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Deciphering the possible mechanism of exogenous NO alleviating alkali stress on cucumber leaves by transcriptomic analysis

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ABSTRACT

Alkali stress is a major factor that limits crop yield, and nitric oxide (NO) is involved in the regulation of plants tolerant to abiotic stress. In the present study, sodium nitroprusside (SNP), a NO donor, reversed the chlorosis of cucumber leaves caused by alkali stress. Physiological analysis indicated that application of SNP protected mesophyll cell ultrastructure from damage by alkali stress. SNP increased nutrient element utilization, pigment content, photosynthetic capacity and accumulation of organic acids. In this study, Solexa sequencing was used to investigate the effect of SNP on expression of genes involved in cucumber response to alkali stress. About 5.9 million (M) and 5.8 M 21-nt cDNA tags were sequenced from the cDNA library of the alkali treatment and SNP treatment, respectively. When annotated, a total of 10,271 genes for the alkali stress treatment from the Solexa sequencing tags and 10,288 genes for SNP treatment were identified. We detected 901 differentially expressed genes in two samples, of which 437 and 464 of them were up- or down-regulated by SNP under alkali stress, respectively. The expression levels of 11 differentially expressed genes were confirmed by real-time RT-PCR. The trends observed agreed well with the Solexa expression profiles, although the degree of change was diverse in amplitude. Gene ontology analysis revealed that differentially expressed genes were mainly involved in response to abiotic stress, cellular metabolic process, photosynthesis, transmembrane transportation and organelle development, which were in accordance with physiological results.

1. Introduction

Soil salinity and alkalinity have greatly limited crop production in semi-arid and arid regions. There are 831 million ha of soils affected by excessive salinity and alkalinity around the world. Of those, 434 million ha are sodic soils (alkaline soils) and 397 million ha are saline soils (Jin et al., 2008). It is well known that soil salinity is mainly due to the accumulation of NaCl and Na_2SO_4, while soil alkalinity is mainly caused by the accumulation of $NaHCO_3$ and Na_2CO_3 (Shi and Sheng, 2005; Yang et al., 2009). The mechanism of higher plant tolerance to salinity stress has been widely studied. However, the mechanism of adaptation to alkalinity in plants is in need of deeper investigation (Jin et al., 2008). Osmotic stress and ion-induced injury are generally considered to be involved in salt stress of plants, while there is the additional influence of high-pH on plants under alkaline conditions (Liu et al., 2010). The most conspicuous symptoms of alkaline salt stress on plants are the induction of leaf chlorosis and stunted growth, which is strongly related to the precipitation of metal ions and the disruption of ionic balance and pH homeostasis in tissues caused by high-pH environment (Yang et al., 2007, 2008).

Nitric oxide (NO) is a highly diffusible gas that is also a ubiquitous bioactive molecule. Its chemical properties make NO a versatile signal molecule that functions through interaction with cellular targets via either redox or additive chemistry (Lamattina et al., 2003). Recent studies provided evidence that NO is involved in many key physiological processes of plant tolerance to salt, drought, temperature, UV-B, heavy metals and other abiotic stresses (Siddiqui et al., 2011). However, there have been few investigations on NO modulating plant tolerance to alkaline stress. Cellini et al. (2011) observed that endogenous NO content was associated with tolerance to bicarbonate-induced chlorosis in micropropagated *Prunus* explants. The research indicated that NO was also involved in plant tolerance to alkaline stress, although the mechanism was not clear.

Recently, high throughput technologies, such as microarray and RNA-Seq, have been applied to investigation of global expression profiles of genes and to identify functional genes under various

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environmental stresses (Zeller et al., 2009; Yao et al., 2011). Jin et al. (2008) examined the response of gene expression profiles in *Leymus chinensis* to Na_2CO_3 and NaCl stresses. They identified many genes regulated by the two stresses, while only 37 genes in the leaves and 6 genes in the roots showed the same expression change patterns between Na_2CO_3 and NaCl stresses. Thus, genetic control mechanisms in *L. chinensis* differed for tolerance to alkaline stress and salt stress. Ge et al. (2010) studied global transcriptome profiling of the roots of wild soybean under $NaHCO_3$ treatment using the Affymetrix Soybean GeneChip. They identified 3307 genes up-regulated and 5720 genes down-regulated at various time points. Those results extend the current understanding of wild soybean response to alkaline stress. Meng et al. (2011) observed that 164 proteins were regulated by NO in cotton leaf using label-free quantitative proteomics analysis.

Cucumber (*Cucumis sativus* L.) is an economically important crop as well as a model system for cucurbit plant study because of its worldwide cultivation and the publication of its genome (Huang et al., 2009). Cucumber is sensitive to salt and alkaline stress. Previous research indicated that exogenous NO alleviated NaCl stress on cucumber by increasing mitochondrial antioxidant capacity and activities of H^+-ATPase and H^+-PPase (Shi et al., 2007). However, to our knowledge, there were no reports on NO alleviating alkaline stress in plants. In this study, cucumber was chosen as the experimental plant material to investigate the physiological process and gene expression regulation by SNP (a NO donor) under alkalinity stress. We were also interested in determining the function of NO in metabolism and how it improves tolerance to alkalinity stress in cucumber and other cucurbits.

2. Materials and methods

'Jinchun 5' cucumber seeds were germinated on moist filter paper in the dark at 28 °C for 2 days. Germinated seedlings were transferred to flats containing vermiculite, and grown in the greenhouse for 8 days. They were then transplanted into 5 l black plastic containers with 3 seedlings each. Bags contained aerated full nutrient solution: 4 mM $Ca(NO_3)_2$, 4 mM KNO_3, 2.5 mM KH_2PO_4, 2 mM $MgSO_4$, 29.6 μM H_3BO_3, 10 μM $MnSO_4$, 50 μM Fe-EDTA, 1.0 μM $ZnSO_4$, 0.05 μM H_2MoO_4, 0.95 μM $CuSO_4$. After 9 days of pre-culture, the treatments were started. The experiment had 2 treatments: 30 mM $NaHCO_3$ (alkali stress treatment, indicated as Na) and 30 mM $NaHCO_3$ + 100 μM sodium nitroprusside (indicated as SNP). The nutrient solution was renewed every 3 days. Plants were cultivated under natural conditions in a glass greenhouse. After 12 days of treatment, the leaves were sampled for testing.

2.1. Determination of photosynthetic parameters and pigment contents

Net photosynthetic rate (P_N) was measured using a portable photosynthesis system Li-6400 (Li-Cor, Lincoln, USA) at ambient CO_2 concentration of 340 μmol mol^{-1} and a photon flux density of 1000 μmol m^{-2} s^{-1}. Fv/Fm was measured with a pulse amplitude modulated system (model FMS2, Hansatech Instrument, UK) according to Burzyński and Klobus (2004). The content of photosynthetic pigments was measured according to the method of Arnon (1949).

2.2. Determination of nitrogen (N), iron (Fe) and magnesium (Mg) concentration

Cucumber leaves were dried at 70 °C to constant weight before weighing. The dry leaves were ground to a powder, and digested in a mixture of H_2SO_4–H_2O_2, N content was determined by the Kjeldahl method, and contents of Fe and Mg were measured with atomic absorption spectrometry.

2.3. Determination of organic acid content

Determination of organic content was carried out through HPLC (High Performance Liquid Chromatography) and measurements were taken with three independent biological replicates. Each leaf sample (2 g) was ground with 3 ml 80% ethanol. The mixture was heated at 75 °C for 30 min, centrifuged at 8000 × *g* for 10 min, and the supernatant transferred to a 25 ml volumetric flask. The precipitate was suspended with 5 ml 80% ethanol and the above procedure repeated. The resulting supernatant was transferred to the same 25 ml volumetric flask and made up to volume with ultrapure water, and evaporated to dryness in a freeze drier (Speed Vic, Savant Instruments, Holbrook, NY), and redissolved with 3 ml ultrapure water. The solution was passed through a 0.22 μm membrane filter. Ten microliters of the filtrate was analyzed using HPLC (Waters 510, Waters Co.) that was fitted with a Thermo Hypersil GOLD AQ column (250 mm × 4.6 mm i.d., 5 μm particle size). The mobile phase was 10 mm $NH_4H_2PO_4$ (the pH value was adjusted to 2.5 with phosphoric acid):methanol (98:2, v:v) run at 0.8 ml min^{-1} at room temperature. The organic acids were detected at 210 nm with a dual λ absorbance detector (Waters 2478). A mixed standard solution for oxalate, succinate, acetate, malate and citrate were made in ultrapure water at concentrations within the ranges found in the plant samples. As with the sample test solution, 10 μl of the mixed standard was run through the HPLC system. Linear standard curves of the five organic acids were generated from the areas under the peaks in line with different concentrations. The standard curves were used for quantification of organic acids in the samples. In order to verify running conditions, a mixture of the standard organic acids was run before each batch of samples was analyzed.

2.4. Determination of cell ultrastructure

Cell ultrastructure was determined by TEM according to the method of Xu et al. (2008). The cucumber leaves were fixed in 3.5% glutaraldehyde for 24 h, washed with 0.1 M phosphate buffer (pH 7.2) and then postfixed with 1% osmic acid at 4 °C for 4 h. Cells were dehydrated with ascending concentrations (from 30% to 100%) of ethanol and embedded in spur resin at 60 °C for 24 h. After thin sections were cut with an LKB Ultratome Nova (Bromma, Sweden) and picked on 250-mesh grids, leaf cells were post-stained with uranyl acetate and lead citrate and then were observed using a transmission electron microscope (JEM-1200EX; JEOL Ltd., Tokyo, Japan) at 80 kV.

2.5. Preparation of digital expression libraries

Samples from Na- and SNP-treated leaves, used for RNA isolation and library construction, were harvested and immediately frozen in liquid nitrogen, and stored at −80 °C. Total RNA was isolated from the frozen leaf with an RNA simple Total RNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Sequence tag preparation was done with the Digital Gene Expression Tag Profiling Kit (version 2.1 B, Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. Biotin-Oligo(dT) magnetic bead adsorption was used to purify the mRNA contained in 6 μg of total RNA extraction. The first- and second-strand cDNA were synthesized using biotin-Oligo(dT) as primers. Subsequently, the double cDNA was digested with restriction enzyme NlaIII, which recognizes and cuts off the CATG sites, to produce a cDNA fragment containing sequence from CATG to the poly (A)-tail. These 3′ cDNA fragments were purified with the method of magnetic bead precipitation and the Illumina adaptor 1 (GEX adapter 1) was ligated to the

Table 1
Effects of SNP on photosynthesis as well as pigment, N, Fe and Mg content in cucumber leaves under $NaHCO_3$ stress. The third unfolded leaves from the apex of the $NaHCO_3$-treated and $NaHCO_3$ plus SNP treated cucumber plants were harvested and net photosynthesis rate, Fv/Fm, chlorophyll, carotenoid and N, Fe and Mg content were measured. Means ± SD ($n = 5$ for Fv/Fm, $n = 3$ for the other parameters) follow by different letters indicate a statistical difference at $P < 0.05$.

	Net photosynthesis rate (CO_2 mol m^{-2} s^{-1})	Fv/Fm	Chlorophyll (mg g^{-1} FW)	Carotenoid (mg g^{-1} FW)	N content (g kg^{-1} DW)	Fe content (μg ml^{-1})	Mg content (μg ml^{-1})
$NaHCO_3$	10.08 ± 0.29b	0.76 ± 0.02b	0.58 ± 0.08b	0.21 ± 0.04b	3.86 ± 0.16b	0.33 ± 0.02b	1.19 ± 0.11b
$NaHCO_3$ + SNP	14.95 ± 0.40a	0.83 ± 0.03a	1.28 ± 0.13a	0.32 ± 0.03a	4.74 ± 0.26a	0.49 ± 0.02a	1.33 ± 0.09a

new 5′ end. The junction of Illumina adaptor 1 and CATG site was recognized by *Mme*I, which was an endonuclease with separated recognition sites and digestion sites that cuts 17 bp downstream of the CATG site, producing tags with adaptor 1. After elimination of 3′ fragments with magnetic bead precipitation, the Illumina adaptor 2 (GEX adapter 2) was added to the 3′ ends of tags, to obtain the tag library.

2.6. Solexa sequencing

Sequencing was carried out by "HuaDa Gene" (HuaDa Gene [http://www. genomics.org.cn/]) using sequencing by synthesis. After a linear PCR amplification with 15 cycles using Phusion polymerase (Finnzymes, Espoo, Finland) and primers complementary to the adapter sequence, the consequent 105 bp fragments were purified by 6% TBE PAGE electrophoresis. These fragments were then denatured, and the single-chain molecules were fixed onto the Illumina/Solexa Sequencing Chip (flow cell). Each molecule grew into a single-molecule cluster sequencing template through in situ amplification. Four types of nucleotides that were labeled by four colors that were added, and sequencing was carried using sequencing by synthesis (SBS). The resulting image was transformed into sequence data, referred to as raw read. Each tunnel generated millions of raw reads with sequencing length of 49 bp (target tags plus 3′ adaptor) and each read in the library represented a single tag came from a single transcript.

2.7. Sequencing annotation

By removing the 3′ adaptor of the raw reads, filtering off low quality tags (containing ambiguous bases), and filtering off tags that were longer or shorter than 21 nt and tags with copy number less than 2, "clean tags" were obtained. Clean tags were annotated based on cucumber reference genes (http://cucumber.genomics.org.cn), and only sequences that were matched to one gene with perfect homology or 1 nt mismatch (unambiguous tags), for conservative and precise annotation, were considered further. The number of unambiguous, clean tags for each gene was calculated and then normalized to the number of transcripts per million clean tags (TPM) ('t Hoen et al., 2008; Morrissy et al., 2009).

2.8. Identification of different expressed genes (DEGs)

Using the methods of Audic and Claverie (1997), a strict algorithm to identify differentially expressed genes between two samples was developed. In the formula below the *P* value was to test differential gene expression; N_1 and N_2 represent the total clean tag number of the Na and SNP library, respectively; gene A holds x tags in the Na and y tags in the SNP library. The probability of gene A expressed equally between two samples can be calculated with:

$$P(y|x) = \left(\frac{N_2}{N_1}\right)^y \frac{(x+y)!}{x!!y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}!}$$

The false discovery rate (FDR) was used to determine the threshold of *P* value in multiple tests and analyses (Benjamini et al., 2001). We used FDR < 0.001 and the absolute value of $\log_2^{Ratio} \geq 1$ as the threshold to estimate the significance of gene expression difference. Additionally, except the sequenced genome of Chinese fresh market inbred line '9930', another cucumber genotype 'Gy14' were also sequenced. In order to improve the reliability of the following analysis, all reads with significant differential expressed are blasted to 203 Mbp assembled Gy14 genome sequence and Gy14 EST sequences from phytozome 8.0 (http://www.phytozome.net) (Goodstein et al., 2012). 901 genes can be mapped to Gy14 assembly that covered over 99% of the genome. These genes were first annotated using Cucumber Genome Database (http://cucumber.genomics.org.cn), and then classified according to the most homologous genes from other species.

2.9. Gene ontology (GO) analysis

In order to facilitate the functional analysis of the preferentially expressed genes in the SNP-treated tissue, AgriGO (Du et al., 2010), a comprehensive GO analysis tool, was adopted for annotation. The GO terms for cucumber genes in this study were drawn from the most paralogous genes to cucumber in Arabidopsis.

2.10. Real time RT-PCR analysis

Total RNA was extracted from the samples that were prepared using the method described above. Experiments were performed on three independent replicate reactions per sample to increase statistical significance. First-strand cDNA was synthesized from 2 μl DNase I (EN0521, Fermentas)-treated 2 μg total RNA using Reverse Transcription Kit (K1622, Fermentas) according to the manufacturer's instructions. Specific primer sequences for the eleven randomly selected target genes are listed (Table 1). Primers specific for cucumber actin (Forward: GCTGGCATATGTTGCTCTTG; Reverse: GAATCTCTCAGCTCCGATGG) were used for the normalization of reactions. Real-time RT-PCR was performed using Maxima® SYBR Green/ROX qPCR Master Mix (2×) (K0222, Fermentas) in an optical 96-well plate with ABI PRISM® 7300 Real Time PCR System (Applied biosystems). The reaction volume was 20 μl, including 10 μl Maxima® SYBR Green/ROX qPCR Master Mix (2×), 0.2 μl 10 μM primer, 1 μl cDNA template and 8.6 μl nuclease-free water. The following thermal cycling profile was used: 95 °C 10 min; 40 cycles of 95 °C for 15 s, 61 °C for 31 s; 95 °C for 15 s, 61 °C for 30 s, 95 °C for 15 s. Data were analyzed using 7300 system software. Actin expression was used as an internal control to normalize all data. The fold change in gene expression was determined using threshold cycles, by the $2(-\Delta\Delta Ct)$ method (Livak and Schmittgen, 2001).

3. Results

3.1. Phenotype, ultrastructure of mesophyll cells and organic acids

Alkali stress significantly induced cucumber leaf chlorosis, and SNP treatment reversed the chlorosis (Fig. 1), in accordance the color of leaf, chlorophyll and carotenoids contents were significantly increased by application of SNP under alkali stress (Table 1).

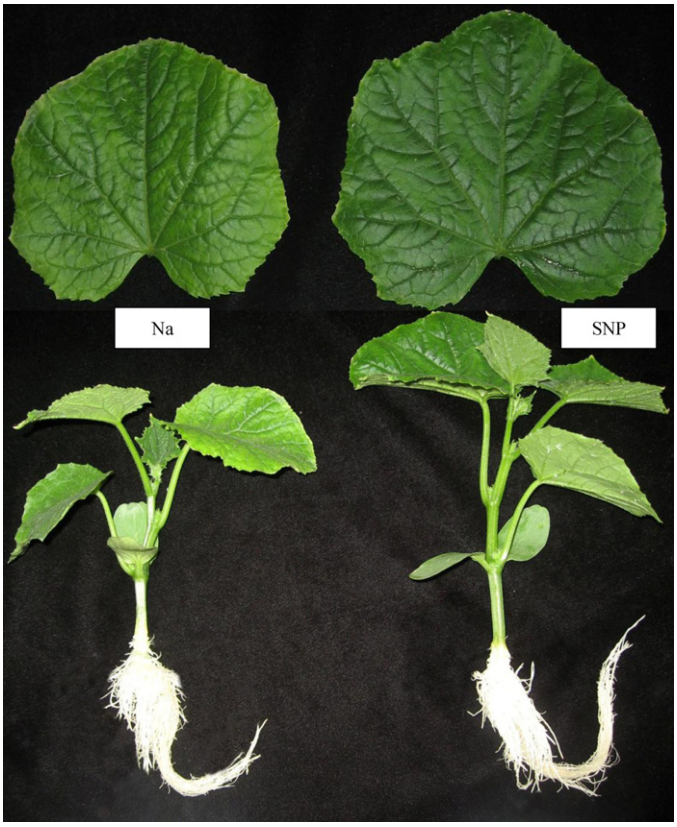

Fig. 1. Effects of SNP on the phenotype of $NaHCO_3$-stressed cucumber plants. Na, 30 mM $NaHCO_3$ treatment; SNP, 30 mM $NaHCO_3$ + 100 μM SNP treatment. (For interpretation of the references to color in the text, the reader is referred to the web version of the article.)

From Fig. 1, it was also discovered that plant height and leaf area of cucumber in alkali stress treatment were much lower than when treated with SNP.

Alkali stress induced obvious damage in mesophyll cells. It can be seen in Fig. 2A that plasma, chloroplast and mitochondria membranes had visible dissolutions and obvious inflation, and a fuzzy cell wall in cucumber leaves that had been treated with alkali. The application of SNP kept the membrane system and cell wall in good condition (Fig. 2B).

In the present study, the contents of acetate, malate and citrate were significantly increased by SNP treatment under alkaline stress ($P < 0.05$), with citrate showing the greatest increase. Unlike acetate, malate and citrate, the contents of oxalate and succinate were not significantly influenced by application of SNP (Fig. 3).

3.2. Photosynthetic parameters and contents of N, Fe, Mg

SNP treatment significantly increased the net photosynthesis rate and the maximum photochemical efficiency of PSII (Fv/Fm) of cucumber leaves under alkali stress (Table 1). At the same time, it was also found that SNP treatment significantly increased accumulation of N, Fe and Mg, all of which play important roles in pigment synthesis.

3.2.1. Saturation analysis of sequencing

Saturation of the library was determined by checking whether the numbers of detected genes continued to increase as sequencing (total tag numbers) progressed. Na and SNP libraries were sequenced to saturation, producing a complete representation of the transcripts in the conditions tested (Fig. 4). In the two libraries, the increasing rate of percentage of genes identified slowed as the number of sequencing tags increased, reaching a plateau shortly after 4 M tags were sequenced. No new genes were identified as the total tag number approached 5.8 M and 5.9 M in the Na and SNP libraries, respectively.

Table 2
Solexa tags in $NaHCO_3$-treated (Na) and $NaHCO_3$ plus SNP-treated (SNP) libraries.

	Na	SNP
Total tag	6,072,989	5,950,246
Clean tag	5,908,585	5,789,862
Distinct tag	91,783	89,864
Distinct tag copy number >5	43,177	41,744
Distinct tag copy number >10	30,662	29,343
Distinct tag copy number >20	21,460	20,267
Distinct tag copy number >50	12,660	11,710
Distinct tag copy number >100	7802	6994

3.2.2. Tag identification and quantification

For RNA-Seq analysis, the third expanded leaf from the shoot apex was used. A total of 6,072,989 and 5,950,246 tags were sequenced in Na and SNP treatment, respectively (Table 2) (the detailed data have been submitted to Sequence read archive, the GEO accession number: GSE42439). The tags were transformed into clean tags after certain steps of data-processing, and a total of 5,908,585 and 5,789,862 clean tags remained from the two libraries, from which 91,783 (Na treatment) and 89,864 (SNP treatment) unique tags were obtained. Furthermore, with the copy number increasing, the percentage of unique tags declined rapidly.

3.2.3. Annotation analysis of the clean tags

All clean tags were mapped to the reference gene sequences and the genome of Cucumber Genomic Database using blastN (http://www.icugi.org). Clean tags with a full match or only one base pair mismatch were studied further, and tags mapped to a single reference gene sequence were annotated and designated as unambiguous tags. A substantial proportion of clean tags had a match in the cucumber genome, including total tags (68.61% in Na library and 64.55% in SNP library) and unique tags (56.62% in Na library and 56.02% in SNP library) (Table 3). On the other hand, the percentage of clean tags mapped to genes was smaller both in total tags (23.23% in the Na library and 24.46% in the SNP library) and in unique tags (28.39% in the Na library and 30.10% in the SNP library). Further analysis carried out on unambiguous tags revealed that 1,233,576 (20.88%) in the Na library and 1,416,013 (24.46%) in the SNP library matched to one gene sequence in the cucumber genome, and the corresponding data for unique tags were 24,713 (26.92%) in the Na library and 25,697 (28.60%) in the SNP library. There were no evident differences in all clean tag-mapped genes between the two libraries [11,176 (41.89%) in the Na library and 11,199 (41.97%) in the SNP library]. Results were similar for unambiguous tag-mapped genes [10,271 (38.49%) in the Na library and 10,288 (38.56%) in the SNP library] (Table 3).

There were still some unknown total tags that were 482,105 (8.16%) in the Na library and 476,931 (8.24%) in the SNP library, and some unknown unique tags that were 13,758 (14.99%) in the Na library and 12,476 (13.88%) in the SNP library that had no homology with cucumber in the two libraries.

3.3. Comparison of gene expression level between the two libraries

Tag copy numbers identified in the Na and the SNP libraries were used for estimating gene expression levels under the testing conditions. Transcripts having at least 2-fold differences (FDR ≤ 0.001)

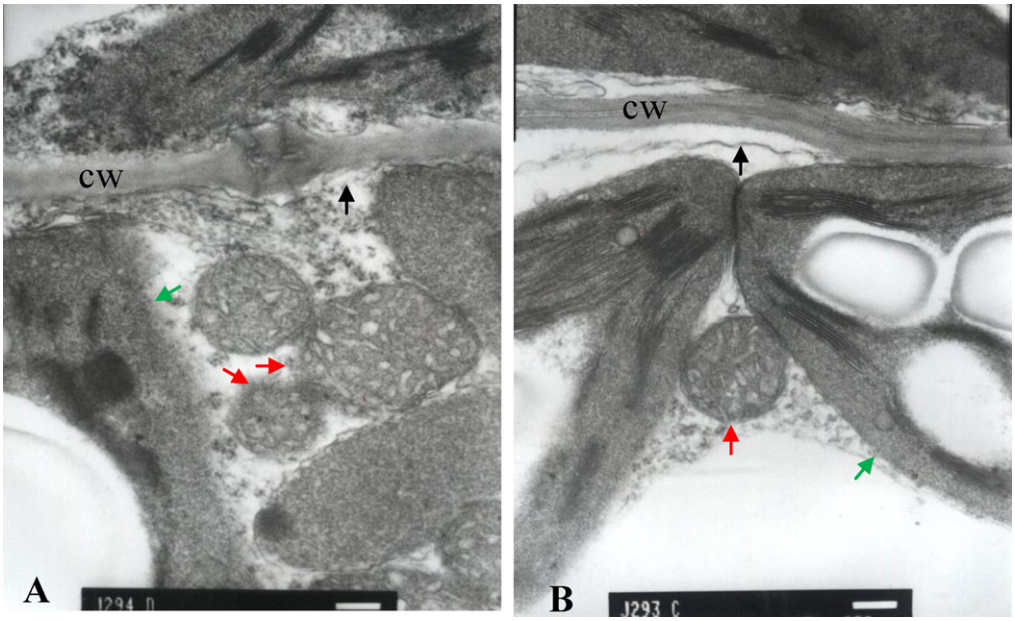

Fig. 2. Effects of SNP treatment on ultrastructure of cucumber mesophyll cells under alkali stress. (A) 30 mM $NaHCO_3$ treatment; (B) 30 mM $NaHCO_3$ + 100 μM SNP treatment; CW, cell wall; the black arrows indicate cell member; the red arrows indicate mitochondrial membrane; the green arrows indicate chloroplast membrane. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in the two libraries are shown in Fig. 5. Red dots and green dots were used to represent the up- and down-regulated differentially expressed genes in the SNP treatment library and the Na treatment library, respectively. The blue dots represent genes with less than 2-fold difference between the two libraries, considered to have no difference in expression. After comparison of the two libraries, we found that 437 (red dots) and 464 (green dots) genes were up and down-regulated by SNP under $NaHCO_3$ stress (Fig. 5; supplement 1). The DEGs with 5-fold or greater differences in accumulation were shown in Fig. 6. Approximately 90 genes (0.16% of the total unique tags) increased by at least 5-fold, and 257 genes (1.10% of total unique tags) were decreased by at least 5-fold in the SNP libraries compared to the Na libraries, while the expression level of 98.74% of unique tags was less than 5-fold difference between the two libraries.

3.4. Real-time RT-PCR analysis

In order to evaluate the validity of Solexa expression profiles, seven up-regulated genes (Csa001726, Csa008595, Csa008597, Csa011385, Csa013551, Csa018505, Csa013740) and four down-regulated genes (Csa000881, Csa004896, Csa017274, Csa00938) were selected and detected by the real-time quantitative PCR (QRT-PCR) with gene-specific primers (Table 4). Actin was used as a reference gene for data normalization. After comparison, we observed that different scales of DEGs varied between QRT-PCR (2–8-fold differences) and the Solexa sequencing (3–14-fold difference). The apparent discrepancies could be due to the different algorithms used by the two techniques (Ekman et al., 2003). The similar patterns provided basic confirmation for the reliability of our DEGs analysis (Fig. 7).

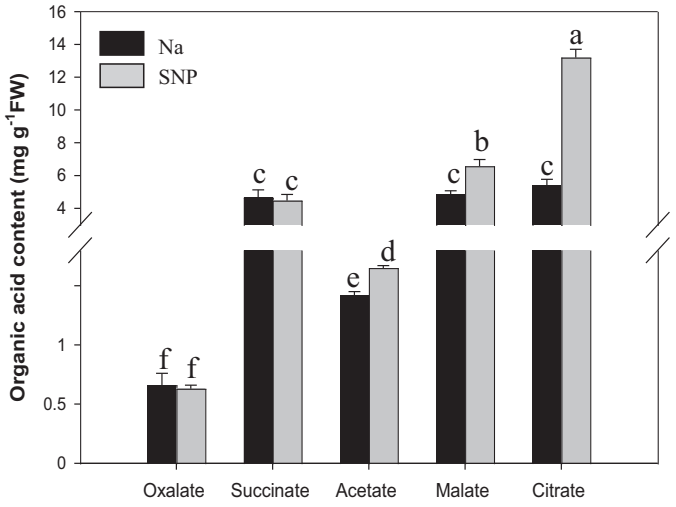

Fig. 3. Effects of SNP on organic acid content in cucumber leaves under $NaHCO_3$ stress. The third unfolded leaves from the apex of the $NaHCO_3$-treated (black) and SNP-treated (gray) cucumber plants were harvested for organic acid analysis. Data represent the means of three replicate experiments (±SD). Means denoted by different letters show significant differences at $P < 0.05$.

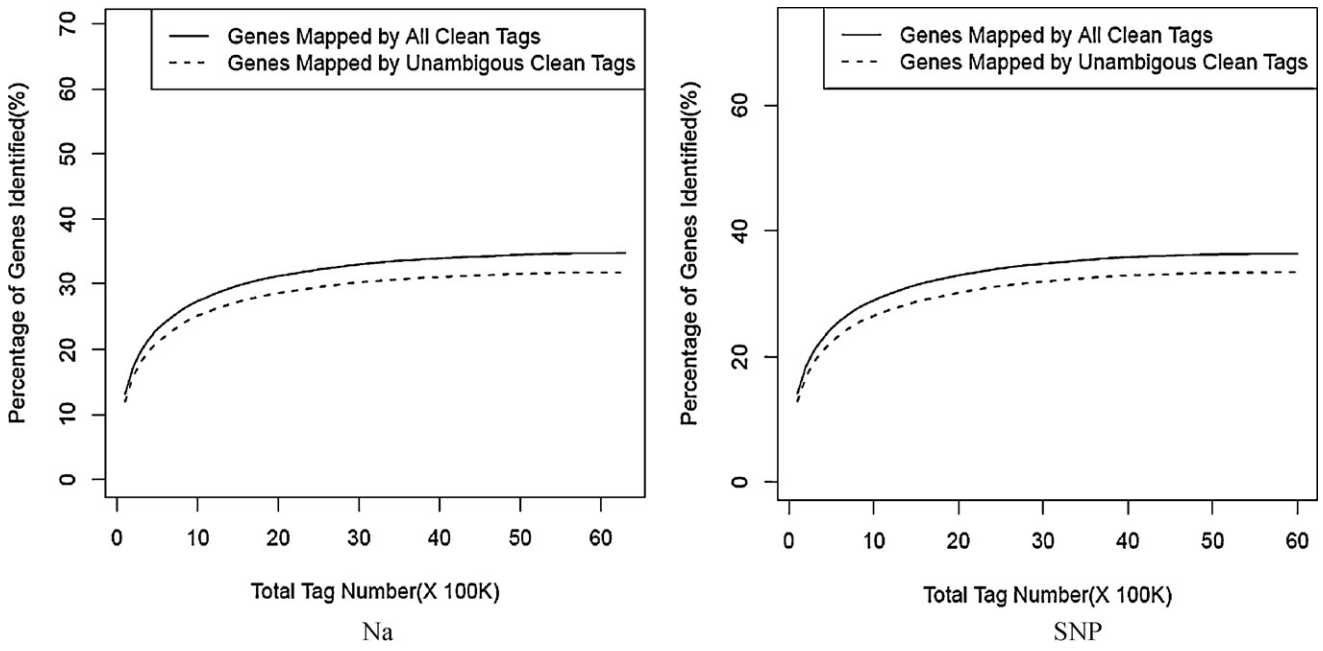

Fig. 4. Accumulation of Solexa total tag and identified genes in the two libraries. New-identified genes ("*y*" axis) of Na and SNP libraries decreased as the Solexa sequencing increased ("*x*" axis). The percentage of genes mapped by all clean tags was 35.76% in Na and 36.35% in SNP library. And the corresponding percentage of genes mapped by unambiguous clean tags in two libraries was 32.85% and 33.39%, respectively. Na, 30 mM $NaHCO_3$ treatment; SNP, 30 mM $NaHCO_3$ + 100 μM SNP treatment.

3.5. Function analysis of differentially expressed genes

To investigate the function of exogenous NO in modulating the patterns of gene expression under alkali stress, GO enrichment for $P < 0.001$ was analyzed (Table 5). According to the biological process, the molecular function, and the cellular components, the number of enriched GO terms was 34, 13, and 41, respectively. Besides, there were other GO terms also affected to varying degrees (results shown in supplement 2).

4. Discussion

Solexa sequencing can provide a comprehensive and unbiased dataset in the global analysis of gene expression. The major goal of the present experiment was to explore transcripts involved in the process of NO modulating cucumber seedlings adaptive to alkali stress, providing groundwork for investigating the mechanism of NO alleviating alkali stress on cucumber plants. In this study, 901 DEGs were identified, among which 437 were up-regulated and 464 were down-regulated by SNP under alkali stress. In order to elucidate the mechanisms underlying the data, GO enrichment was employed.

Photosynthesis is among the primary processes to be affected by salinity and alkalinity stress (Munns et al., 2006). It has been reported that the genes associated with photosynthesis are significantly down-regulated in Na_2CO_3 stressed leaves of *L. chinensis* (Jin et al., 2008), and that the up-regulation of photosynthesis genes contribute to the recovery of photosynthetic ability in the halophyte, *Burma mangrove* (Miyama and Tada, 2008). In the present study, SNP treatment significantly increased net photosynthesis rate and Fv/Fm of cucumber leaves under alkali stress. Similar effect of SNP was observed in Cd-treated barley (Chen

Table 3
Annotation of Solexa tags against the cucumber genomic sequence. Na, 30 mM $NaHCO_3$ treatment; SNP, 30 mM $NaHCO_3$ + 100 μM SNP treatment.

		All clean tags		Unambiguous tags	
		Total tags	Distinct tags	Total tags	Distinct tags
Na	Map to genome	4,053,647 (68.61%)[a]	51,969 (56.62%)[a]		
	Map to gene	1,372,833 (23.23%)[a]	26,058 (28.39%)[a]	1,233,576 (20.88%)[a]	24,713 (26.92%)[a]
	Mapped genes		11,176 (41.89%)[c]		10,271 (38.49%)[c]
	Unknown	482,105 (8.16%)[b]	13,758 (14.99%)[b]		
SNP	Map to genome	3,737,444 (64.55%)[a]	50,338 (56.02%)[a]		
	Map to gene	1,575,487 (27.21%)[a]	27,050 (30.10%)[a]	1,416,013 (24.46%)[a]	25,697 (28.60%)[a]
	Mapped genes		11,199 (41.97%)[c]		10,288 (38.56%)[c]
	Unknown	476,931 (8.24%)[b]	12,476 (13.88%)[b]		

[a] Percentage of matched tags/total tags.
[b] Percentage of unmatched tags/total tags.
[c] Percentage of matched genes/total reference genes of Cucurbit Genomic Database.

Table 4
Genes selected randomly from our findings for QRT-PCR.

Gene ID	Annotation	Primer sequence (5′–3′)	Target size	Solexa fold	RT-PCR fold
Csa001726	Glutathione synthase	F: GTGCTGTTATTTTGTTTGTGGTTC R: CGGAGAGTTTTGACGTTATGTGT	97	4	4
Csa008595	Cycloartenol synthase 1	F: CCCTTGGTTGGAGTTGATC R: CCTATGCCCTGGATGTAAC	130	3	5
Csa008597	Cytochrome P450	F: ATCAGGATACGACATACCAC R: GATCTTCCCACAATACAGG	83	5	7
Csa011385	ATP-citrate synthase	F: ATGCCATTTGGTAGGGTTATG R: GATGCGCTGGTCTTTTCA	71	3	8
Csa013551	Hydroperoxide lyase 1	F: CCCAGTTCCCTCCCAATAC R: AGCGGCTGATACCCACAAAG	110	6	4
Csa018505	P450 reductase 2	F: ACGGGGTTGGCTCCTTTCA R: AGTTGTTCAGCTCATCCTCGTAGA	142	4	2
Csa013740	Chloroplast precursor	F: ATTCTTCTGCTTATCGGTGTAT R: CGTTGTTCGGTTAGTCTCA	130	3	2
Csa000881	Galactinol synthase 1	F: ACGCCGTAATGGACTGCTTTTG R: CGGGTGGCCACTTGACTC	103	−14	−2
Csa004896	WRKY transcription factor, putative	F: GTGGAGGAAATACGGGCAGAA R: ACTTGTTTTGTGGCTTGGCAGGT	105	−10	−2
Csa009383	Unknown protein	F: GAAGCGCCCGAACTCAGC R: TAAGGCAGATAAGGATTGGAAGGA	126	−8	−3
Csa017274	NAC transcription factor	F: ACTGGGTGCTATGTCGTATCTA R: CATGTCGTTGTTGGTGGTAA	131	−9	−4

et al., 2010). In accordance with the effects of SNP, photosynthesis (GO: 0015879), a GO term of biological process, was mostly dominantly enriched in cucumber leaves, which included 13 up-regulated genes by SNP. Among them, six genes (Csa012562, Csa019740, Csa015190, Csa013074, Csa005159, Csa018819) coded for the light-harvesting complex chlorophyll a/b binding protein, which belonged to PSII or PSI and bounded by 'antenna' chlorophylls (Caffarri et al., 2007; Wientjes et al., 2011), suggesting that SNP treatment contributed to the improving ability to harvest light energy of the cucumber leaves under alkali stress. Other 7 up-regulated genes by SNP were involved in cellular Fe homeostasis (Csa018291) (Tarantino et al., 2010), photosystem II repair (Csa019224) (Luciński et al., 2011), photosynthetic electron transport (Csa015782, Csa003352, Csa020828) (Lehtimäki et al., 2010; Ifuku et al., 2011), chloroplast organization (Csa016781) (Chen et al., 2010) and phosphorylation of the major antenna complex (LHCII) between PSII and PSI (Csa012585) (Samol et al., 2012). The damage of photosynthetic apparatus was one important factor for salt-induced decrease in photosynthesis for cucumber plants (Shu et al., 2012), and chloroplast was the organelle of photosynthesis. GO analysis of cell components showed that many cell components involved in chloroplast were enriched by SNP treatment under alkali stress, which mainly included "chloroplast part (GO: 0044434)", "chloroplast envelop (GO: 0009941)", "photosynthetic membrane (GO: 0034357)", "chloroplast thylakoid (GO: 0009543)", "chloroplast thylakoid membrane (GO: 0009535) and chloroplast stroma (GO: 0009570)". Fe was important for chloroplast development and chlorophyll metabolism, and its deficiency in plants was often considered to be induced by high pH in soil (Mori, 1999). The present study indicated that application of SNP significantly increased Fe content in cucumber leaves, which might be responsible for the SNP function in reversing chlorosis and

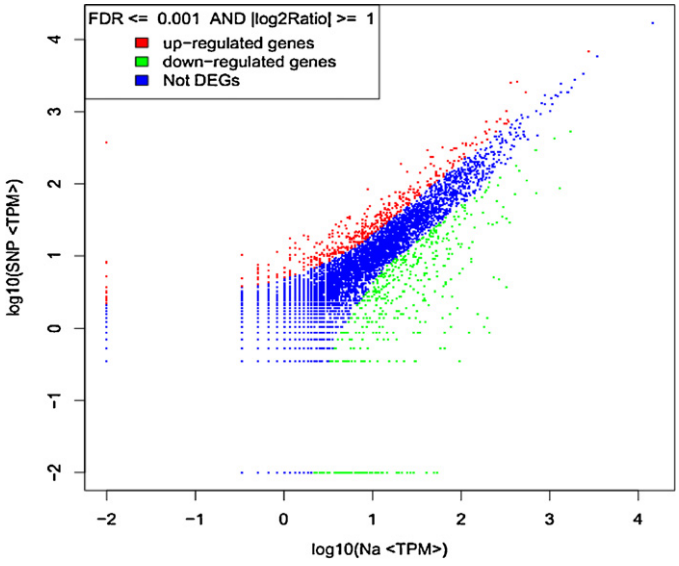

Fig. 5. Comparison of gene expression level between the two libraries. Red dots stand for genes that have higher expression levels in the SNP library, green dots represent those present less expression quantities in the SNP-alleviated tissue and blue dots show genes that did not alter dramatically. The parameters "FDR ≤ 0.001" and "$\log_2^{Ratio} = 1$" were used as the threshold to decide the significance of gene expression difference. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

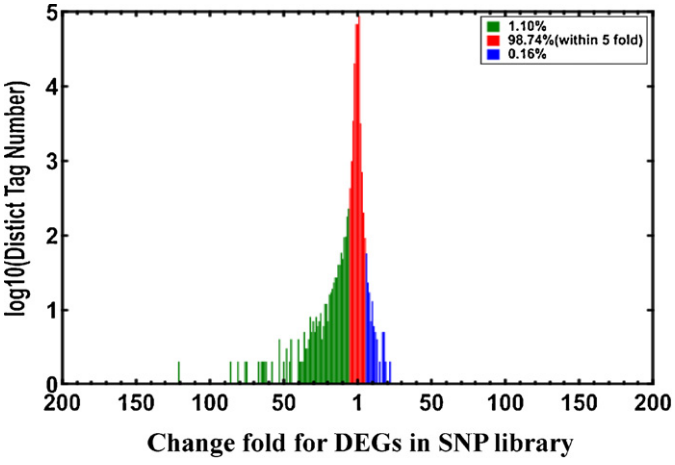

Fig. 6. Differentially expressed tags in the SNP library. The "*x*" axis represents fold-change of differentially expressed distinct tags in the SNP library. The "*y*" axis represents the number of distinct tags (log 10). The red region (98.74%) shows the distinct tags that with a 5-fold difference between two libraries. Distinct tags that are up- and down-regulated for more than 5-fold in the SNP library are represented in the blue (0.16%) and green (1.10%) regions, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 5
GO enrichment analysis of the differentially expressed genes in cucumber leaves exposed SNP treatment after $NaHCO_3$ stress ($P < 0.001$).

Biological process	
Photosynthesis (GO: 0015879)	Oxoacid metabolic process (GO: 0043436)
Response to abiotic stimulus (GO: 0009628)	Cellular lipid metabolic process (GO: 0044255)
Lipid localization (GO: 0010876)	Organic acid metabolic process (GO: 0006082)
Response to temperature stimulus (GO: 0009266)	Carboxylic acid metabolic process (GO: 0019752)
Metabolic process (GO: 0008152)	Isoprenoid metabolic process (GO: 0006720)
Response to cold (GO: 0009409)	Organic acid biosynthetic process (GO: 0016053)
Cellular metabolic compound salvage (GO: 0043094)	Carboxylic acid biosynthetic process (GO: 0046394)
Response to stimulus (GO: 0050896)	Cellular ketone metabolic process (GO: 0042180)
Response to radiation (GO: 0009314)	Isoprenoid biosynthetic process (GO: 0008299)
Response to light stimulus (GO: 0009416)	Sulfur metabolic process (GO: 0006790)
Photosynthesis, light reaction (GO: 0019684)	Transport (GO: 0006810)
Response to nematode (GO: 0009624)	Establishment of localization (GO: 0051234)
Cellular metabolic process (GO: 0044237)	Peptide transport (GO: 0015833)
Carbohydrate biosynthetic process (GO: 0016051)	Oligopeptide transport (GO: 0006857)
Photorespiration (GO: 0009853)	Cellular nitrogen compound metabolic process (GO: 0034641)
Generation of precursor metabolites and energy (GO: 0006091)	Cellular nitrogen compound biosynthetic process (GO: 0044271)
Lipid biosynthetic process (GO: 0008610)	Carbohydrate metabolic process (GO: 0005975)
Molecular function	
Transporter activity (GO: 0005215)	Oxidoreductase activity, acting on CH—OH group of donors (GO: 0016614)
Catalytic activity (GO: 0003824)	Chlorophyll binding (GO: 0016168)
Substrate-specific transmembrane transporter activity (GO: 0022891)	Isomerase activity (GO: 0016853)
Ion transmembrane transporter activity (GO: 0015075)	Oxidoreductase activity, acting on the CH-OH group of donors, NAD and NADP as acceptor (GO: 0016616)
Transmembrane transporter activity (GO: 0022857)	Cation transmembrane transporter activity (GO: 0008324)
Substrate-specific transporter activity (GO: 0022892)	Transferase activity, transferring hexosyl groups (GO: 0016758)
Oxidoreductase activity (GO: 0016491)	
Cellular component	
Plastid part (GO: 0044435)	Intracellular membrane-bounded organelle (GO: 0043231)
Chloroplast part (GO: 0044434)	Plastid stroma (GO: 0009532)
Plastid (GO: 0009536)	Intracellular part (GO: 0044424)
Chloroplast (GO: 0009507)	Intracellular organelle (GO: 0043229)
Cytoplasmic part (GO: 0044444)	Chloroplast envelop (GO: 0009941)
Thylakoid (GO: 0009579)	Organelle (GO: 0043226)
Cytoplasm (GO: 0005737)	Intracellular (GO: 0005622)
Thylakoid part (GO: 00044436)	Envelope (GO: 0031975)
Thylakoid membrane (GO: 0042651)	Organelle envelope (GO: 0031967)
Plastid envelope (GO: 0009526)	Light-harvesting complex (GO: 0030076)
Photosynthetic membrane (GO: 0034357)	Membrane (GO: 0016020)
Chloroplast thylakoid (GO: 0009543)	Microbody (GO: 0042579)
Plastid thylakoid (GO: 0031976)	Peroxisome (GO: 0005777)
Organelle subcompartment (GO: 0031984)	Stromule (GO: 0010319)
Plastid thylakoid membrane (GO: 0055035)	Plastoglobule (GO: 0010287)
Organelle membrane (GO: 0031090)	Cell part (GO: 0044464)
Chloroplast thylakoid membrane (GO: 0009535)	Cell (GO: 0005623)
Organelle part (GO: 0044422)	Vacuolar membrane (GO: 0005774)
Intracellular organelle part (GO: 0044446)	Vacuole (GO: 0005773)
Membrane-bounded organelle (GO: 0043227)	Vacuolar part (GO: 0044437)
Chloroplast stroma (GO: 0009570)	

retaining chloroplast integrity under alkali stress. Involvement of NO in Fe metabolism has been observed in several plants. Graziano et al. (2002) found that that exogenous NO improves internal Fe availability in maize and promoted chlorophyll synthesis and chloroplast development, and he attributed the mechanism to the formation of $Fe^{III}NO$ complex (Graziano et al., 2002). In Arabidopsis some key genes related to Fe acquisition and homeostasis were up-regulated by application of exogenous NO (García et al., 2010).

Accumulation of organic acids was one regular defense response in plants exposed to stress conditions, especially in salt and alkali-induced osmotic stress or nutrient deficiency (Li and Shi, 2010; Carvalhais et al., 2011). In the present study, SNP treatment significantly increased contents of acetate, malate and citrate in cucumber leaves under alkali stress, which was exactly in accordance with the enrichment of organic acid metabolic process (GO: 0006082) and carboxylic acid biosynthetic process (GO: 0046394). It has been reported that citrate production in white lupin grown under P-deficient conditions was positively correlated with endogenous NO concentrations (Wang et al., 2010). In common, Fe and Mg deficiencies shared the same causes with P deficiency, which are mainly induced by high pH in growth media (Tewari et al., 2006; Oburger et al., 2011). Accumulations of organic acid play important roles in plant tolerance to these adverse factors (López-Bucio et al., 2000). In the present study, it was observed that higher organic acid accumulation in cucumber leaves was accompanied with higher Fe and Mg contents by SNP treatment under alkali stress. Therefore, it could be concluded that the organic acid metabolism modulated by NO from SNP might be one important mechanism in increasing cucumber tolerance to alkali stress.

Ultrastructural alterations are frequent observations in salt-treated plants due to excess ions or osmotic stress (Pareek et al., 1997). In the present study, membrane systems including plasma membrane, chloroplast and mitochondria in cucumber leaves were dramatically induced by alkali stress, which might be caused by serious osmotic stress, ion toxicity, or alkali-induced specific adverse factors such as direct high pH damage, Fe, Mg and N

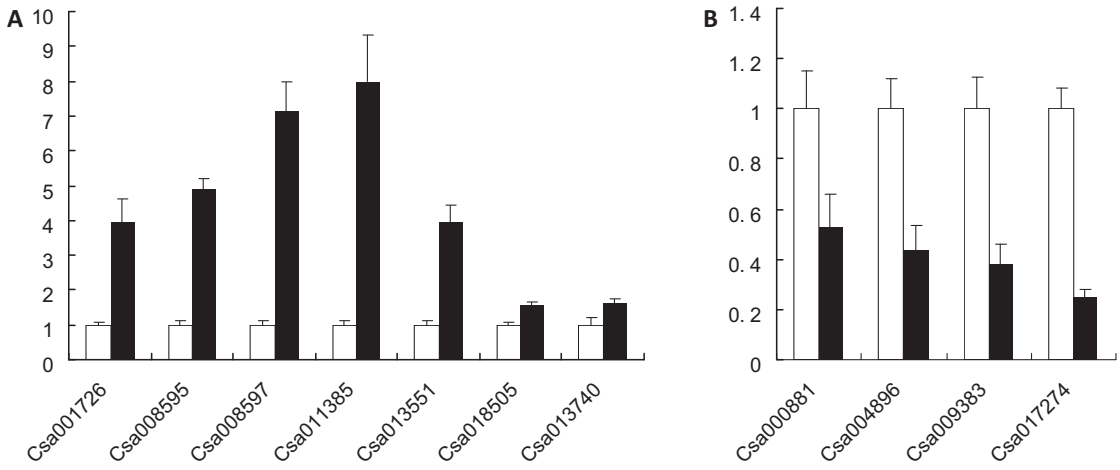

Fig. 7. QRT-PCR analysis for eleven transcripts differentially expressed genes in $NaHCO_3$-stress treatment (white) and SNP treatment (black), seven were up-regulated (A) and four were down-regulated (B) in the SNP library as identified by Solexa expression profile. All data were normalized to the actin expression level. Data represent fold change of RQ (relative quantification) in SNP treatment compare to $NaHCO_3$-stress treatment. Bars represent RQ standard deviation calculated from three biological replicates.

physiological deficiency. It is well known that lipid is very important for cell membrane formation, and transporters or channels imbedded in membranes play key role in plant tolerance to salt, osmotic and other abiotic stress through modulating intracellular ion or organic solutes distribution (Mahajan and Tuteja, 2005; Conde et al., 2011). The present study provided that lipid biosynthetic process (GO: 0008610) according to the GO term of biological process, and some transporter activities including ion transmembrane transporter activity (GO: 0015075), substrate-specific transporter activity (GO: 0022892) and cation transmembrane transporter activity (GO: 0008324) according to the GO term of molecular function were positively enriched by SNP treatment under alkali stress. Transmembrane transportations are energy-demanding processes, which greatly depend on respiration and photosynthesis in plant leaves (Bukhov et al., 2008), therefore, the integration of chloroplast and mitochondria by SNP under alkali stress could be particular relevant with the enrichment of above GO terms. Furthermore, a GO term directly related with energy production called generation of precursor metabolites and energy (GO: 0006091) was enriched in the present study. Involvement of Fe in chloroplast formation has been known for a long period of time, while recently increasing discovers indicated that mitochondria also represented a crucial target of Fe homeostasis, and Fe deficiency could dramatically affect mitochondrial development, metabolism and morphology (Vigani, 2012). Based on this, it could be concluded that higher Fe efficiency by SNP treatment might be a dominant role in keeping both photosynthetic apparatus and respiratory machinery from alkali damage. Mitochondria were the energy resource in plants, and their metabolism was closely linked with N assimilation in leaf by amino acid, carbon (C) and redox metabolism (Szal and Podgórska, 2012). In the present study, the higher accumulation of N in cucumber leaves by SNP treatment under alkali stress might be partly attributed to increasing mitochondrial metabolism, resulting in higher N assimilation.

5. Conclusion

Exogenous NO could significantly reversed alkali-induced chlorosis in cucumber leaves, and promoted cucumber plant growth. Analysis of physiological metabolism and gene ontology indicated that the function of NO might greatly depend on the modulation in organic acid accumulation, photosynthetic process and nutrient utilization. Based on Solexa sequencing, a large number of different expression genes were recognized by SNP treatment under alkali stress, providing a basis for the further investigation about the mechanism of NO alleviating alkali stress on cucumber.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scienta.2012.11.033.

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