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Expression profiling of the genes induced by Na_2CO_3 and NaCl stresses in leaves and roots of *Leymus chinensis*

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ABSTRACT

Alkaline soil is more challenging factor to grow plants than saline soil based on the size of its affected area. To reveal genetic expression changes by alkaline and saline soil, *Leymus chinensis* (Trin.) Tzvel. was examined response to Na_2CO_3 and NaCl stresses by using microarray chips comprising 1642 cDNA clones, previously reported by us. A total of 536 genes were responsive to Na_2CO_3 and NaCl stresses by up-regulation or down-regulation. We observed transcriptional changes arose more to Na_2CO_3 stress, or leaves than to NaCl stress or roots, respectively, in *L. chinensis* genome. Overall, 99 and 59 genes were up-regulated, while 365 and 176 genes were down-regulated in leaves and roots, respectively; demonstrating down-regulation occurs more as the response to the Na_2CO_3 and NaCl stress. The majority of the down-regulated genes (30.1%) were classified to photosynthesis related proteins while that of the up-regulated genes were categorized into metabolism proteins or noble proteins. Only 37 genes in the stressed leaves and 6 genes in the stressed roots were showed the same expression changing patterns between Na_2CO_3 and NaCl stresses during three time points of detections. Among those genes, 70% were constantly up-regulated or down-regulated during our detection presenting difference in the gene regulation systems against two difference stress, the saline-alkali stress (Na_2CO_3) and saline stress (NaCl). In addition, a total of 87 genes detected in this study were characterized to the unclassified proteins, whereas 72 had no similarity to the current GenBank databases which were considered as novel proteins detected in the alkali and saline stressed *L. chinensis* genome. The four important genes to abiotic stress tolerance, ACC, GDP, hsp70, and elF1 were detected constantly for all of three time points of stressed which are the grand candidates for establishing stress tolerance plants by developing transgenic.

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1. Introduction

A number of abiotic stresses including water, salt and temperature affect plant growth and productivity. Among these, saline and alkaline soils are the major constraints limiting economic yields of crop plants. Based on the world soil map, the area of 831 million hectares is affected by salt stress. Of this, area under sodic soils (alkaline soils) is 434 million hectares, compared to 397 million hectares of saline soils (www.fao.org/ag/agl/agll/spush/intro.htm). Unfortunately alkalinity tolerance in plants has not been studied in depth although salinity tolerance has been extensively studied using *Arabidopsis* and rice [1]. Soil alkalinity is generally associated with the presence of sodium in soil such as Na_2CO_3 or $NaHCO_3$ which damages plants by sodium toxicity as well as high pH. Therefore, it is very rare the high alkaline tolerant plants reported, with the consequence that studies on sodium abiotic stress has been biased to salinity tolerance. However, *Leymus chinensis* (Trin.) Tzvel, was recognized to survives in high alkaline soil (pH 8.5–11.5) naturally. *L. chinensis* is a rhizomatous perennial forage native in grassland of northern China, known to be drought tolerant [2] and was studied on alkaline tolerance by using cDNAs of Na_2CO_3 stressed *L. chinensis* identifying putative ESTs associated with adaptation of itself under sodic stress [3].

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The EST analysis, however, does not provide information about the levels of genes expressed by stresses. The microarray technology has become an important tool to study the genome-wide expression profiling of the genes responding to a particular stress. A number of studies have been carried out to understand the mechanisms of stress tolerances and to determine levels of gene expression [4–6]. The stress inducible genes are grouped into two: protecting and regulating genes to stresses [7–9]. Most of the studies on stress-regulated transcriptomes have been conducted on leaves [10] and rarely on roots [5]. Because roots and leaves contain different sets of specialized cells, there should be different stress response programs between these two tissues. Moreover, the roots contact directly to the stressful environment factors, like Na^+, Cl^- and CO_3^{2-}. Accordingly, understanding the expression changes of the genes in stressed roots will add more comprehensive clues to the mechanisms of stress tolerance established by studying leaf transcriptomes.

In this study, the genes differentially expressed during Na_2CO_3 and NaCl stresses were profiled by using the microarray constructed of *L. chinensis* cDNAs [3]. The expression of the transcripts was monitored by tissue types and time points. The changes of gene expression by saline-alkali stress and saline stress were compared by evaluating gene regulation during Na_2CO_3 and NaCl stresses. In addition, three genes were examined for salt resistance by express them in yeast.

2. Materials and methods

2.1. Plant materials

A *L. chinensis*, growing naturally under alkaline sodic condition in 'An Da' grasslands of northern China (44°1′N latitude and 119°28′E longitude) [3], was used for this study.

2.2. Amplification of cDNA inserts

Inserts of 1642 cDNA clones, previously characterized [3], were amplified by polymerase chain reaction (PCR) using primers, T3: 5′-AATTAACCCTCACTAAAGGG and T7: 5′-GTAATACGACTCACTATAGGGC. Plasmid template (20 ng) was used to 100 μl of PCR mixture containing 0.25 mM of each dNTP, 0.2 μM primer, 1× reaction buffer (Bioneer), and 1 unit of *Taq* DNA polymerase (Bioneer). PCR was performed with following conditions: 5 min at 94 °C, 33 cycles of 1 min at 95 °C, 45 min at 56 °C, and 2 min at 72 °C followed 10 min at 72 °C. The PCR products were purified by precipitation in sodium acetate and isopropanol, and resolved in 3× SSC and 1.5 M betaine. The purified cDNA inserts were confirmed the amplification quality and quantity by electrophoresis in 1% agarose gel.

2.3. cDNA chip preparation

cDNA inserts that PCR amplified and purified were arrayed from 384-well microtiter plates onto CMT-GAPSTM glass slide (Corning) using chip builder (Cartecian PixSYS 5500). Slides contained 869 leaf and 773 root cDNAs spotted in duplicate. *Arabidopsis* α-tubulin gene [11] served as an internal control, and the mouse nicotinic acetylchiline receptor epsilon-subunit (*nAChRE*) gene and the mouse glucocortical receptor homolog gene, which have no substantial homology to any sequences of the *L. chinensis*, as negative control to assess for nonspecific hybridization.

2.4. Stress treatments and RNA isolation

L. chinensis seedlings were hydropoincally grown in Hoagland nutrient solution in a growth chamber under 13 h light/11 h dark periods at 22 °C for 30 days. The plants were subjected to NaCl and Na_2CO_3 treatment for 1, 3 and 6 h by adding NaCl (200 mM) or Na_2CO_3 (100 mM) to the Hoagland medium. Leaves and roots were harvested and RNA was extracted.

2.5. Preparation of fluorescent probes

Total RNA samples were reverse transcribed in presence of Cy3-dCTP (Control) or Cy5-dCTP (Target). Reverse transcription reaction was performed in 30 μl volume containing 50 μg RNA with 2 μg of 18-mer oligo (dT), 100 μM DTT, 10 μM dNTP, 50 μM each of Cy3 and Cy5, 200 U reverse-transcriptase (GIBCO BRL). After incubation at 42 °C for 2 h, the reaction products of two samples (one labeled with Cy3 and the other with Cy5) were combined, treated with 15 μl of 0.1 M NaOH and 1.5 μl of 20 mM EDTA, and incubated at 65 °C 10 min. The cDNA was concentrated and cleaned by ethanol precipitation. The pellets were dissolved in 21 μl hybridization buffer (1.2× Denhardt's Solution, 4.5% SDS, 1× SSC, 1 mM EDTA, 0.25 M Na_2HPO_4). The probe samples were denatured by placing them in a 100 °C water bath for 2 min, incubated at 45 °C for 20 min, and then used for hybridization.

2.6. Hybridization, scanning and data acquisition

A cover slip was placed over the entire array surface to avoid the formation of bubbles. Slides were placed in a sealed hybridization cassette and submerged in a 65 °C water bath for 12 h. After hybridization, the slides were placed in slide rack submerged in washing solution I (2× SSC, 0.1% SDS) for 2 min, washing solution II (1× SSC) for 3 min, and washing solution III (0.2× SSC) for 2 min, spun at 1000 rpm for 2 min, and dried.

The cDNA microarrays were scanned at two different wavelengths (channels), one for Cy3-labeled (green, 532 nm) samples and another for Cy5-labeled (red, 635 nm) samples, were analyzed using Axon GenePix scanarray 4000 (Axon 4000) software. To normalize the two channels with respect to signal intensity, we adjusted photo-multiplier and laser power settings so that the signal ratio of house-keeping gene was close to 1 ± 0.05.

Spot intensities were extracted from a scanned image with ImaGene 2.0 (BioDiscovery) and GeneSpring (Silicon Genetics) Software. The raw intensity data was global normalized by intensity dependent normalization on Lowess method [12], then was normalized by with-print-tip group normalization method for each print-tip, eight tips was used for making 1.5 K cDNA microarray. We gathered fold change from M, $\log_2 R/G$ (R is Cy5 signal and G is Cy3 signal). We have chose gene expression of 1.5-fold changed, over 1.5 and down 0.67 ($M \geq 0.58$ and $M \leq -0.58$). If gene expression of 1.5-fold changed, the $\log_2 R/G$ values of transcripts showed ≥ 0.58 and ≤ -0.58, they were considered up-regulated and down-regulated, respectively (Supplementary Fig. 1). Hybridizations were performed twice for each sample, and average values worked out.

2.7. Expression of L. chinensis cDNA in yeast

Yeast *Saccharomyces cerevisiae* Y2805 [13,14] was used to express selected cDNAs. Yeasts were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) for normal growth and YPRG (1% yeast extract, 2% peptone, 2% raffinose, 2% galactose) for expression of selected cDNA.

Among 1642 cDNAs, 3 cDNA clones were selected based on increased expression level of >3-fold by Na_2CO_3 or NaCl stresses. The following primer sets were used to amplify the cDNA inserts from CD809126, CD809168, and CD809218: 15′-GCGAATTCATGTCTGATCTCGACAT CCAG/5′-TCGTCGACCTAAAATCCGTGAAT-

CTTGATG (CD809126), 5′-GCGAATTCATGGT GCTTCACGACTCCCT/5′-TCGTCGACCTACCATGGCTCGGAGTTTTTG (CD809168), and 5′-GCGAATTCATGGCTTCCAGTTTCCAACTGC/5′-TCGTCGACT-CAGCTGTTGACGACCC TGC (CD809218). The PCR products were purified using Qiaquick PCR purification kit, (Qiagen) and digested with EcoRI, SalI. The samples were loaded in an agarose gel and the target bands were purified using Qiaquick Gel extraction kit (Qiagen) and cloned into pYEGα-hir252 [14], the yeast expression plasmid. Yeasts were transformed with the constructed plasmids and selected on UD plate (0.67% yeast nitrogen base without amino acid, 0.2 mM uracil, 2% agar, 2% glucose, pH 5.6–6.0).

Yeast grew in YPD medium overnight and diluted to be $OD_{600} = 1$. The diluted cultures were serially diluted (1:10, 1:100 and 1:1000) and spotted on YPRG plate containing NaCl or Na_2CO_3. Growth was examined after 4 days at 30 °C [15,16].

3. Results

3.1. Na_2CO_3 and NaCl stress responsive genes in *L. chinensis* leaves and roots

We have developed cDNA microarray containing 1642 *L. chinensis* ESTs, previously reported [3]. To monitor expression changes of those transcriptomes during saline-alkali and saline stresses, total RNA samples from leaves and roots at three time points (1, 3 and 6 h) after NaCl and Na_2CO_3 treatments were extracted and reverse transcribed into microarray probes. A total of 536 transcriptomes (Supplementary Table 1) were detected to be differentially expressed all over the three time points of Na_2CO_3 and NaCl treatments. The transcriptomes expressed by Na_2CO_3 and NaCl stresses were categorized by the function (Table 1, Supplementary Table 1). As the response to Na_2CO_3 and NaCl stress, 27–87 genes were up-regulated, 91–357 genes were down-regulated, and 1198–1503 genes were unchanged in roots and leaves. The majority of the down-regulated genes (30.1%) were classified to photosynthesis related proteins while that of the up-regulated genes were categorized into metabolism proteins or noble proteins. Interestingly, the genes functioned for cell division, cell wall, chromosome metabolism, and cytoskeleton were responded significantly low (one to three genes) to Na_2CO_3 and NaCl stresses.

The expression patterns of the different stress types (Na_2CO_3 and NaCl) and tissue types (leaf and root) were compared to analyze the common features of the up- or down-regulated genes (Fig. 1.). Overall, 99 and 59 genes were up-regulated, while 365 and 176 genes were down-regulated in leaves and roots, respectively; demonstrating down-regulation occurs more as the response to the Na_2CO_3 and NaCl stresses. Approximately, twice more genes were changed in the stressed leaves (464 up- or down-regulated genes) than in the stressed roots (235 up- or down-regulated genes). The most change of the gene expression was detected from Na_2CO_3 stressed leaves by showing 357 down-regulated genes and 87 up-regulated genes. It revealed more genes react to Na_2CO_3 stress than NaCl stress in *L. chinensis* genome with exception of up-regulation in roots. In leaves and roots, 38%/25% of the up-regulated genes and 36%/33% of the down-regulated genes, respectively, were regulated in common by both of Na_2CO_3 and NaCl stresses. This implies up-regulation may arise more in leaves than in roots when *L. chinensis* is in saline-alkali and saline stresses. However, only 37 genes in leaves (4 up-regulation, 33 down-regulation), and 6 genes in roots (0 up-regulation, 6 down-regulation) were showed the same expression changing patterns between Na_2CO_3 and NaCl stresses for all three time points. Among those genes, 70% were detected to be constantly up-regulated or down-regulated during our detection. In conclusion, our results revealed the existence of different gene regulation systems to the

Table 1
Summary of genes responsive to Na_2CO_3 and NaCl stresses in leaves and roots of *Leymus chinensis*

Functional category[a]	Na_2CO_3 stressed						NaCl stressed					
	Leaves			Roots			Leaves			Roots		
	Up-regulated	Down-regulated	Unchanged	Up-regulated	Down-regulated	Unchanged	Up-regulated	Down-regulated	Unchanged	Up-regulated	Down-regulated	Unchanged
Cell division	0 (0%)	1 (0.3%)	6 (0.5%)	0 (0%)	0 (0%)	7 (0.5%)	0 (0%)	0 (0%)	7 (0.5%)	0 (0%)	1 (1.1%)	6 (0.4%)
Cell wall	0 (0%)	1 (0.3%)	13 (1.1%)	0 (0%)	0 (0%)	14 (1.0%)	1 (2.0%)	0 (0%)	13 (0.9%)	0 (0%)	0 (0%)	14 (0.9%)
Chromosome metabolism	0 (0%)	1 (0.3%)	12 (1.0%)	0 (0%)	0 (0%)	13 (1.0%)	0 (0%)	0 (0%)	13 (0.9%)	0 (0%)	0 (0%)	13 (0.9%)
Cytoskeleton	0 (0%)	3 (0.8%)	9 (0.8%)	0 (0%)	1 (0.7%)	11 (0.8%)	0 (0%)	0 (0%)	12 (0.8%)	0 (0%)	0 (0%)	12 (0.8%)
Defense	4 (4.6%)	10 (2.8%)	27 (2.3%)	0 (0%)	6 (4.2%)	35 (2.4%)	4 (8.0%)	4 (2.9%)	33 (2.3%)	3 (6.3%)	3 (3.3%)	35 (2.3%)
Gene expression and RNA metabolism	3 (3.4%)	20 (5.6%)	71 (5.9%)	2 (7.4%)	5 (3.5%)	87 (5.9%)	1 (2.0%)	7 (5.1%)	86 (6.1%)	3 (6.3%)	2 (2.2%)	89 (5.9%)
Membrane transport	4 (4.6%)	11 (3.1%)	45 (3.8%)	0 (0%)	10 (7.0%)	50 (3.4%)	3 (6.0%)	2 (1.5%)	55 (3.8%)	3 (6.3%)	4 (4.4%)	53 (3.5%)
Metabolism	18 (20.7%)	55 (15.4%)	159 (13.3%)	7 (25.9%)	26 (18.2%)	199 (13.4%)	12 (24.0%)	17 (12.2%)	203 (14.0%)	10 (20.8%)	12 (13.2%)	210 (14.0%)
Photosynthesis	5 (5.8%)	115 (32.2%)	75 (6.3%)	3 (11.1%)	26 (18.2%)	166 (11.3%)	6 (12.0%)	61 (43.7%)	128 (8.8%)	6 (12.5%)	26 (28.6%)	163 (10.8%)
Protein metabolism and sorting	12 (13.8%)	15 (4.2%)	98 (8.2%)	3 (11.2%)	17 (11.8%)	105 (7.0%)	4 (8.0%)	4 (2.5%)	117 (8.1%)	5 (10.4%)	4 (4.4%)	116 (7.7%)
Signaling components	3 (3.4%)	5 (1.4%)	47 (3.8%)	1 (3.7%)	4 (2.8%)	50 (3.4%)	1 (2.0%)	2 (1.4%)	52 (3.5%)	1 (2.1%)	1 (1.1%)	53 (3.5%)
Unclassified proteins	18 (20.7%)	69 (19.6%)	261 (21.7%)	5 (18.5%)	21 (14.7%)	322 (21.8%)	7 (14.0%)	21 (15.0%)	320 (22.0%)	10 (20.8%)	21 (23.1%)	317 (21.1%)
No hit	20 (23.0%)	51 (14.3%)	375 (31.3%)	5 (18.5%)	27 (18.9%)	414 (28.1%)	11 (22.0%)	22 (15.7%)	413 (28.3%)	7 (14.5%)	17 (18.7%)	422 (28.1%)
Total	87	357	1,198	27	143	1,472	50	140	1,452	48	91	1,503

[a] Based on Jin et al. [3].

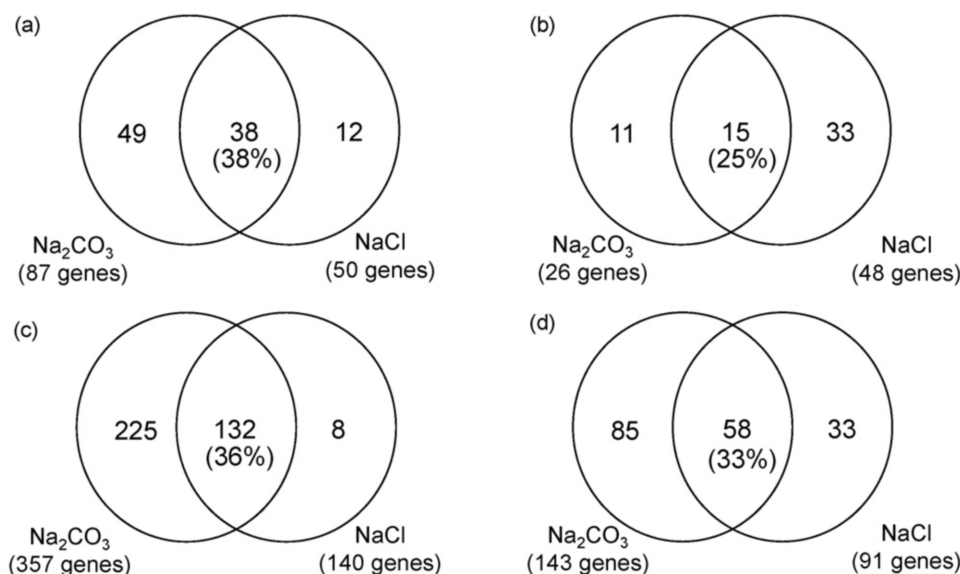

Fig. 1. Na_2CO_3 and/or NaCl stress-induced genes in leaves and roots: up-regulated genes in leaves (a) and roots (b); down-regulated genes in leaves (c) and roots (d).

two different stresses, the saline-alkali stress (Na_2CO_3) and saline stress (NaCl).

3.2. Kinetics of Na_2CO_3 and NaCl stress responsive genes

To examine *L. chiniensis* gene expression changes during the stresses, the number of genes up- or down-regulated by Na_2CO_3 and NaCl stresses at the three time points (1, 3, and 6 h) were compared (Fig. 2, Supplementary Figs. 1 and 2). It was detected the responsive genes to the different stresses (NaCl or Na_2CO_3) were differently changed the expression level by time (1, 3, and 6 h) and space (leaves or roots). The genes up-regulated by Na_2CO_3 in leaves and roots (73%, and 93% of the up-regulation genes) were turned on at the time point of 6 h or continuously expressed during the three time points. The up-regulated genes by NaCl stress showed different expression changes from those by Na_2CO_3 as well as different patterns in the stressed leaves from the roots. The genes were turned on during NaCl stress showing the up-regulation of 56%/46%/35% or 36%/64%/28% of the total up-regulated genes in leaves and roots at 1 h/3 h/6 h of time points, respectively. The down-regulation patterns for the stresses at the detected time points were varied by the stress (Na_2CO_3 or NaCl) and tissue types (leaves or roots) (Fig. 2, Supplementary Fig. 2). The same down-regulation pattern was detected from Na_2CO_3 stressed roots and NaCl stressed leaves presenting 34%/73%/42% and 46%/76%/52% of the total down-regulated expression at 1 h/3 h/6 h of time points, respectively. The down-regulations in the Na_2CO_3 stressed roots and NaCl stressed roots showed unique pattern of gene expression changes compared to the other gene expression changes in this study; 73% of the total down-regulated genes were down-regulated at the time point of 3 h in the Na_2CO stressed roots and the two time points of 1 and 6 h were detected as the major down-regulation points of the reacted genes in the NaCl stressed roots.

3.3. Comparative expression profiles of transcripts in Na_2CO_3 and NaCl stressed leaves and roots

The different number of transcripts and their expression were observed in the stressed leaves and roots (Supplementary Table 1) as expected since a leaf and a root are specialized cells. Among the 140 of the total up-regulated genes, 87 were able to be characterized their functions based on our previous report [3]. Thirty-two genes out of 87 functionally characterized genes were expressed in the stressed leaves while 21 genes were expressed in the stressed roots. Only 15.6% of the characterized up-regulated genes (11 genes) were co-expressed in the stressed leaves and roots which might be governing a common mechanism in the salt stress response in the both organs. The number of up-regulated genes by NaCl stress was much higher in roots (22 genes) than in leaves (8 genes). Na_2CO_3 stress, on the other hand, up-regulated 31 genes in leaves and 2 in roots.

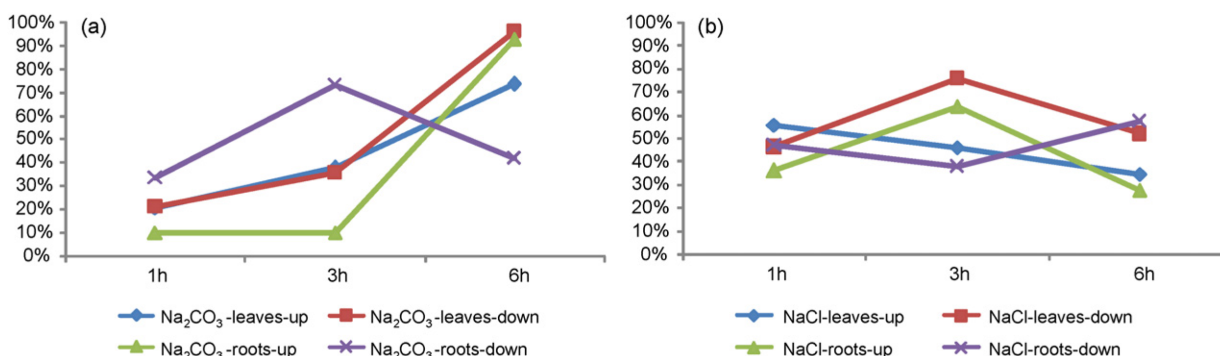

Fig. 2. Comparison of gene expression changes in Na_2CO_3 (a) and NaCl (b) stressed leaves and roots of *L. chinensis* during the three time points (1, 3, and 6 h) of observation.

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Table 2
Functional categorized summary of up-regulated genes by Na_2CO_3 and/or NaCl stressed leaves and roots of *L. chinensis*

Transcript (EST) ID	Functional category
Na_2CO_3-leaves	
CD808983, CD809218, CD809219, CN466364	Defense
CD808880, CD808932, CD808961	Gene expression and RNA metabolism
CN466421, CN465835, CD808916, CN465792	Membrane transport
CD808664, CN465984, CN466172, CN466090, CD808970, CD808569, CD808842, CD809057, CD808941, CD808535, CD809030, CN466292, CN466379, CD809275, CN466300, CN466429, CN466301, CD808834	Metabolism
CD808785, CD809067, CD808565	Photosynthesis
CN465776, CD808728, CD808533, CD808703, CN466270, CN466187, CN466325, CN466293, CN466293, CD809115, CD808843, CN466192	Protein metabolism and sorting
CN465837, CN465837, CD809317	Signaling components
CN466214, CN466245	Miscellaneous
CD808595, CD808611, CD808612, CN466456, CD808677, CD809187, CD809077, CD808617, CD808460, CD809012, CN465961, CN466183, CN466447, CD808497, CD808687, CD808647, CN466365, CD808760	Unclassified protein
CD808500, CD808524, CD808540, CD808525, CD808592, CD808624, CD808992, CD809036, CD808771, CD809186, CD809226, CN466151, CN466185, CN466220, CN466291, CN466296, CN466459, CN466420, CN466440, CN466476	No hit
Na_2CO_3-roots	
CD808881, CD808586	Gene expression and RNA metabolism
CD808581	Membrane transport
CD808664, CD808941, CD808535, CN466292, CN466429, CN466301, CD808798, CD808834	Metabolism
CD808731, CD808514, CD808551	Photosynthesis
CN466270, CD809139, CD809126	Protein metabolism and sorting
CD809181	Signaling components
CD809187, CD808617, CD808738, CD808687, CD809168	Unclassified protein
CD808607, CD809034, CD808720, CD808771, CD809186, CD808717	No hit
NaCl-leaves	
CD808914	Cell wall
CD808983 CD809218, CD809219, CD809031	Defense
CD809200, CD808932	Gene expression and RNA metabolism
CN466264, CN465835, CD808916	Membrane transport
CD808664, CD809309, CN466090, CN465980, CD808569, CD808842, CD808535, CD809030, CN466292, CD809005, CN466300, CD808576	Metabolism
CD808785 CD808731, CD809204, CD809205	Photosynthesis
CD808533, CN466270, CN466187, CD809126	Protein metabolism and sorting
CD809317	Signaling components
CN466214, CN466245, CN465878	Miscellaneous
CD808907, CD808611, CD808612, CD809187, CD808460, CD809012, CD808497	Unclassified protein
CD808883, CD808500, CD808524, CD808540, CD808525, CD808624, CD809036, CD809226, CN466220, CN466440, CN466476	No hit
NaCl-roots	
CD809041, CD809031, CD808890	Defense
CD808821, CD808881, CD808586	Gene expression and RNA metabolism
CD808902, CD808581, CD809170	Membrane transport
CN466273, CD808970, CD808941, CD809030, CN466069, CN466292, CD808903, CD808798, CD808598, CD808834	Metabolism
CD808835, CD809113, CD808864, CD808539, CD809073	Photosynthesis
CD809085, CD808566, CN465753, CN466187, CD808554	Protein metabolism and sorting
CD809181	Signaling components
CD809119, CD808838, CD809187, CD808935, CD808738, CD808460, CD808869, CD808687, CD808757, CD809168	Unclassified protein
CD808520, CD808592, CD808607, CD809034, CD809066, CD809186, CN466461	No hit
Na_2CO_3-leaves and roots	
CD808834, CD808941, CD808535, CD808664, CN466292, CN466301, CN466429	Metabolism
CD809126, CN466270	Protein metabolism and sorting
CD808617, CD808687, CD809187	Unclassified protein
CD808771, CD809186, CN466291	No hit
NaCl-leaves and -roots	
CD809031	Defense
CD809030, CN466292	Metabolism
CN466187	Protein metabolism and sorting
CD808460, CD8091874	Unclassified protein
Na_2CO_3- and NaCl-leaves	
CD808983, CD809218, CD809219	Defense
CD808932, CD808881, CD808586	Gene expression and RNA metabolism
CD808916, CD808864, CD808581	Membrane transport
CD808842, CD808535, CD808569, CD809030, CD808664, CN466090, CN466292, CN466300, CD808798, CD808834, CD808941, CN466292	Metabolism
CD808785	Photosynthesis
CD808533, CD809126, CN466187, CN466270	Protein metabolism and sorting
CD809317	Signaling components
CN466214, CN466245	Miscellaneous

Table 2 (*Continued*)

Transcript (EST) ID	Functional category
CD808460, CD808497, CD808611, CD808612, CD809012, CD809187	Unclassified protein
CD808500, CD808540, CD808525, CD808624, CD809036, CD809226, CN466220, CN466440, CN466476, CD808607, CD809034, CD809186	No hit
Na_2CO_3- and NaCl-roots	
CD809181	Signaling components
CD809168, CN466240, CD808738, CD809187	Unclassified protein
Na_2CO_3- and NaCl-leaves and roots	
CD808719	Metabolism
CD809187	Unclassified protein

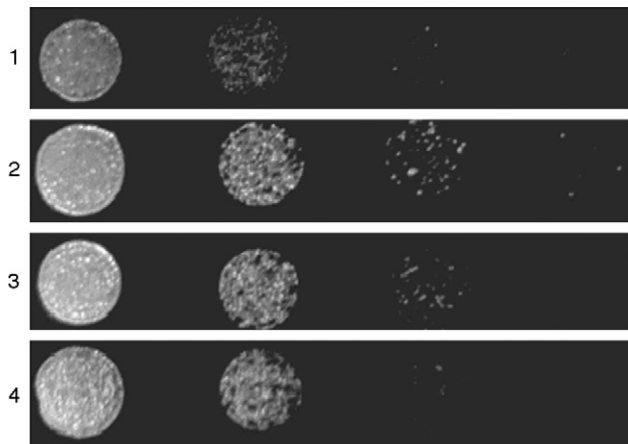

Fig. 3. NaCl resistance of the selected genes expressed in yeast. The yeast was grown on plates containing 0.75 M of NaCl. Lanes: (1) control (yeast *S. cerevisiae* Y2805 containing empty plasmid); (2) yeast containing expressed cDNA of CD809126; (3) CD809168; (4) CD809218. Medium: 0.3% yeast extract, 0.5% peptone, 2% raffinose, 2% galactose, and 2% agar.

Information pertaining to down-regulated genes is as important as up-regulated ones. A total of 458 genes were found to be down-regulated, of which 376, 180 and 98 were detected from the stressed leaves, roots, and from the both tissues, respectively. Approximately 87% of the down-regulated genes were annotated and 56% of those down-regulated genes were characterized to photosynthesis (30%), metabolism (20%) and protein metabolism (6%) related proteins. Four hundred forty, 211 and 193 genes were down-regulated by Na_2CO_3 stress, NaCl stress and the both stresses, respectively.

A total of 87 genes detected in this study were characterized to the unclassified proteins, whereas 72 had no similarity to the current GenBank databases which were considered as novel proteins detected in the saline-alkali and saline stressed *L. chinensis* genome. Those proteins might hold the key to elucidate the mechanism of salt tolerance in general and alkali and/or saline soil tolerance in particular.

3.4. Tolerance genes implicated in Na_2CO_3 and NaCl stresses

We identified 140 transcripts exhibiting up-regulation by detecting genes expressed over 1.5-fold or more at least at one time course point during the Na_2CO_3 and NaCl stresses in leaves and roots (Table 2, Supplementary Table 1). Eighty-seven out of 140 transcripts were assigned the functions based on the previous report [3]. We identified 6 and 5 of continuously up-regulated genes by Na_2CO_3 and NaCl stresses, respectively and 4 and 3 of them were annotated. CD808664 was annotated to 1-aminocyclopropane-1-carboxylate (ACC) oxidase and detected to be continuously co-expressed in Na_2CO_3 stressed leaves and roots. The remaining three genes were glyceraldehyde 3-phosphate dehydrogenase (GPD) (CD809030, CN466292) heat shock protein cognate 70 (hsp70) (CN466270) and translational initiation factor eIF1 (CD809126) which were up-regulated in Na_2CO_3 stressed leaves. Of these, translation initiation factor *elF1* has been reported to increase tolerance to salt in yeast [17] and heat tolerance in *Arabidopsis* [18]. GPD (CN466292) was also detected to be expressed continuously in NaCl stressed leaves supporting the

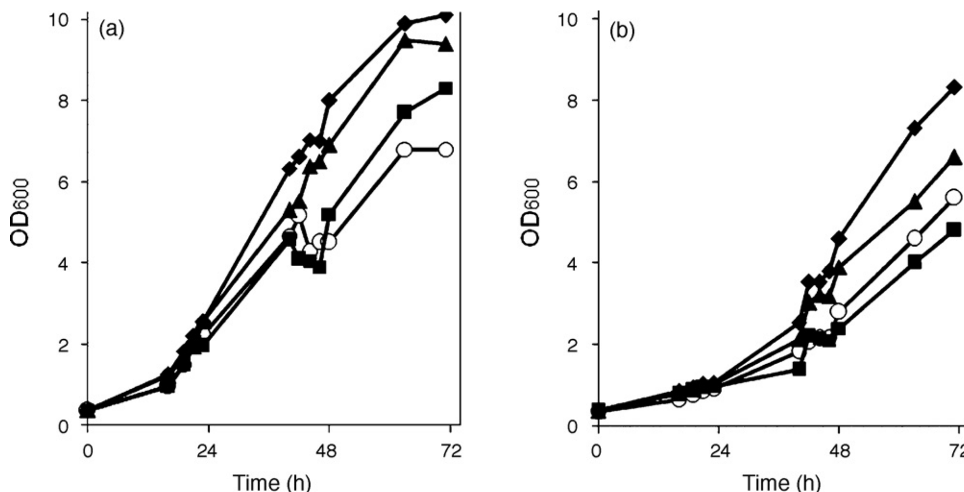

Fig. 4. Yeast growth in liquid culture containing NaCl. Panels: (a) NaCl 0.5 M; (b) NaCl 1 M. Symbols: (○) control (Y2805 with empty plasmid); (■) yeast containing LcA13C06 cDNA expression plasmid; (▲) LcA13G06; (◆) LcA17C06. Medium: 0.3% yeast extract, 0.5% peptone, 2% raffinose, and 2% galactose.

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GPD expression is induced in yeast by different abiotic stresses, including heat, salt, dehydration and cold [19]. The other annotated genes constantly up-regulated by NaCl stress were lectin-like protein kinase (CD809031) and phosphoglycerate kinase (CD808798) which were expressed in the roots.

3.5. Increased NaCl resistance in yeast by introducing up-regulated genes of L. chinensis

To validate DNA chip results presented in this study, three up-regulated cDNAs (CD809126, CD809168, and CD809218) by Na_2CO_3 or NaCl were expressed in yeast. The expression of CD809126 (translational initiation factor eIF1) in *L. chinensis* genome was 13-fold increased in Na_2CO_3 stressed roots. That of CD809126 (unnamed protein product) was 8-fold increased by NaCl stress in roots. CD809218 (cationic peroxidase) expression was 3.2-fold and 4.4-fold increased by NaCl stress in leaves and by Na_2CO_3 stress in roots, respectively. The three cDNAs were cloned into the plasmid where the expression is controlled by galactose promoter.

All three genes containing yeasts showed increased growth on plates containing NaCl when those genes were expressed (Fig. 3). Yeast growth was also measured in liquid culture. When yeast grew in medium containing 0.5 M of NaCl, all three clones showed higher growth (Fig. 4a) than in medium containing 1.0 M of NaCl. The expression of CD809218 (cationic peroxide) showed no effect on increasing the yeast growth at 1.0 M of NaCl (Fig. 4b), indicating this gene is involved in NaCl resistance at a low concentration. Yeast growth in Na_2CO_3 was poor and also there were no difference in growth of yeast by the expression of those genes (data not shown).

4. Discussion

We reported herein the comparative analysis of expression changes in the *L. chinensis* genome by saline-alkali and saline stresses that can be used as an embarkation basis to address questions in alkali stress. The crucial resources developed in this study were cDNA microarray containing 1642 *L. chinensis* transcripts [3], their expression changes monitored, and their functional assignments. To our knowledge this study is the important attempt to describe the expression changes of the genes by Na_2CO_3 and NaCl stresses in plants. A total of 87 unclassified and 72 of 'no hit' transcripts detected in this study will be the useful resources for the researches on alkali and saline stresses in plant system.

4.1. Up-regulated transcripts

Plant cell walls are directly exposure to the environmental stress. Therefore, genes involved in cell wall formation, modification, and maintenance of cell wall architecture are responsible to stress. In this study, it was not detected that the major up-regulation of cell wall related genes response to salt stresses. This is contrast to the previous report that salinity stress resulted in major cell wall restructuring in both salt-sensitive and salt-tolerant rice [20]. It is not known whether *L. chinensis* has the same cell wall structure with rice, however, it appears that cell wall-related gene responses to the salt stresses were distinguished between two species. Expansins are known to cell wall proteins that induce acidic pH-dependent cell enlargement and stress relaxation [21]. Salinity is known to suppress the expression of expansins due to the reduced cell growth. The expression of it was down-regulated in the salt stressed leaves in agreement with the previous report in the rice [20] whilst it was not affected or 1.5-fold up-regulated in the stressed *L. chinensis* root showing the different responses by the stressed tissue types as well as the possibility of salt tolerance aspects.

Most of the Na_2CO_3 specific up-regulated genes were responded after 6 h of stress and mostly in the leaves which are distinguishing saline-alkali stress reactions from saline stress reactions in the *L. chinensis* genome. Those late responding genes only by Na_2CO_3 are supposed to be up-regulated by the secondary reaction to the damage of Na_2CO_3. Much more number of genes was up-regulated by saline-alkali stress than saline stress indicating there might be more physiological responses by saline-alkali stress. We also observed much more severe physical damages by Na_2CO treatment than NaCl in *L. chinensis*. One of the Na_2CO_3 specific up-regulated genes, S-like RNase was detected from the initial stage of the stress. This gene is known to be induced by senescence and phosphate starvation in plants by participating in the nutrient recycling during senescence and scavenging phosphate sequestered in RNA, in combination with the actions of phosphatases during starvation for phosphate [22–24]. In this study, acid phosphatase was also detected at 6 h of time point as a Na_2CO_3 specific up-regulated gene. This enzyme is known to free the attached phosphate groups from other molecules during digestion and the induction of it is a universal response to phosphate starvation in higher plants. Based on these, we hypothesize that saline-alkali stress inhibit the uptake of phosphate resulting the severe physical damages in plants.

We found five transcripts encoding fructose-bisphosphate aldolase (ALD) showing distinct expression patterns. Clone CD809057 encoding ALD was the other Na_2CO_3 specific up-regulated genes detected from the initial stage of the stress in this study. It is known that plants have isoenzymes of ALD in the cytosolic (AldC) and plastidic (AldP) compartments encoded by separated nuclear genes [25,26]. Generally, AldC and ALDp2 were known to be up-regulated by salt stress while the AldP1 was negatively affected by salt stress [27]. ALD was reported to be induced by oxidative stress and up-regulated by salt stressed *Nicotiana paniculata* [28]. In contrast, we only detected the up-regulation of this transcript of CD809057 from the saline-alkali stressed leaves of *L. chinensis*.

In this study, more than half (58.4%) of the classified up-regulated transcripts were involved in metabolisms of RNA, protein and chemicals which were responded tissue-, time- and stress-specific manners. This abundance and diversity of salt-inducible transcripts suggests that metabolic readjustment is a typical feature of the salt stress response in the *L. chinensis* genome in agreement with the previous data from *Oryza sativa* and *Avicennia marina* [5,29].

4.2. Constantly up-regulated transcripts

Four out of the six transcripts showing constant up-regulation by Na_2CO_3 stress were annotated to ACC oxidase, GPD, hsp70, and eIF1. Three transcripts by NaCl stress (GDP, lectin-like protein kinase and phosphoglycerate kinase) annotated were also exhibit continuous up-regulation. Those steady up-regulation transcripts are grand candidates for establishing stress tolerance plants by developing transgenic. ACC oxidase is known to be involved in the ethylene biosynthesis which is induced by biotic and abiotic stresses [30] including ozone and UV radiation [31,32]. It is reported that ACC can rescue the salt-stressed phenotype in Arabidopsis, implying that ethylene signaling is beneficial for plant survival under salt stress [33]. GPD detected from the both of saline-alkali and saline stresses is known to be increased the transcription in all kinds of organisms, including plants, fungi, and mammals by heat shock, anaerobiosis, and salt stress [34,35]. The

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GPD transgenic potato was reported and demonstrated the improved salt tolerance [36]. Heat shock proteins are known to be molecular chaperones regulating the folding, localization, accumulation, and degradation of protein in both plant and animal [37] suggesting to impart a generalized role in tolerance to multiple environmental stress. Revealing the function of heat shock proteins thus may provide insight into multiple stress tolerance mechanisms in plants. The translation initiation factor eIF1 was known as an important determinant of salt tolerance [17] by playing a critical role in cell growth, proliferation and development as an adaptive regulation strategy to stresses. The most up-regulated gene, translational initiation factor eIF1 (CD809126), by the stress was detected from Na_2CO_3 stressed leaves showing 13-fold of increased expression. Increased NaCl concentrations result in drought, ion toxicity and oxidative stresses [1,38–40] to plants causing growth inhibition or death. Consequently, molecules leading tolerance and regulatory proteins controlling the tolerance molecules are actively induced as an adaptation mechanism to salt stress. The protein synthesis is initiated by ribosome recruiting to an mRNA initiation codon. The translation initiation factors participate in the assembly of the ribosomal subunits that is competent for protein synthesis. The role of eIF1 is multiple such as stabilizing binding of Met-tRNA to 40S ribosomal subunits, promoting mRNA binding and preventing premature of 40S ribosomal subunits [41]. eIF1 is also function for the scanning of the mRNA and location of the initiation codon [42]. We have tested *L. chinensis* genes (CD809126) that confer increased salt tolerance when expressed in yeast. Our result provides excellent evidence implicating translation initiation is one of the major physiological targets to salt toxicity in plants.

4.3. Down-regulated transcripts

It was reported that up-regulation of photosynthesis genes contribute to the recovery of the photosynthetic ability in the halophyte, Burma mangrove [43]. In contrast, we detected that photosynthesis related genes were strongly affected, especially down-regulated by salt stress suggesting photosynthesis regulation may play an important role for salt stress regulation. The decreased growth in many plants in salt stress is often associated with a reduced photosynthetic capacity [44,45]. This declined photosynthetic capacity is related to the partial stomatal closure and/or the non-stomatal limitation, such as the decrease in Rubisco activity and content [46–48]. The rate of down-regulation of photosynthesis related transcripts by the time frame was more sever in saline-alkali stressed leaves than in saline stressed leaves. This severe down-regulation might be involved in the physical damages by the inhibition of the uptake of phosphate which we hypothesized.

The strongest down-regulated gene by salt stress was β-glucosidase (CD809001) showing 5-, 7- and 18-fold of down-regulation at the three time points of the detection in Na_2CO_3 stressed leaves. β-Glucosidase is involved in cell wall hydrolysis as well as many of roles in defense, phytohormone regulation, oligosaccharide catabolism, scent production and other functions. Plant β-glucosidase is still not fully understood although some of that have been purified and confirmed the trend [49–51]. The β-glucosidase is known to be optimal at pH 5.0 [51] implicating the down-regulation of β-glucosidase in this detection was the reaction to alkalinity of Na_2CO_3.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2008.07.016.

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