolerant (Xiaoyan60) and salt-sensitive (Lumai21) wheat cultivars using a high-throughput sequencing approach. The salt-responsive wheat lncRNA candidates were predicted and their target genes, biological processes and significant metabolic pathways were assessed using bioinformatics tools. We further analyzed the salt-stress-responsive pathways of lncRNAs participation. We identified a total of 746 candidate lncRNAs that were both up and down-regulated and validated expression levels of 6 lncRNAsusing qRT-PCR.

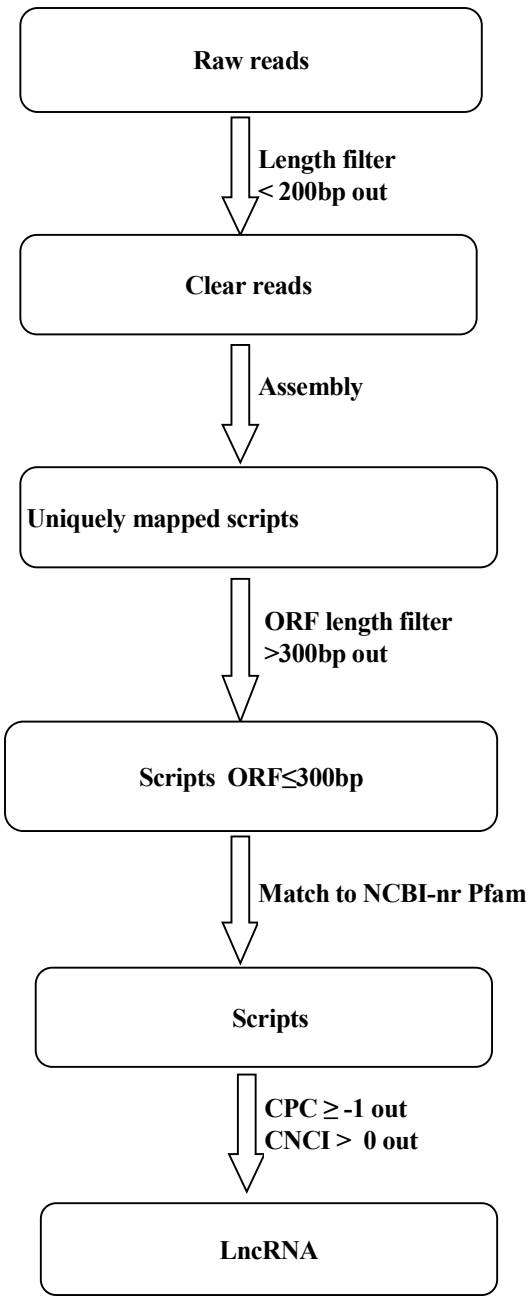

Fig. 1. The flow chart for predicting LncRNA responses to salt stress.

Materials and Methods

Plant materials: The seeds of salt-tolerant wheat cultivar Xiaoyan60 (XY) (Chen *et al.,* 2015) and salt-sensitive wheat cultivar Lumai21 (LM) (Fang & Liu, 1996) were treated with hydrochloric acid and then thoroughly rinsed with water. Then seeds were placed on culture dishes for germination at 25°C. After 7 days seedlings were transferred to a hydroponic box containing Hoagland's solution for two weeks at 25°C with 12-h photo periods. The seedlings at the three true-leaf stage were selected for salt stress treatments.

Stress treatments: Salt treatments were performed by soaking plant roots in Hoagland's solution (200 mMNaCl) for 3, 12, or 24h. All the plant materials were cultured in the greenhouse at Qingdao Agricultural University (36°N, 120°40'E). Leaves of five plants for each treatment were sampled for RNA extraction. Three biological replicates were used for high-through put sequencing and quantitative real-time PCR. Samples labeled as XY1, XY2, XY3, XY4 were designated for samples of cultivar XY at salt stresses for 0, 3, 12, and 24 h, respectively. Similarly, LM1, LM2, LM3, LM4 indicated the samples of cultivar LM at salt stresses for 0, 3, 12, and 24 h, respectively.

Measurement of osmotic potential: Osmotic potential was measured to evaluate the degree of damage to the plants. The sampling time was fixed as the end of salt treatment. The materials (1-2 g of the third leaves) were collected on July 15, 2018. The leaves were placed in aluminum foil immediately to minimize water loss and placed at -20°C for 4 hours. The materials were then crushed in a syringe and about 8-10μLsapof materials was used to test for osmotic content using a WescorVapro 5520 Vapor Pressure osmometer (Logan, UT, USA) (Ji *et al.,* 2012). Osmotic potential was obtained using the following formula:

$$\text{Osmotic potential (Mpa) } \psi s = - iCRT$$

where: i =1; C = Osmotic content, $mol.kg^{-1}$; R=0.08314 $Mpa.L.mol^{-1}.K^{-1}$; T= 273 + t°C

LncRNA library construction and sequencing: Total RNA was extracted from leaves and roots of seedlings from the two varieties derived from different treatments using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols, with three biological replicates for each treatment. A small fraction of the RNA was run in a 1% agarosegel to check for quality. The RNA integrity and purity were checked by an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). The sequencing was constructed using the IlluminaHiseq platform with a paired-end sequencing length of 150 base pairs (bp).

Quality checking, gene modeling, expression estimation and identifying novel LncRNAs: Clean reads were mapped to the wheat reference genome (TGACv1) (ftp://ftp.ensemblgenomes.org/pub/release-27/plants/fasta/triticum_aestivum/dna) using Top Hat (version 2.0.12) with default parameters (Trapnell *et al.,* 2009). The transcriptome of each treatment was assembled separately using Cufflinks version 2.2.1 (Deng *et al.,* 2015). The sequences of total assemble transcripts were subjected to analysis using CNCI (Sun *et al.,* 2013), and CPC (Kong *et al.,* 2007) tools, respectively. A transcript with a CPC value < –1 and a CNCI value < 0, respectively, were taken as non-coding. Differential gene expression was analyzed using 10 comparison schemes: XY2_XY1, XY3_XY1, XY4_XY1, LM2_LM1, LM3_LM1, LM4_LM1, LM1_XY1, LM2_XY2, LM3_XY3, LM4_XY4, which were performed using DESeq (version 1.18.0) (Wang *et al.,* 2010). Genes with $q<0.05$ and $|log2\_ratio| > 1$ were identified as differentially expressed genes (DEG) (Fig. 1). LncRNA functions were predicted from the target gene using RNA flex (Tafer & Hofacker, 2008).

Bioinformatics: DEGs were mapped to the GO (Gene Ontology, http://geneontology.org/) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. GO terms with q<0.05 for differentially expressed genes were considered significantly enriched. A path that conformed to this condition was defined as a pathway of significant enrichment in differentially expressed genes (Zhang *et al.*, 2016; Zhu *et al.*, 2017).

Quantitative real-time PCR analysis: Six lncRNAs that were differentially expressed were randomly chosen for qRT-PCR validation. Total RNA was isolated using Spin Column Plant Total RNA Purification Kit (Sangon Biotech, Shanghai, China) and was reverse-transcribed into cDNA following the manufacturer's instructions using a Prime Script RT reagent kit with gDNA Eraser (Takara, Beijing, China).All primers were designed and synthetized by QingdaoTsingke Biological (Qingdao, China) (Table S1). The method of $2^{-\Delta\Delta C\ t}$was used to determine target gene differential expression (Livak *et al.*, 2001). The experiments were performed in triplicate.

Results

Identification of lncRNAs responding to salt stress by high-throughput sequencing: The damage effects by salt to the two wheat cultivars were determined by measuring the osmotic potential of plant sap after salt exposure. Under control conditions, the osmotic potential of the salt tolerant cultivar XY60 (-1.01Mpa) did not differ significantly from the salt-sensitive cultivar LM21 (-0.92MPa). However, 24 h exposure to salt stress resulted in an osmotic pressure that was 31.8% less for XY60 (-1.45MPa) than controlLM21 (-1.10MPa). These results indicated that 200mM NaCl was sufficient to induce a severe degree of salt stress compared to control plants.

Using these experimental conditions we generated total genomic RNA expression libraries and compared assembly genome transcripts with those of the reference genomes. Transcripts of ≥200 bp with exon numbers at ≥2 and no coding potential were chosen as lncRNA. The lncRNAs were further identified by positional mapping to the genome and >50% of the filtered reads were mapped to intergenic regions. About 30% and 2.5% of filtered reads were mapped to exons and introns, respectively (Tables 1 and 2). We obtained 828 and 601 potential lncRNAs transcripts from cultivars XY1 and LM2, respectively. The number in XY1 was the largest followed by LM2. The number of other transcripts from XY were >LM at the same duration of salt stress, except that XY2 was <LM2 (Fig. 2b).

Differential expression analysis of lncRNAs: We found 746 lncRNAs that were altered ≥ 2-fold after salt stress. The number of up-regulated lncRNAs was maximal in the absence of NaCl treatments and the number of down-regulated lncRNAs was maximal at 3 h (Fig. 3a). In cultivar XY, 505lncRNAswere up- and 449 were down-regulated as compared with cultivar LM. We identified 9 up-regulated and 4 down-regulated lncRNAs in common between the XY experimental groups, and there were675 and 592 differentially expressed lncRNAs in XY and LM, respectively (Fig. 3b). The numbers of up-regulated lncRNA in XY under stress for 3, 12 and 24 h were >150. Compared with XY1, the largest numbers of up-regulated lncRNAs were detected in XY3; while the largest numbers of down-regulated lncRNAs were detected in XY4. We detected 25 up-regulated lncRNAs and 138 down-regulated lncRNAs among the XY treatment groups (Fig. 3c). As the exposure time to salt stress increased, the numbers of up-regulated lncRNAs in LM decreased and the number of down-regulated increased. Furthermore, 119 up and 49 down-regulated lncRNA in LM2 and LM4 were the maximum values in the each class of lncRNAs, respectively. A total of 8 up-regulated and 104 down-regulated lncRNAs were predicted in cultivar LM (Fig. 3d).

Supplementary Table 1. Primers used for RT-PCR.

Gene name	Forward primer (5'→3')	Reverece primer (5'→3')
β-actin	TGCTATCCTTCGTTTGGACCTT	AGCGGTTGTTGTGAGGGAGT
lnc_364	GGTGATTAGCGGCAAGGC	GGCGAGCACGATGGGAAA
lnc_521	CTTGATGTGCTGCCTTTC	TTTATGGGACTGTTACGC
lnc_593	GAAGACGGGAGAAGGAGT	CGAGTGATGTAACCGATGT
lnc_693	CCCCAGTCCACCTCCAAGA	GCTATCACCGCTCCAACC
lnc_726	TAGAGCAGTCGGACCCTT	CGGACACCCAACTTCAATA
lnc_743	GAAAGAAGGACCAGGATG	GAGGGTGGAAACAGAACTA

Table 1. Number and proportion of the sequences that mapped to the reference genome.

Sample	Exon		Intron		Intergenic	
	Sequence numbers	Ratio (%)	Sequence numbers	Ratio (%)	Sequence numbers	Ratio (%)
XY1	9,952,948	23.95	1,212,589	2.92	30,385,319	73.13
XY2	10,114,095	26.38	904,505	2.36	27,324,031	71.26
XY3	13,682,584	40.73	945,172	2.81	18,964,390	56.45
XY4	6,217,632	22.56	546,112	1.98	20,791,105	75.45
LM1	11,470,143	32.75	930,770	2.66	22,625,198	64.60
LM2	12,001,294	33.93	1,085,894	3.07	22,283,235	63.00
LM3	12,482,994	37.17	798,840	2.38	20,297,311	60.45
LM4	2,850,785	12.76	306,931	1.37	19,187,620	85.87

Table 2. Expression level of selected LncRNAs based on sequencing results.

LncRNA	XY1	XY2	XY3	XY4	LM1	LM2	LM3	LM4
364	0.00	0.00	6.80	8.88	0.00	0.00	0.00	7.86
521	0.00	3.43	0.00	0.00	0.00	0.00	3.06	0.00
593	0.00	2.90	0.00	0.00	0.00	2.97	0.00	0.00
693	0.00	6.33	9.33	7.55	0.00	3.71	5.99	4.57
726	0.00	0.00	3.01	9.52	0.00	0.00	5.32	11.01
743	0.00	0.00	5.46	3.24	0.00	0.00	0.00	0.00

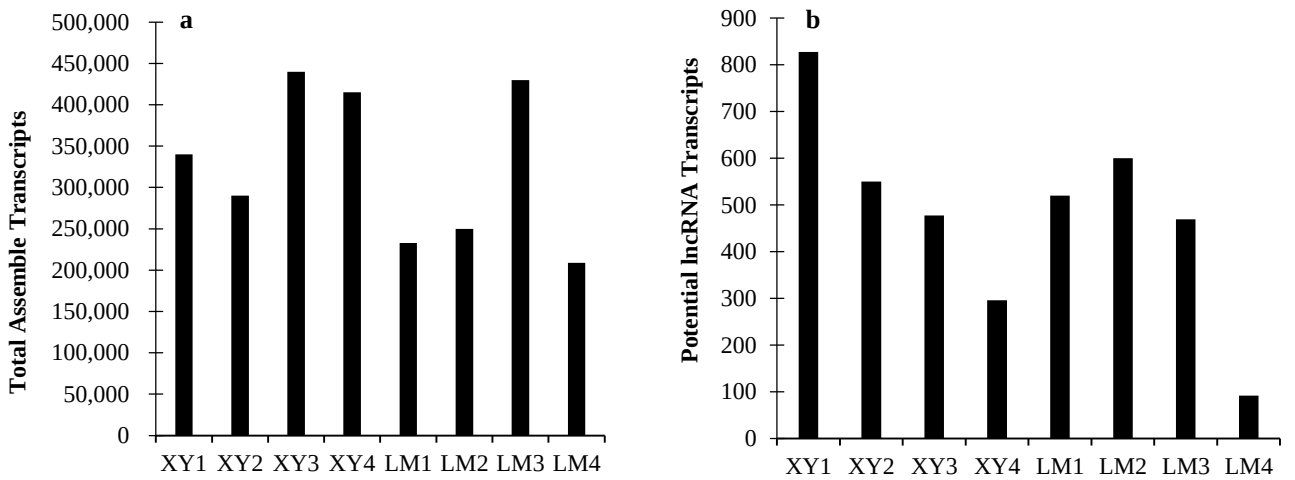

Fig. 2. The number of total assembled transcripts and potential lncRNAtranscripts.
a: The numbers of total assembled transcripts from 8 samples; and **b:** The numbers of potential lncRNA transcripts screened.
Note: XY1, XY2, XY 3 and XY4 indicated Xiaoyan 60 (XY) under salt stress for 0, 3, 12 and 24 hours; LM1, LM 2 LM3 and LM4 indicated Lumai 21 (LM) under salt stress for 0, 3, 12 and 24 hours.

GO term enrichment of differential genes between XY and LM: The differential genes of test modules XY and LM were assigned and enriched to different GO terms and covered3 domains including BP, CC and MF. Functional enrichments of 488 significant XY genes were associated with photosynthesis. These enrichments of genes included photosystem I (GO:0009522), light harvesting (GO:0009765), chlorophyll binding (GO:0016168), plastid thylakoid (GO:0031976), photosynthesis (GO:0015979), electron transport chain (GO:0022900), thylakoid part (GO:0044436), and chloroplast thylakoid (GO:0009534). However, 2185 significant genes of LM were enriched in items related to cellular structure, secondary metabolism, and redox. For example, intrinsic to membrane (GO:0031224), integral to membrane (GO:0016021), secondary metabolite biosynthetic process (GO:0044550), and oxidoreductase activity (GO:0016491) were all enriched in LM (Table S2).

Pathways analysis of stress-responsive lncRNA participation: To examine the function of lncRNAs related to salt stress, we analyzed relationships between gene function and trans-lncRNAs using KEGG analysis. We identified 52 paths of significant enrichment with 25 and 30 pathways significantly enriched in XY and LM, respectively. In cultivar XY, at least 98 differentially expressed lncRNAs were involved in the significant pathways and 116 for LM (Fig. 4). We also identified lncRNA target genes associated with salt stress. In the plant hormone signal transduction category, the results were only significant at the start of the experiments (3 h). However, we identified 31 lncRNAs in XY (29 up and 2 down) and 25 lncRNAs in LM (22 up and 3 down) (Table S3).

In cultivar XY, we identified 5 pathways associated with the salt stress response at 12 and 24 h with correlation pathways including photosynthesis (5 lncRNAs, 4 up and 1 down), oxidative phosphorylation (17 lncRNAs, 8up and 9 down), glycine, serine and threonine metabolism (21 lncRNAs, 20 up and 1 down) in XY3. Photosynthesis (7 lncRNAs, 4 up and 3 down), oxidative phosphorylation (20 lncRNAs, 13 up and 7 down), flavonoid biosynthesis (21 lncRNAs, 18 up and 3 down), and photosynthesis-antenna proteins (1 up lncRNA) correlated with the salt stress in XY4 (Table S3).

There were4 significant pathways in LM. Only the glutathione metabolism pathway (43 lncRNAs, 40 up and 3 down) was related to salt stress (12 h). The related pathways are plant hormone signal transduction (20 lncRNAs, 10 up and 10 down), peroxisome (10 lncRNAs, 5up and 5 down), glutathione metabolism (15 lncRNAs, 12 up and 3 down) and arginine and proline metabolism (6 lncRNAs, 5 up and 1 down) in LM4.

Significantly enriched pathways of lncRNA participation in two cultivars: We compared the different expression levels of lncRNA of LM *vs* XY, and then identified 4 commonly up-regulated lncRNAs. A total of 111 differentially expressed lncRNAs were involved in significant KEGG pathways. We found 28 pathways that were significantly enriched in LM, including systemic lupus erythematosus, photosynthesis-antenna proteins, phenylpropanoid biosynthesis, alcoholism, and biosynthesis of secondary metabolites.

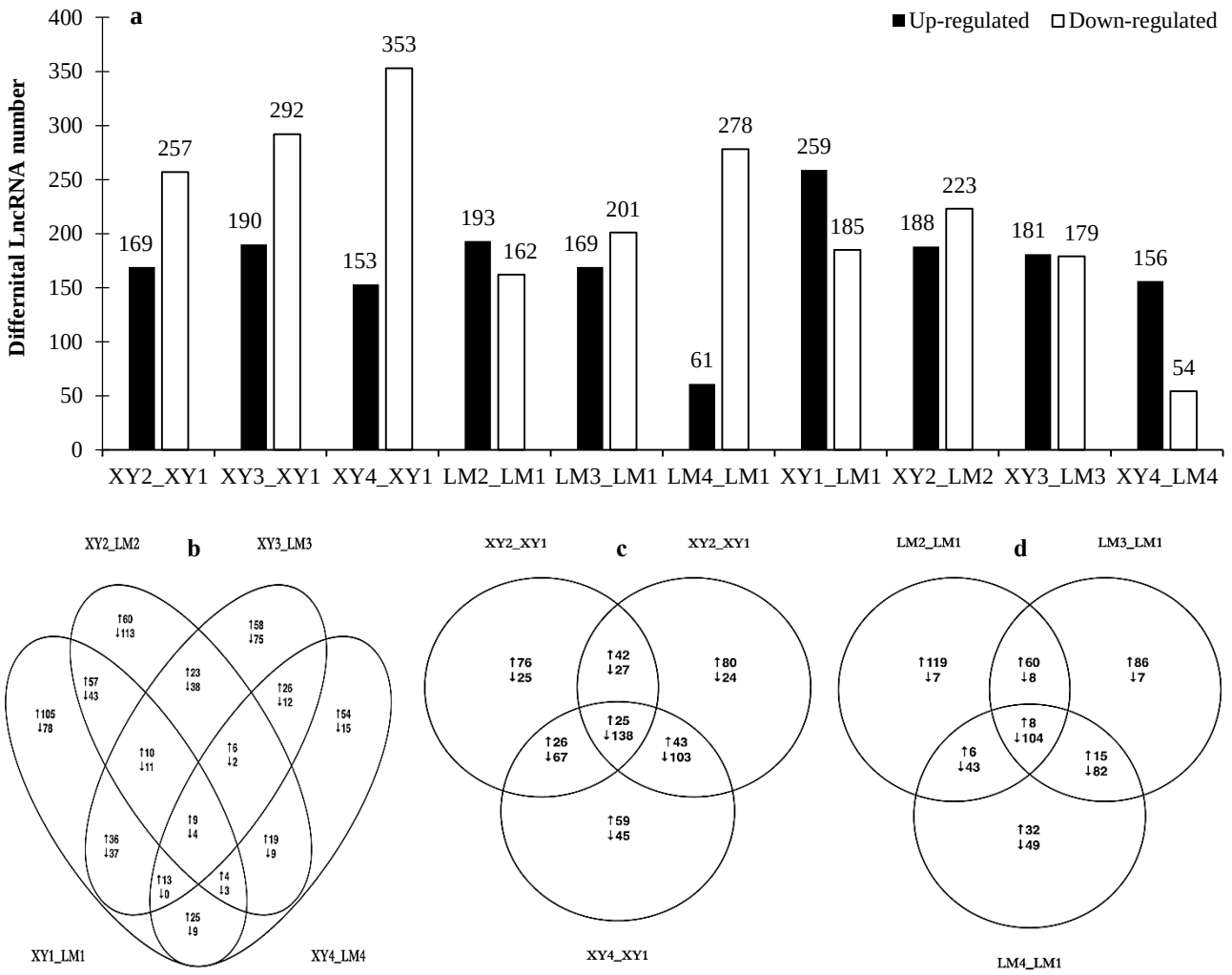

Fig. 3. Expression pattern and venn diagram of lncRNAs under salt stress at different time points.
a: The numbers of up and down-regulated lncRNA by differential analysis; **b:** the numbers of common/specific lncRNA in four comparisons of Lumai 21 and Xiaoyan 60; **c:** The numbers of common/specific lncRNA in Xiaoyan 60; and **d:** The numbers of common/specific lncRNA in Lumai 21.
Note: The XY2_XY1, XY3_XY1 and XY4_XY1 indicated that Xiaoyan 60 (XY) under salt stress for 3, 12 and 24 hours were compared with the control group, respectively. For Lumai 21 (LM), the same as shown in XY. XY1_LM1, XY2_LM2, XY3_LM3, XY4_LM4 indicated that XY and LM were under different salt stress, respectively. Upward and downward arrows indicate the number of up-regulated and down-regulated lncRNA, respectively.

Compared with XY, the photosynthesis pathway was significant in cultivar LM1 and included 3 lncRNAs (1 up and 2 down). DNA replication was significant in LM2 with 23 lncRNAs participating in this pathway (22 up and 1 down). There were three pathways associated with salt stress in LM3 and the correlation pathways were flavonoid biosynthesis (1 up), starch and sucrose metabolism (3 up) and glutathione metabolism (10 lncRNAs, 9 up and 1 down) (Table S4).

Real-time PCR detection of differentially expressed lncRNA: We next chose 6 salt-responsive lncRNAs to validate using qRT-PCR and included lnc_364, 521, 593, 693, 726 and 743 selected from the significant pathway involved in salt stress after 0, 3, 12 and 24 h of salt treatment (Fig. 5). From the sequencing data, cultivar XY displayed higher levels of lnc_364, 521, 593 and 743 than LM at 3 and 12 h of salt treatment. Lnc_726 and lnc_693 were significantly expressed by both cultivars after 12and 24 h of salt treatment, respectively. When we examined these same lncRNAs using qRT-PCR we found that lnc-364, 693 and 726 were consistent with the sequencing results. However, we detected significant differences between qRT-PCR and sequence results for lnc_521,593 and 743 (Fig. 5).

Discussion

Numerous studies have identified the presence of lncRNAs using RNA-Seqanalysis and thousands of putative lncRNAs are expressed in plants in response to stress conditions including drought, fungal infection, phosphate starvation and nitrogen stress (Liu *et al*., 2012; Shuai*et al.*, 2014; Yuan *et al.*, 2016; Zhang *et al*., 2016). In this study, RNA-Seq sequencing technology identified 746 salt-responsive lncRNAs. In addition, we analyzed target gene function and path enrichment that may be related to potential regulatory mechanisms.

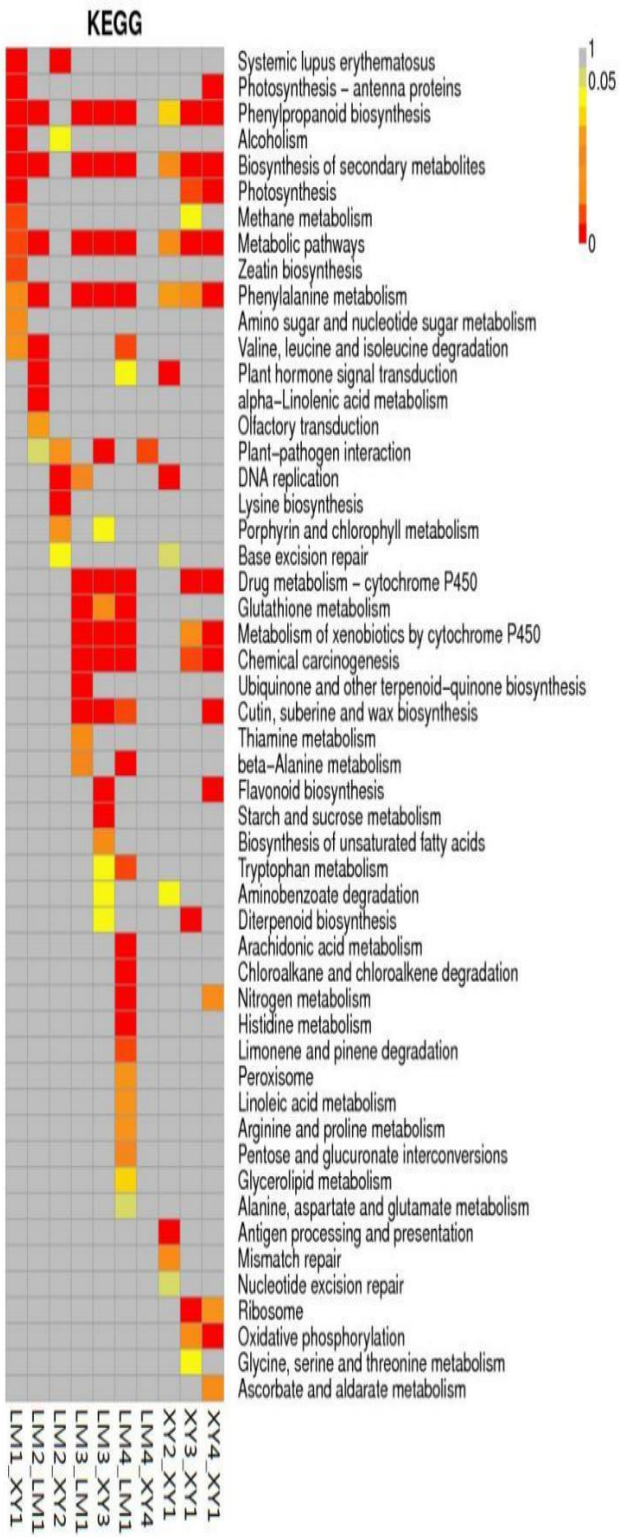

Fig. 4. Heat map shows relative expression profile of different lncRNA
Note: The darker the color, the more significant it is.

The putative roles of lncRNA in salt stress responses in this study were also compared to a previous study (Shumayla *et al.*, 2017). We found that lncRNAs were differentially expressed during abiotic stresses treatment in both studies. The number of lncRNAs increased significantly with the duration of stress treatment. Furthermore, co-expression analysis revealed that lncRNAs were involved in numerous biological processes such as photosynthesis and respond to biotic and abiotic stimuli including organic acid and mono acid metabolism.

Previous studies have shown that several abiotic stresses are significantly enriched for related GO categories such as photosynthesis, light harvesting, photosynthesis and oxidoreductase activity (Li *et al.*, 2014; Lv *et al.*, 2016; Song *et al.*, 2016). We found that the top GO categories of photosynthesis and light harvesting predominated in the current study. (Gondor *et al.*, 2016; Song *et al.*, 2016). There are many endogenous hormone related proteins or receptors in plant hormone signal transduction pathways including the ABA responsive element binding factor and SAUR family proteins. We also identified these in response to salt stress (Hirayama *et al.*, 2010; Ismail *et al.*, 2012; Kumar *et al.*, 2012). The differentially expressed genes were significantly enriched in the glycine, serine and threonine metabolism pathway under salt stress. Among them, the gene encoding D-3-phosphoglycerate dehydrogenase and serine hydroxymethyltransferase has been previously implicated in salt stress tolerance (Kito *et al.*, 2017).

We verified that 6 lncRNAs involved in gene regulation were also present in the significantly enriched pathways. Lnc_726, an up-regulated lncRNA regulates *ERF1* in the plant signal transduction pathway. A previous study identified that *TdERF1* was increased in leaves after salt treatment for 6 h while it was decreased in a sensitive cultivar (Makhloufi *et al.*, 2014). The expression level of *TdERF1* displayed a dramatic increase in the current study in the two varieties after 24h. The expression level of lnc_726 increased over time, which was verified by qRT-PCR that its target gene (*ERF1*) was enriched in this pathway. The expression level of *npc536* was changed after 3h salt treatment and remained unchanged after 24 h in another study (Amor *et al.*, 2009). We found 25 lncRNAs in XY (salt tolerant) were up regulated after salt stress, and one (lnc_693) was verified to be up-regulated after salt stress. The expression levels of four lncRNAs fluctuated after inoculation with stripe rust pathogens (Zhang *et al.*, 2013). Interestingly, we found that the expression levels of several lncRNAs (i.e., lnc_521, 593, 693 and 743) also fluctuated after salt treatment (Fig. 4). Actually, lncRNAs responding to salt stress in wheat have not been yet reported. We identified lncRNAs responding to salt stress at the regulatory level and studied the pathways related to these lncRNAs. Our future work will investigate the function of target genes related to lncRNAs to examine mechanisms involved in regulation of the salt stress response by lncRNAs in wheat.

Acknowledgements

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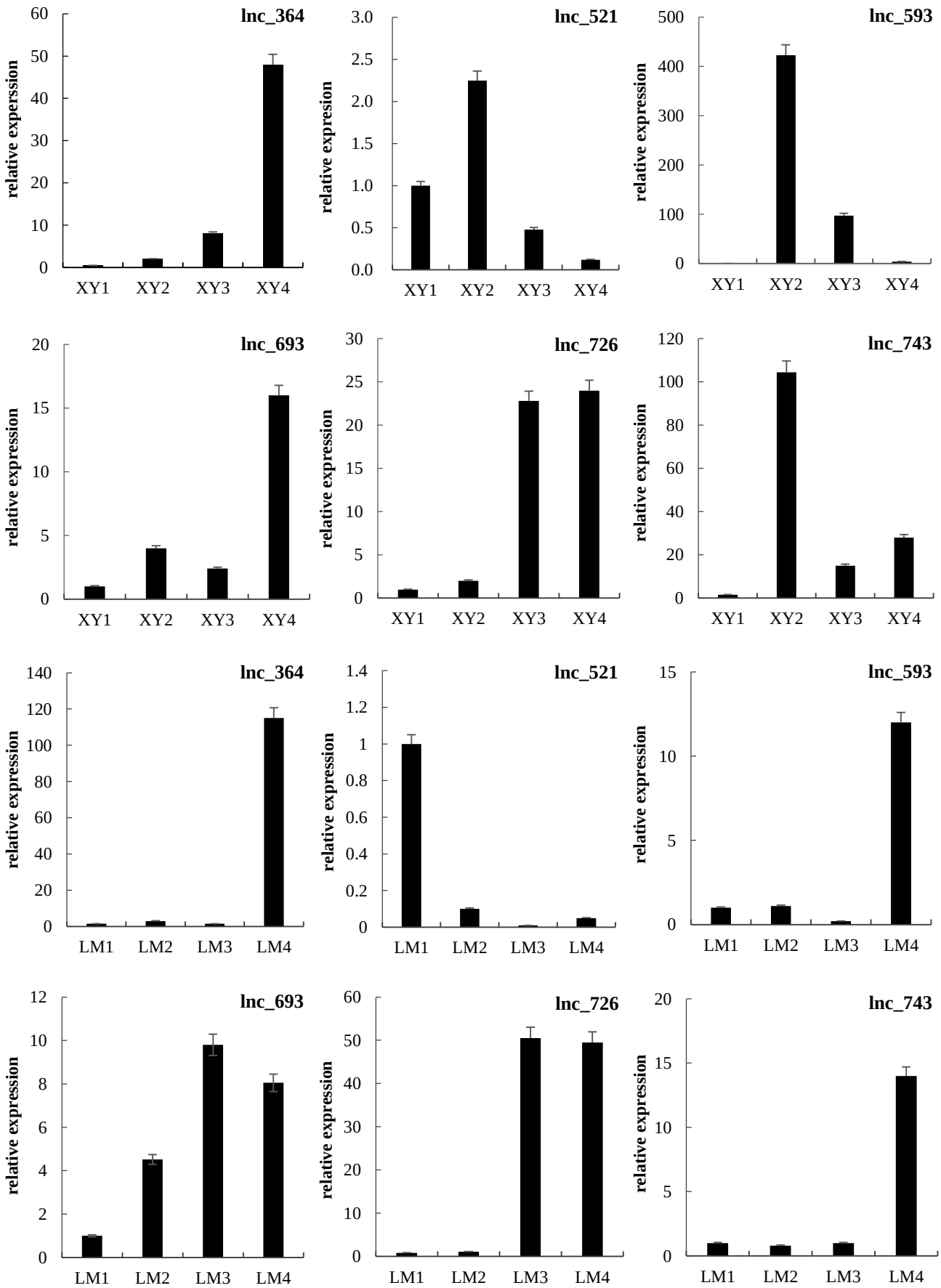

Fig. 5. Expression patterns of 6 lncRNAs in response to salt treatment using qRT-PCR
Note: XY1, XY2, XY3 and XY4 representing the salt stress for 0,3, 12 and 24 hours of Xiaoyan60; and LM1, LM2 LM3 and LM4 representing the salt stress for 0, 3, 12 and 24 hours of Lumai21.

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