Transcriptional Responses of a Bicarbonate-Tolerant Monocot, *Puccinellia tenuiflora*, and a Related Bicarbonate-Sensitive Species, *Poa annua*, to NaHCO₃ Stress

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**Abstract:** *Puccinellia tenuiflora* is an alkaline salt-tolerant monocot found in saline-alkali soil in China. To identify the genes which are determining the higher tolerance of *P. tenuiflora* compared to bicarbonate sensitive species, we examined the responses of *P. tenuiflora* and a related bicarbonate-sensitive *Poeae* plant, *Poa annua*, to two days of 20 mM NaHCO₃ stress by RNA-seq analysis. We obtained 28 and 38 million reads for *P. tenuiflora* and *P. annua*, respectively. For each species, the reads of both unstressed and stressed samples were combined for *de novo* assembly of contigs. We obtained 77,329 contigs for *P. tenuiflora* and 115,335 contigs for *P. annua*. NaHCO₃ stress resulted in greater than two-fold absolute expression value changes in 157 of the *P. tenuiflora* contigs and 1090 of *P. annua* contigs. Homologs of the genes involved in Fe acquisition, which are important for the survival of plants under alkaline stress, were up-regulated in
*P. tenuiflora* and down-regulated in *P. annua*. The smaller number of the genes differentially regulated in *P. tenuiflora* suggests that the genes regulating bicarbonate tolerance are constitutively expressed in *P. tenuiflora*.

**Keywords:** *Puccinellia tenuiflora*; *Poa annua*; RNA-seq; alkaline salt stress; bicarbonate

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1. **Introduction**

Soil salinity and sodicity are major environmental stresses faced by crops. In Songnen Plain in Northeast China, about $3.73 \times 10^6$ ha contain elevated levels of alkaline salt, and the area is expanding at a rate of 1.4% annually [1,2]. In alkaline soils, plant stress factors include excess Na$^+$ and HCO$_3^-$/CO$_3^{2-}$ ions and high pH. In highly alkaline areas, only a small number of plants can grow.

Recently some transcriptome analyses under bicarbonate stress have been performed on both bicarbonate-tolerant and bicarbonate-sensitive species. The bicarbonate tolerant species include *Leymus chinensis* [3], *Puccinellia tenuiflora* [4–7], *Tamarix hispida* [8,9], *Limonium bicolor* [10] and the sensitive species include soybean [11], maize [2], *Lotus japonicus* [12] and flax [13]. However, most of these studies examined only a single species, which makes it difficult to compare the responses of bicarbonate-tolerant and bicarbonate-sensitive species.

*Puccinellia tenuiflora* (Griseb.) Scrib. et Merr. is a graminaceous plant found in saline-alkali soil in Songnen Plain, China. It is tolerant to both neutral and alkaline salts, and its tolerance mechanisms have been extensively studied. *P. tenuiflora* is able to maintain a high K$^+$/Na$^+$ ratio due to a high K$^+$/Na$^+$ selectivity of its plasma membrane [14] and a high ability to limit Na$^+$ influx in the roots [15]. In addition, its leaves exude salts with wax through the stomata [16]. *P. tenuiflora* is able to maintain high photosynthetic activity under low NaCl stress, possibly through the activity of antioxidant enzymes [17]. *P. tenuiflora* also accumulates and exudes citric acid under alkaline salt stress, and secretes it from the roots, where it may adjust the pH of the rhizosphere [18]. Several *P. tenuiflora* genes that are presumably involved in the response to alkaline salt stress have been cloned and characterized [19–26]. EST [4,6,7], microarray [5,6] and proteomics [27] analyses have identified genes in various categories (metabolism, transcription regulation, signal transduction, transport etc.) that are presumably involved in the responses of *P. tenuiflora* to salt stress. However, it is unclear whether these responses are specific to *P. tenuiflora* or are shared with sensitive species.

Here, to obtain insights into the molecular mechanisms of alkaline salt tolerance, we compared the transcriptomes of *Puccinellia tenuiflora* and the salt-sensitive *Poa annua* L. (both of the tribe Poeae).

2. **Results**

2.1. Bicarbonate Stress Tolerance Test of *P. annua*

*P. annua* grew faster than *P. tenuiflora* under the control condition. Under the stress of 300 mM NaHCO$_3$, *P. tenuiflora* survived but *P. annua* did not (Figure 1B), indicating that *P. annua* was a suitable species for a comparison of bicarbonate stress tolerance.
Figure 1. Stress tolerance of *P. tenuiflora* (left) and *P. annua* (right). Seedlings of *P. annua* and *P. annua* were hydroponically grown for nine days and were transferred to the nutrient solution containing 0 (A); or 300 mM NaHCO₃ (B), and were grown for another five days. White bars = 1 cm

*P. annua* roots continued to elongate at NaHCO₃ concentrations up to 30 mM, but stopped growing and the root tips turned black at 40 mM NaHCO₃ (Figure 2A). Root growth of *P. tenuiflora* was reduced at NaHCO₃ concentrations as low as 10 mM, but was not stopped by concentrations up to 40 mM NaHCO₃. Based on these results, we used 20 mM NaHCO₃ for the RNA-seq analysis.

Figure 2. Root elongation during NaHCO₃ treatment (A) and shoot length after five days of treatment (B) of *P. tenuiflora* and *P. annua* under the indicated concentrations of NaHCO₃. Values show the average of three plates, each containing 7–10 seedlings. The error bars represent standard error (SE) and * indicate values that are significantly different from those under 0 mM NaHCO₃ (*p* < 0.05 in Student’s *t* test).

2.2. De Novo Assembly of *P. tenuiflora* and *P. annua* Transcripts

After two sequencing runs using the same cDNA libraries (see Experimental Section 4.3 for details), we obtained 29,619,901 reads for *P. tenuiflora* and 47,700,089 reads for *P. annua*. After *de novo* assembly, 77,329 and 115,335 contigs were obtained for *P. tenuiflora* and *P. annua*, respectively. Setting
stringent parameters for assembly to avoid trans chimeras resulted in relatively short contigs. Species distributions of the top BLASTX hits for the contigs of each species are shown in Figure S1. For both species, the grass *Aegilops tauscieii* was the species with the most hits and the species distributions were very similar.

2.3. Read Mapping and Gene Annotation

The reads from the second run were mapped to the assembled contigs to calculate the expression values, and the expression values were compared between the control and the stressed samples. The numbers of the contigs whose expressions changed >2- or <0.5-fold under NaHCO₃ stress compared to the control were 1090 in *P. annua* and 157 in *P. tenuiflora* (Table S2). In this table, fold changes of $\infty$ and $-\infty$ indicate transcripts that were expressed only under the NaHCO₃ condition or only under the control condition, respectively. However, transcripts detected only under one condition or the other may include artifacts caused by misassembled contigs.

The expressions of some of the genes which were differentially regulated in the RNA-seq analysis were checked by qRT-PCR. For each of the genes, the qRT-PCR results confirmed the RNA-seq results (Figure 3).

![Figure 3](image)

**Figure 3.** Fold change in expression values (expressed as Log₂ values) of selected genes as determined by RNA-seq analysis and real-time quantitative RT-PCR. RNAs extracted from the same plant samples were used for the two analyses and *tubulin* was used as an internal control for the RT-PCR.

3. Discussion

3.1. Effects of NaHCO₃ Stress on *P. tenuiflora* and *P. annua*

*P. annua* was sensitive to NaHCO₃ (Figures 1 and 2). NaHCO₃ concentrations as low as 40 mM killed the root tip of *P. annua* and totally blocked its elongation (Figure 2). On the other hand, roots of *P. tenuiflora* continued to elongate under 40 mM NaHCO₃ treatment. The plants could survive 300 mM
NaHCO₃ stress treatment for five days, suggesting that the ability to protect root tips from bicarbonate stress is important for survival.

Twenty mM NaHCO₃ treatment on agar plates had less effect on shoot growth than on root growth in both species. This finding is contrary to the finding that carbonate stress increased the root/shoot ratio of pea [28]. However, it should be noted that the stress treatment in this study was performed in sealed plates. The air inside the plates would have had a high CO₂ concentration generated from bicarbonate, which might have affected the respiration and photosynthesis of the shoots.

3.2. RNA-Seq Analysis and de Novo Assembly

De novo assembly of the reads obtained in the first RNA-seq revealed the presence of many trans chimeras [29] that were later incorrectly assigned as up- or down-regulated genes, probably as the result of high expressions of rRNAs. Although rRNA removal tools and PCR duplicate removal tools have not been commonly used in other transcriptome studies, in the present study, they seemed to reduce the number of trans chimeras in the up- or down-regulated contigs. Setting stringent parameters for assembly also helped to reduce the number of chimeras, although it also resulted in the frequent appearance of possibly the same gene products in the up- and down-regulated genes lists. Because our goal was to identify the kinds of genes that are differentially regulated by bicarbonate stress, we concluded that multiple counts of the same gene products would have less effect on our results than the occurrence of trans chimeras. It is also possible that the contigs that yielded the same BLAST hits are actually orthologs or splice variants.

3.2.1. Genes Differentially Regulated in P. annua under NaHCO₃ Treatment

Significantly more genes were differentially regulated in P. annua than in P. tenuiflora (Table S2), suggesting that P. annua was more severely stressed by NaHCO₃ than P. tenuiflora. Some of the up-regulated genes were probable homologs of genes involved in stress tolerance and defense mechanisms such as glutathione S-transferase, disease resistance protein RPM1 and mitochondrial chaperone BCS1-B as well as genes involved in metabolism such as aspartic proteinase nepenthesin-2 and patatin group A-3 (Table 1 and “poupa_hit” tab in Table S2). Homologs of genes encoding flavonoid 3′-monooxygenase-like protein and anthocyanidin 5,3-O-glucosyltransferase-like protein were up-regulated, suggesting that secondary metabolites have a role in protecting against bicarbonate stress.

Table 1. Genes that were up-regulated under NaHCO₃ treatment (fold change > 2, FDR-corrected p-value < 0.05). The annotations were assigned by BDA (Blast2GO Descriptor Annotator), showing the words most frequently counted of all blast hit descriptions (https://www.blast2go.com/b2gsupport/faqs).

| Contig    | Annotation                                         | Fold Change (EDGE Test) |
|-----------|----------------------------------------------------|--------------------------|
| Poa_27190 | Glutathione S-transferase                          | 11.34                    |
| Poa_3657  | High affinity nitrate transporter                  | 53.21                    |
| Poa_12387 | Anthocyanidin 5,3-O-glucosyltransferase-like       | 5.09                     |
High affinity nitrate transporters and predicted inorganic phosphate transporters were up-regulated in both *P. annua* and *P. tenuiflora* under bicarbonate stress. There have been conflicting reports on the effect of HCO$_3^-$ on the uptake of NO$_3^-$ [31–37] and on phosphorus status in plants [38,39], and the roles of these transporters under bicarbonate stress are not clear. However, the genes for nitrate transporters in alkaline salt-tolerant ecotype of *Lotus japonicus* has been reported to be up-regulated under bicarbonate stress [40]. Phosphoenolpyruvate carboxykinase (*PEPCK*) was strongly induced by NaHCO$_3$ in both species, in agreement with previous studies [11,41], and may have a role in maintaining homeostasis of intracellular CO$_2$/H$_2$CO$_3$/HCO$_3^-$ and/or organic acids. However, its function in the response to NaHCO$_3$ stress is unclear.

Transcripts involved in metal acquisition and homeostasis were down-regulated only in *P. annua* (Table S2). Down-regulated transcripts included homologs of iron-phytosiderophore transporter, *nicotianamine aminotransferase A* (*NAAT-A*) and *nicotianamine synthase* (*NAS*). Bicarbonate stress induces Fe chlorosis in plants [42,43]. High pH and HCO$_3^-$ ion have been suggested to impede the solubility of Fe in the rhizosphere and plant apoplast, making Fe unavailable for plant cells [42–44].

### Table 1. Cont.

| Contig   | Annotation                                             | Fold Change (EDGE Test) |
|----------|--------------------------------------------------------|-------------------------|
| Poa_688  | ABC transporter b family member 4-like                 | 62.27                   |
| Poa_2744 | Aspartic proteinase nepenthesin-2                       | 7.68                    |
| Poa_18988| Cytochrome p450                                         | 54.50                   |
| Poa_15731| Disease resistance protein RPM1                         | 18.52                   |
| Poa_4898 | Flavonoid 3-monoxygenase-like                           | 14.47                   |
| Poa_6299 | Mitochondrial chaperone BCS1-B                         | 2.93                    |
| Poa_8595 | Patatin group A-3                                      | 3.97                    |
| Poa_2480 | Phosphate transporter                                   | 176.58                  |
| Poa_8940 | Phosphoenolpyruvate carboxykinase                       | 54.00                   |
| Poa_12214| Potassium channel SKOR                                 | 6.51                    |
| Poa_25791| Probable WRKY transcription factor 70-like              | 16.62                   |
| Poa_19228| Subtilisin-like protease                                 | 9.35                    |

*P. tenuiflora*

| Contig   | Annotation                                             | Fold Change |
|----------|--------------------------------------------------------|-------------|
| Put_2050 | Boron transporter                                      | 3.58        |
| Put_11515| Carbonic anhydrase                                     | 3.90        |
| Put_1357 | High-affinity nitrate transporter-like                  | 3.53        |
| Put_763  | Leucine-rich repeat receptor-like protein kinase at2g19210-like | 3.48        |
| Put_2064 | Long chain acyl-CoA synthetase 4-like                  | 5.03        |
| Put_4625 | Metal-nicotianamine transporter YSL6                   | 3.68        |
| Put_22802| Nicotianamine aminotransferase A-like                  | 4.65        |
| Put_1383 | Phosphate transporter                                   | 25.87       |
| Put_1973 | Phosphoenolpyruvate carboxykinase                      | 7.47        |
| Put_7021 | Sucrase-like protein                                    | 6.08        |
| Put_3399 | Sulfate transporter                                     | 3.21        |
| Put_4027 | Thionin-like peptide                                    | 6.82        |
| Put_10418| Zinc transporter                                        | 5.74        |

EDGE = Empirical analysis of digital gene expression [30].
NASs and NAATs are involved in synthesis of phytosiderophores, which are members of the mugineic acid family. Overexpression of OsNAS2 led to a higher tolerance of Fe deficiency and to high pH [45], and gene expression of NAAT was reported to be induced by Fe deficiency in barley [46]. Metal-nicotianamine transporter YSL (yellow stripe 1-like) transports Fe-chelated phytosiderophore complexes from rhizosphere into root cells. Putative homologs of transporters such as high-affinity potassium transporters [47] and zinc-induced facilitator-like 1 [48] were also down-regulated in *P. annua*, suggesting that NaHCO₃ disrupts the ion homeostasis in *P. annua*. Down-regulation of β-expansin 1a precursor and cellulose synthase only in *P. annua* may partly account for the reduced root growth of the species under NaHCO₃ stress.

3.2.2. Genes Differentially Regulated in *P. tenuiflora* under NaHCO₃ Treatment

Metal-nicotianamine transporter and NAAT-A, which were down-regulated in *P. annua*, were up-regulated in *P. tenuiflora*. The difference in the responses of genes related to Fe-acquisition in *P. tenuiflora* and *P. annua* (Tables 1 and S2) suggests that *P. tenuiflora* is better able to cope with Fe deficiency under alkaline salt stress. A putative homolog of BOR-like 2 was also induced in *P. tenuiflora*. BOR is involved in the export of boron from the cytoplasm to the apoplast [49]. Although 10 mM NaHCO₃ treatment did not reduce the concentration of boron in leaves of *Lotus tenuis* [40], boron transporters may have an important function in saline-alkali fields since boron is said to be adsorbed to the soil in the presence of CaCO₃ under high pH [50]. Other genes that were up-regulated only in *P. tenuiflora* included putative homologs of genes involved in metabolism (e.g., sucrase-like protein) and defense (e.g., thionin-like peptide [51]). Metabolic responses and defense responses must be important in both *P. annua* and *P. tenuiflora* under NaHCO₃ stress, but the genes involved seem to be different.

Transcripts down-regulated in *P. tenuiflora* include members of families that are known to be involved in other stress responses (e.g., cytochrome p450 and WRKY transcription factors; Tables 2 and S2). *PutHKT2;1*, which is described as a “salt transporter” in Table 2 and which has a high-affinity Na⁺-K⁺ symport activity [21], was also down-regulated. Thus, down-regulation of *PutHKT2;1* might contribute to maintaining intracellular ion homeostasis under weak bicarbonate stress conditions.

| Contig     | Annotation                                      | Fold Change (EDGE Test) |
|------------|------------------------------------------------|-------------------------|
| Poa_40361  | 2'-Deoxymugineic-acid 2'-dioxygenase-like       | −3.90                   |
| Poa_7047   | Actin                                          | −3.31                   |
| Poa_13081  | Ammonium transporter                           | −2.94                   |
| Poa_24077  | β-Expansin 1a precursor                        | −8.33                   |
| Poa_8466   | Cellulose synthase                             | −3.48                   |
| Poa_9099   | GDSL esterase lipase At5g45910-like            | −2.24                   |
| Poa_2507   | High-affinity potassium transporter             | −2.80                   |
| Poa_21856  | Iron-phytosiderophore transporter              | −3.51                   |
| Poa_36815  | Mate efflux family protein chloroplastic-like  | −6.77                   |
| Poa_2955   | Nicotianamine aminotransferase A-like          | −4.99                   |
Table 2. Cont.

| Contig      | Annotation                          | Fold Change (EDGE Test) |
|-------------|-------------------------------------|-------------------------|
| Poa_64935  | Nicotianamine synthase 3            | −90.37                  |
| Poa_58054  | Protein zinc induced facilitator-like 1-like | −50.8                  |
| Poa_41522  | STOP1                               | −9.44                   |
| Poa_6256   | Urea active transporter 1           | −2.47                   |
| Poa_9416   | Wall-associated receptor kinase 2-like | −3.47                  |
| Put_32521  | Cytochrome p450 716b1-like          | −3.72                   |
| Put_28631  | Sodium transporter                   | −8.04                   |
| Put_52287  | WRKY transcription factor           | −5.15                   |

4. Experimental Section

4.1. Plant Materials, Growth Conditions and Stress Treatments

Seeds of *P. tenuiflora* were collected in an alkaline soil area located in North-East China. Seeds of *Poa annua* were kindly provided by Masaru Ogasawara at University of Utsunomiya, Japan. Seeds were surface-sterilized by washing with 70% v/v ethanol for 5 min. and subsequently with 50% v/v NaClO for 15 min. Growth chamber was maintained at 28 °C during the day and 22 °C at night while the daily photoperiod of 350–400 µmol·m⁻²·s⁻¹ was 12 h.

To compare the survival rates under strong alkaline salt stress, the surface-sterilized seeds were sown in tap water and were grown for nine days. The seedlings were transferred to nutrient solution containing 6 mg/L (NH₄)₂SO₄, 2 mg/L K₂SO₄, 8.2 mg/L MgSO₄, 2.3 mg/L KNO₃, 7.5 mg/L Ca(NO₃)₂, 3.1 mg/L KH₂PO₄, 10 mg/L Fe-EDTA with 0 or 300 mM NaHCO₃. Photos were taken after 5 days. The experiment was repeated twice and similar results were obtained.

For measuring the shoot and root length under weak alkaline salt stress, the surface-sterilized seeds were sown on 0.8% w/v agar plates containing the nutrient solution described above. After 4 days (*P. annua*) and 9 days (*P. tenuiflora*) of germination, the plants were transferred to 0.8% w/v agar plates containing the nutrients mentioned above and 0, 10, 20, 30 or 40 mM NaHCO₃. The *P. tenuiflora* seedlings were allowed to germinate longer because they grow more slowly than *P. annua* seedlings. Shoot length and root elongation were measured after 5 days.

For RNA-seq analysis, surface-sterilized seeds were hydroponically grown for 19 days. Water was changed every 3–4 days. The plants were transferred to the nutrient solution containing 0 or 20 mM NaHCO₃ and harvested after 48 h. Three biological replicates with >100 plants each were obtained.

4.2. RNA Extraction and cDNA Library Construction

Plants harvested in the previous subsection were separated into shoots and roots, frozen immediately using liquid nitrogen and ground to fine powder. In the first sequencing, mRNA was extracted directly from powdered tissue samples with a Dynabeads® DIRECT™ Micro Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer’s instructions. In the second sequencing, total RNA was extracted from frozen root samples using an RNeasy Plant Mini Kit (QIAGEN, Venlo, The Netherlands). mRNA was then isolated from total RNA with the Dynabeads Kit (See subsection 4.3 for details; Life
Technologies). mRNA samples were fragmented, reverse transcribed and amplified to make barcoded whole transcriptome libraries using Ion Total RNA-seq Kit v2 (Life Technologies). Yield and size distribution of the fragmented RNA and the amplified cDNA were checked using an Agilent 2200 Tapestation with High Sensitivity RNA ScreenTape® and High Sensitivity D1000 ScreenTape® (Agilent Technologies, Palo Alto, CA, USA) at each step. For libraries whose peak sizes of amplified cDNAs were <200 bp (all the libraries in the first run and *P. annua* control #2, *P. tenuiflora* control #3 and *P. tenuiflora* control #1 in the second run), cDNAs with the sizes around 280 bp were selected using E-Gel® SizeSelect™ Agarose Gel (Life Technologies). Ion OneTouch™ System with Ion PI™ Template OT2 200 Kit v3 (Life Technologies) was used to prepare enriched, template-positive Ion PI™ Ion Sphere Particles. The whole library preparation and sequencing were performed twice from the same sample sets, obtaining 24 libraries from 12 sample sets (see the next section for detail).

4.3. Next Generation Sequencing and Data Analysis

Sequencing was performed using the Ion Proton™ System with an Ion PI™ Sequencing 200 Kit v3 (Life Technologies) following the manufacturer’s instructions. The sequencing results are summarized in Table S1. The numbers of raw reads were different in the different libraries, which was caused by an error in diluting the cDNA libraries during the library preparation step. Since the expression values calculated in the RNA-seq analysis described later were normalized to the number of total reads in the given libraries, the difference in the number of total reads should not greatly impact the conclusions. Sequencing results were imported into CLC Genomic Workbench 7.5 (CLC bio, Aarhus, Denmark) as FASTQ files for further analysis. On CLC Genomic Workbench, the raw reads with the quality score less than 0.05 were trimmed using the “Trim Sequences” tool. Reads shorter than 15 bp were discarded. The average quality scores of the trimmed reads were 22.89 for *P. annua* and 23.08 for *P. tenuiflora*. In the first trial of RNA-seq, the trimmed reads were *de novo* assembled just after this step. However, BLASTN analysis revealed that many of the resulting contigs were *trans* chimeras [29] resulting from misassembly of different gene products into one contig. Many of them seemed to be chimeras of rRNA and other transcripts, so in the second trial we tried to reduce rRNA contamination by extracting mRNAs from total RNA instead of directly from tissue samples. This way rRNA contamination was reduced substantially (Table S1), but not totally removed. Thus we used SortMeRNA (ver. 1.99 beta, [52]) to filter out probable rRNA contamination from the trimmed reads. The average quality scores of the trimmed reads from the second run were 23.60 for *P. annua* and 23.27 for *P. tenuiflora*. The trimmed reads were exported as FASTQ files from CLC Genomics Workbench. rRNA was removed from the reads with SortMeRNA with the databases supplied with the software (silva-euk-18s-database-id95.fasta & silva-euk-28s-database-id95.fasta). The “length of the sliding window” option was set (“-L 14”) to allow reads longer than 14 bp. Default settings were used for other parameters. Reads not assigned as rRNA were imported back to CLC Genomics Workbench. The same process was applied to the reads from the first run, and the reads from the two runs were combined to be used for *de novo* assembly on CLC Genomics Workbench. The reads were *de novo* assembled for each plant species using 12 libraries each with the word and bubble size automatically set by the software to yield contigs (word size 23 and 24 bp, bubble size 108 and 119 bp for *P. annua* and *P. tenuiflora*, respectively). Minimum contig length was set to 200 bp. To raise the precision of the contigs, the trimmed reads were mapped back to the
resulting contigs with the following settings: mismatch cost 2, insertion cost 3, deletion cost 3, length fraction 0.8 and similarity fraction 0.95. The contig sequences were updated occasionally according to the mapped reads. After the removal of probable PCR duplicates from the trimmed reads using Duplicate Read Removal plugin (ver. 1.0, beta, CLC bio) of the CLC Genomics Workbench, expression analysis was performed with RNA-Seq Analysis Tool of CLC Genomic Workbench for each sample groups of the second run, using de novo assembled contigs as references. Parameters for read mapping were set as follows: Mismatch cost 2, insertion cost 3, deletion cost 3, length fraction 0.8, similarity fraction 0.8 and maximum number of hits for a read 10. Expression levels were compared between control groups and stressed groups using Empirical Analysis of DGE [30,53] tool on CLC Genomics Workbench 7.5. Reads per kilobase of exon per million mapped reads (RPKM) was also calculated. Contigs whose absolute fold changes were >2 (FDR-corrected \( p \)-values <0.05) by EDGE test were selected as up-regulated or down-regulated transcripts. For those contigs, homology searches using BLASTX (BLAST+ ver. 2.2.30, NCBI) against the \textit{Poaceae} (taxid: 4479) protein sequences from NCBI nr protein database and annotation with Blast2GO (ver. 2.7.2; [54]) were performed. Summary of rRNA removal and duplicate removal are shown in Table S1.

The FASTQ files of the raw reads and the sequences of differentially regulated contigs were deposited to DDBJ Sequence Read Archive (BioProject ID: PRJDB3227).

4.4. Real-Time PCR Analysis

Selected contigs that were differentially regulated by the NaHCO\(_3\) stress were examined by real-time PCR analysis. Total RNA was extracted from the same plant root samples that were used in RNA-seq analysis with RNeasy Plant Mini Kit (QIAGEN), and reverse transcribed using High-Capacity RNA-to-cDNA™ Kit (Life Technologies) following the manufacturer’s instructions. The cDNA was diluted 20 times and 1 \( \mu \)L of the diluted cDNA was used as the template for quantitative RT-PCR analysis. SYBR® Premix Ex Taq™ II (Tli RNase H Plus) (TaKaRa, Shiga, Japan) and StepOne™ Real-Time PCR System (Applied Biosystems, Framingham, MA, USA) were used for the PCR. A \textit{tubulin} gene from \textit{P. tenuiflora} was used as an internal standard to normalize the expression data [21]. The primer pairs (Table S3) were designed to yield products with the sizes of 80–200 bp based on the contig sequences. The PCR was performed as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The experiments were carried out in triplicate. Ten-fold serial dilution of 0.2× cDNA mixture of all the samples were used for drawing the standard curve.

5. Conclusions

The present RNA-seq analysis revealed the transcriptome of \textit{P. tenuiflora} on a larger scale than did previous studies, and showed significant differences in the numbers of genes that respond to NaHCO\(_3\) stress between related NaHCO\(_3\)-tolerant and NaHCO\(_3\)-sensitive species. The tolerant \textit{P. tenuiflora} seemed to adapt to bicarbonate stress by regulating a small number of genes including those important for Fe acquisition, which suggests that \textit{P. tenuiflora} has a high tolerance to NaHCO\(_3\), even when grown under unstressed conditions.
Supplementary Materials

Supplementary materials can be found at http://www.mdpi.com/1422-0067/16/01/0496/s1.

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Author Contributions

Tetsuo Takano and Shio Kobayashi conceived and designed the experiments; Shio Kobayashi, Hina Satone and Engkong Tan performed the experiments; Shio Kobayashi, Hiroyuki Kurokochi and Engkong Tan analyzed the data; Shuichi Asakawa and Shenkui Liu contributed reagents/materials; Shio Kobayashi wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Wang, L.; Seki, K.; Miyazaki, T.; Ishihama, Y. The causes of soil alkalinization in the Songnen Plain of Northeast China. *Paddy Water Environ.* 2009, 7, 259–270.
2. Zhang, L.M.; Liu, X.G.; Qu, X.N.; Yu, Y.; Han, S.P.; Dou, Y.; Xu, Y.Y.; Jing, H.C.; Hao, D.Y. Early transcriptomic adaptation to Na₂CO₃ stress altered the expression of a quarter of the total genes in the maize genome and exhibited shared and distinctive profiles with NaCl and high pH stresses. *J. Integr. Plant Biol.* 2013, 55, 1147–1165.
3. Jin, H.; Plaha, P.; Park, J.Y.; Hong, C.P.; Lee, I.S.; Yang, Z.H.; Jiang, G.B.; Kwak, S.S.; Liu, S.K.; Lee, I.S.; et al. Comparative EST profiles of leaf and root of *Leymus chinensis*, a xerophilous grass adapted to high pH sodic soil. *Plant Sci.* 2006, 170, 1081–1086.
4. Wang, Y.; Chu, Y.; Liu, G.; Wang, M.H.; Jiang, J.; Hou, Y.; Qu, G.; Yang, C. Identification of expressed sequence tags in an alkali grass (*Puccinellia tenuiflora*) cDNA library. *J. Plant Physiol.* 2007, 164, 78–89.
5. Wang, Y.; Yang, C.; Liu, G.; Jiang, J. Development of a cDNA microarray to identify gene expression of *Puccinellia tenuiflora* under saline-alkali stress. *Plant Physiol.* 2007, 45, 567–576.
6. Wang, Y.; Yang, C.; Liu, G.; Zhang, G.; Ban, Q. Microarray and suppression subtractive hybridization analyses of gene expression in *Puccinellia tenuiflora* after exposure to NaHCO₃. *Plant Sci.* 2007, 173, 309–320.
7. Zhang, X.; Wei, L.; Wang, Z.; Wang, T. Physiological and molecular features of *Puccinellia tenuiflora* tolerating salt and alkaline-salt stress. *J. Integr. Plant Biol.* 2012, 55, 262–276.
8. Gao, C.; Wang, Y.; Liu, G.; Yang, C.; Jiang, J.; Li, H. Expression profiling of salinity-alkali stress responses by large-scale expressed sequence tag analysis in *Tamarix hispid*. *Plant Mol. Biol.* **2008**, *66*, 245–258.

9. Wang, C.; Gao, C.; Wang, L.; Zheng, L.; Yang, C.; Wang, Y. Comprehensive transcriptional profiling of NaHCO₃-stressed *Tamarix hispida* roots reveals networks of responsive genes. *Plant Mol. Biol.* **2013**, *84*, 145–157.

10. Wang, Y.; Ma, H.; Liu, G.; Xu, C.; Zhang, D.; Ban, Q. Analysis of gene expression profile of *Limonium bicolor* under NaHCO₃ stress using cDNA microarray. *Plant Mol. Biol. Report.* **2008**, *26*, 241–254.

11. Ge, Y.; Li, Y.; Zhu, Y.M.; Bai, X.; Lv, D.K.; Guo, D.; Ji, W.; Cai, H. Global transcriptome profiling of wild soybean (*Glycine soja*) roots under NaHCO₃ treatment. *BMC Plant Biol.* **2010**, *10*, 153.

12. Babuin, M.F.; Campestre, M.P.; Rocco, R.; Bordenave, C.D.; Escaray, F.J.; Antonelli, C.; Calzadilla, P.; Gárriz, A.; Serna, E.; Carrasco, P.; *et al.* Response to long-term NaHCO₃-derived alkalinity in model *Lotus japonicus* ecotypes Gifu B-129 and Miyakojima MG-20: Transcriptomic profiling and physiological characterization. *PLoS One* **2014**, *9*, e97106.

13. Yu, Y.; Huang, W.; Chen, H.; Wu, G.; Yuan, H.; Song, X.; Kang, Q.; Zhao, D.; Jiang, W.; Liu, Y.; *et al.* Identification of differentially expressed genes in flax (*Linum usitatissimum* L.) under saline-alkaline stress by digital gene expression. *Gene* **2014**, *549*, 113–122.

14. Peng, Y.H.; Zhu, Y.F.; Mao, Y.Q.; Wang, S.M.; Su, W.A.; Tang, Z.C. Alkali grass resists salt stress through high [K⁺] and an endodermis barrier to Na⁺. *J. Exp. Bot.* **2004**, *55*, 939–949.

15. Wang, C.M.; Zhang, J.L.; Liu, X.S.; Li, Z.; Wu, G.Q.; Cai, J.Y.; Flowers, T.J.; Wang, S.M. *Puccinellia tenuiflora* maintains a low Na⁺ level under salinity by limiting unidirectional Na⁺ influx resulting in a high selectivity for K⁺ over Na⁺. *Plant. Cell Environ.* **2009**, *32*, 486–496.

16. Guorong, S.; Yongzhen, P.; Hongbo, S.; Liye, C.; Xining, Z.; Haiyan, M.; Wenzhong, C.; Cunxu, W. Does *Puccinellia tenuiflora* have the ability of salt exudation? *Coll. Surf. B. Biointerfaces* **2005**, *46*, 197–203.

17. Wang, Y.; Sun, G.; Suo, B.; Chen, G.; Wang, J.; Yan, Y. Effects of Na₂CO₃ and NaCl stresses on the antioxidant enzymes of chloroplasts and chlorophyll fluorescence parameters of leaves of *Puccinellia tenuiflora* (Turcz.) scribn.et Merr. *Acta Physiol. Plant.* **2007**, *30*, 143–150.

18. Guo, L.Q.; Shi, D.C.; Wang, D.L. The key physiological response to alkali stress by the alkali-resistant halophyte *Puccinellia tenuiflora* is the accumulation of large quantities of organic acids and into the rhizosphere. *J. Agron. Crop Sci.* **2010**, *196*, 123–135.

19. Ardie, S.W.; Liu, S.; Takano, T. Expression of the AKT1-type K⁺ channel gene from *Puccinellia tenuiflora*, *PutAKT1*, enhances salt tolerance in *Arabidopsis*. *Plant Cell Rep.* **2010**, *29*, 865–874.

20. Ardie, S.W.; Nishiuchi, S.; Liu, S.; Takano, T. Ectopic expression of the K⁺ channel β subunits from *Puccinellia tenuiflora* (*KPutB1*) and rice (*KOB1*) alters K⁺ homeostasis of yeast and *Arabidopsis*. *Mol. Biotechnol.* **2011**, *48*, 76–86.

21. Ardie, S.W.; Xie, L.; Takahashi, R.; Liu, S.; Takano, T. Cloning of a high-affinity K⁺ transporter gene *PutHKT2;1* from *Puccinellia tenuiflora* and its functional comparison with *OsHKT2;1* from rice in yeast and *Arabidopsis*. *J. Exp. Bot.* **2009**, *60*, 3491–502.

22. Bu, Y.; Sun, B.; Zhou, A.; Zhang, X.; Lee, I.; Liu, S. Identification and characterization of a *PutAMT1;1* gene from *Puccinellia tenuiflora*. *PLoS One* **2013**, *8*, e83111.
23. Chang-Qing, Z.; Shunsaku, N.; Shenkui, L.; Tetsuo, T. Characterization of two plasma membrane protein 3 genes (PutPMP3) from the alkali grass, Puccinellia tenuiflora, and functional comparison of the rice homologues, OsLt6a/b from rice. *BMB Rep.* **2008**, *41*, 448–454.

24. Kobayashi, S.; Abe, N.; Yoshida, K.T.; Liu, S.; Takano, T. Molecular cloning and characterization of plasma membrane- and vacuolar-type Na⁺/H⁺ antiporters of an alkaline-salt-tolerant monocot, *Puccinellia tenuiflora*. *J. Plant Res.* **2012**, *125*, 587–594.

25. Liu, H.; Zhang, X.; Takano, T.; Liu, S. Characterization of a *PutCAX1* gene from *Puccinellia tenuiflora* that confers Ca²⁺ and Ba²⁺ tolerance in yeast. *Biochem. Biophys. Res. Commun.* **2009**, *383*, 392–396.

26. Wang, X.; Yang, R.; Wang, B.; Liu, G.; Yang, C.; Cheng, Y. Functional characterization of a plasma membrane Na⁺/H⁺ antiporter from alkali grass (Puccinellia tenuiflora). *Mol. Biol. Rep.* **2010**, *38*, 4813–4822.

27. Yu, J.; Chen, S.; Wang, T.; Sun, G.; Dai, S. Comparative proteomic analysis of *Puccinellia tenuiflora* leaves under Na₂CO₃ stress. *Int. J. Mol. Sci.* **2013**, *14*, 1740–1762.

28. Zribi, K.; Gharssalli, M. Effect of bicarbonate on growth and iron nutrition of pea. *J. Plant Nutr.* **2002**, *25*, 2143–2149.

29. Yang, Y.; Smith, S.A. Optimizing de novo assembly of short-read RNA-seq data for phylogenomics. *BMC Genomics* **2013**, *14*, 328.

30. Robinson, M.D.; Oshlack, A. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol.* **2010**, *11*, R25.

31. Cramer, M.D.; Lewis, O.A.M.; Lips, S.H. Inorganic carbon fixation and metabolism in maize roots as affected by nitrate and ammonium nutrition. *Physiol. Plant.* **1993**, *89*, 632–639.

32. Zioni, A.B.; Vaadia, Y.; Lips, S.H. Nitrate uptake by roots as regulated by nitrate reduction products of the shoot. *Physiol. Plant.* **1971**, *15*, 288–290.

33. Van der Westhuizen, M.M.; Cramer, M.D. The influence of elevated rhizosphere dissolved inorganic carbon concentrations on respiratory O₂ and CO₂ flux in tomato roots. *J. Exp. Bot.* **1998**, *49*, 1977–1985.

34. Van der Merwe, C.A.; Cramer, M.D. Effect of enriched rhizosphere carbon dioxide on nitrate and ammonium uptake in hydroponically grown tomato plants. *Plant Soil* **2000**, *221*, 5–11.

35. Gao, Z.F.; Lips, S.H. Effects of increasing inorganic carbon supply to roots on net nitrate uptake and assimilation in tomato seedlings. *Physiol. Plant.* **1997**, *101*, 206–212.

36. Alhendawi, R.A.; Römhled, V.; Kirkby, E.A.; Marschner, H. Influence of increasing bicarbonate concentrations on plant growth, organic acid accumulation in roots and iron uptake by barley, sorghum, and maize. *J. Plant Nutr.* **1997**, *20*, 1731–1753.

37. Cai, C.; Wang, J.Y.; Zhu, Y.G.; Shen, Q.R.; Li, B.; Tong, Y.P.; Li, Z.S. Gene structure and expression of the high-affinity nitrate transport system in rice roots. *J. Integr. Plant Biol.* **2008**, *50*, 443–451.

38. Kolesch, H.; Oktay, M.; Höfner, W. Effect of iron chlorosis-inducing factors on the pH of the cytoplasm of sunflower (*Helianthus annuus*). *Plant Soil* **1984**, *82*, 215–221.

39. Guo, R.; Shi, L.; Ding, X.; Hu, Y.; Tian, S.; Yan, D.; Shao, S.; Gao, Y.; Liu, R.; Yang, Y. Effects of saline and alkaline stress on germination, seedling growth, and ion balance in wheat. *Agron. J.* **2010**, *102*, 1252.
40. Paz, R.C.; Rocco, R.A.; Reinoso, H.; Menéndez, A.B.; Pieckenstain, F.L.; Ruiz, O.A. Comparative study of alkaline, saline, and mixed saline-alkaline stresses with regard to their effects on growth, nutrient accumulation, and root morphology of Lotus tenuis. J. Plant Growth Regul. 2012, 31, 448–459.
41. Gong, B.; Zhang, C.; Li, X.; Wen, D.; Wang, S.; Shi, Q.; Wang, X. Identification of NaCl and NaHCO3 stress responsive proteins in tomato roots using iTRAQ-based analysis. Biochem. Biophys. Res. Commun. 2014, 446, 417–422.
42. Mengel, K. Iron availability in plant tissues-iron chlorosis on calcareous soils. Plant Soil 1994, 165, 275–283.
43. Bavaresco, L.; Giachino, E.; Colla, R. Iron chlorosis paradox in grapevine. J. Plant Nutr. 1999, 22, 1589–1597.
44. Römheld, V. The chlorosis paradox: Fe inactivation as a secondary event in chlorotic leaves of grapevine. J. Plant Nutr. 2000, 23, 1629–1643.
45. Lee, S.; Kim, Y.S.; Jeon, U.S.; Kim, Y.K.; Schjoerring, J.K.; An, G. Activation of rice nicotianamine synthase 2 (OsNAS2) enhances iron availability for biofortification. Mol. Cells 2012, 33, 269–275.
46. Takahashi, M.; Yamaguchi, H.; Nakanishi, H.; Shioiri, T.; Nishizawa, N.K.; Mori, S. Cloning two genes for nicotianamine aminotransferase, a critical enzyme in iron acquisition (Strategy II) in graminaceous plants. Plant Physiol. 1999, 121, 947–956.
47. Rodríguez-Navarro, A.; Rubio, F. High-affinity potassium and sodium transport systems in plants. J. Exp. Bot. 2006, 57, 1149–1160.
48. Haydon, M.J.; Cobbett, C.S. A novel major facilitator superfamily protein at the tonoplast influences zinc tolerance and accumulation in Arabidopsis. Plant Physiol. 2007, 143, 1705–1719.
49. Miwa, K.; Wakuta, S.; Takada, S.; Ide, K.; Takano, J.; Naito, S.; Omori, H.; Matsunaga, T.; Fujiwara, T. Roles of BOR2, a boron exporter, in cross linking of rhamnogalacturonan II and root elongation under boron limitation in Arabidopsis. Plant Physiol. 2013, 163, 1699–1709.
50. Goldberg, S.; Forster, H. Boron sorption on calcareous soils and reference calcites. Soil Sci. 1991, 152, 304–310.
51. Epple, P.; Apel, K.; Bohlmann, H. Overexpression of an endogenous thionin enhances resistance of Arabidopsis against Fusarium oxysporum. Plant Cell 1997, 9, 509–520.
52. Kopylova, E.; Noël, L.; Touzet, H. SortMeRNA: Fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. Bioinformatics 2012, 28, 3211–3217.
53. Robinson, M.D.; McCarthy, D.J.; Smyth, G.K. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010, 26, 139–140.
54. Conesa, A.; Götz, S.; García-Gómez, J.M.; Terol, J.; Talón, M.; Robles, M. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 2005, 21, 3674–3676.