Developmental Genetic Mechanisms of \( C_4 \) Syndrome Based on Transcriptome Analysis of \( C_3 \) Cotyledons and \( C_4 \) Assimilating Shoots in \textit{Haloxylon ammodendron}

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Abstract

It is believed that transferring the \( C_4 \) engine into \( C_3 \) crops will greatly increase the yields of major \( C_3 \) crops. Many efforts have been made since the 1960s, but relatively little success has been achieved because \( C_4 \) plant traits, referred to collectively as \( C_4 \) syndrome, are very complex, and little is known about the genetic mechanisms involved. Unfortunately, there exists no ideal genetic model system to study \( C_4 \) syndrome. It was previously reported that the \textit{Haloxylon} species have different photosynthetic pathways in different photosynthetic organs, cotyledons and assimilating shoots. Here, we took advantage of the developmental switch from the \( C_3 \) to the \( C_4 \) pathway to study the genetic mechanisms behind this natural transition. We compared the transcriptomes of cotyledons and assimilating shoots using mRNA-Seq to gain insight into the molecular and cellular events associated with \( C_4 \) syndrome. A total of 2959 differentially expressed genes [FDR \( \leq 0.001 \) and abs \( |\log_2(\text{Fold change})| \geq 1 \)] were identified, revealing that the transcriptomes of cotyledons and assimilating shoots using mRNA-Seq to gain insight into the molecular and cellular events associated with \( C_4 \) syndrome. A total of 2959 differentially expressed genes [FDR \( \leq 0.001 \) and abs \( |\log_2(\text{Fold change})| \geq 1 \)] were identified, revealing that the transcriptomes of cotyledons and assimilating shoots are considerably different. We further identified a set of putative regulators of \( C_4 \) syndrome. This study expands our understanding of the development of \( C_4 \) syndrome and provides a new model system for future studies on the \( C_3 \)-to- \( C_4 \) switch mechanism.

Introduction

Photosynthetic \( \text{CO}_2 \) fixation is a fundamental life process involving the conversion of solar energy into chemical energy that can be later released to fuel the activity of an organism. The ancestral photosynthetic \( \text{CO}_2 \)-fixation process is \( C_3 \) photosynthesis. The first organic product of \( \text{CO}_2 \) fixation is a three-carbon compound. \( C_3 \) photosynthesis and its key enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) evolved early in the history of life, when
there was no oxygen in the atmosphere and atmospheric CO₂ levels were significantly high. As atmospheric CO₂ dropped and O₂ accumulated, Rubisco inefficiency began to limit C₃ photosynthesis because the active site of Rubisco does not completely discriminate between CO₂ and O₂, leading to the catalysis of two competitive reactions: photosynthetic CO₂ assimilation and photorespiratory CO₂ loss, especially under hot, dry and/or saline conditions that enhance photorespiration. Approximately 30 million years ago, an abrupt drop in atmospheric [CO₂] further reduced the efficiency of C₃ photosynthesis and triggered the evolution of C₄ photosynthesis, in which CO₂ is initially fixed into a four-carbon compound and concentrated around Rubisco [1–6]. C₄ photosynthesis has evolved >60 times and occurs in approximately 7500 species of flowering plants [7–9]. The transition from C₃ to C₄ plants was the green revolution of nature. Although C₄ plants comprise only 3% of land plant species, they account for some 25% of global terrestrial carbon fixation [3,4,7,10,11].

C₄ photosynthesis is a complex trait that combines biochemical, physiological and anatomical characteristics, the so-called C₄ syndrome [12,13]. Other than four single-celled C₄ lineages, the vast majority of C₄ plants possess Kranz anatomy and C₄ syndrome [8,9], indicative of convergent evolution [7]. Traditional biochemical and modern molecular biological studies showed that all of the proteins required for the core C₄ cycle are present in C₃ plants [14]. Based on this evidence, it is hypothesized that no significant genetic changes are required for the transition from C₃ to C₄ photosynthesis [15,16]. The first attempt at introducing C₄ photosynthesis into C₃ plants was made in Atriplex through conventional interspecific hybridization of photosynthetic types to reduce photorespiration and increase photosynthetic capacity [17–22]. The results indicated that F₁ hybrids of Atriplex rosea (C₄, NAD-ME type) × Atriplex triangularis (C₃) were more similar to their C₃ parents in physiology and failed to form well-developed Kranz anatomy. Similar hybridization studies were conducted in the genera Flaveria, Panicum, Moricandia, and Brassica, but none proved fruitful at converting C₃ species into functional C₄ types [23]. Due to infertility, few hybrids have been developed beyond the F₁ generation. In the few advanced generations studied in Atriplex and Flaveria hybrids, correlations among photosynthetic traits were low, indicating that C₄ photosynthesis is a combination of independent biochemical, physiological and anatomical characteristics [23]. Recently, a progress report was released on oat-maize addition lines showing that the addition of individual maize chromosomes to the C₃ species oat caused increases in vein density but did not confer functional C₄ photosynthesis [24]. Therefore, introducing a fully developed C₄ photosynthesis pathway into C₃ plants through interspecific hybridization or even genetic engineering is far more practical than previously thought [25].

In nature, there exist examples of switches from a C₃ pathway to a two-celled C₄ pathway triggered by external or internal signals. The former example includes the freshwater amphibious leafless sedge Eleocharis vivipara, which can switch from a C₃ pathway to a C₄ pathway with Kranz anatomy after induction by environmental changes and exogenous application of abscisic acid (ABA) [26–28]. The latter examples were reported in Haloxylon and Salsola species that have different photosynthetic pathways in different photosynthetic organs [29,30]. Haloxylon aphyllum and H. persicum of Chenopodiaceae have C₃ photosynthesis in cotyledons and C₄ photosynthesis in assimilating shoots (the main photosynthetic organs) with a typical Salsolid-type Kranz anatomy [29]; the same phenomenon has been observed in Salsola gern-mascens of the genus Salsola (Chenopodiaceae), in which cotyledons exhibit C₃-type photosynthesis, while leaves perform NAD-malic enzyme (NAD-ME) C₄-type photosynthesis with a Salsolid-type Kranz anatomy [30]. However, this manner of C₃-to-C₄ switches has not received much attention, and there were no follow-up studies after their discovery. Investigations into the developmental genetic mechanisms controlling the different photosynthetic types in cotyledons and leaves or other photosynthetic organs will illuminate the genetic regulatory network of C₄ syndrome.
With the development of new technologies, more studies have begun to analyze C₄ syndrome at the systems biology level. To understand C₄ formation, mesophyll cells and bundle sheath cells in the leaf blade of maize were used as a model system for C₄ differentiation. The cells were separated and analyzed for transcriptional changes by microarray analysis and next-generation sequencing [31–33]; 21% of genes were differentially expressed between mesophyll cells and bundle sheath cells [32]. Recently, John et al. sequenced RNA isolated from the mesophyll cells and bundle sheath cells of *Setaria viridis* and found the significant convergence of cell-specific gene expression in *S. viridis* and maize [34]. Such studies deepen our understanding of C₄ syndrome. In 2011, two research groups used mRNA-Seq analysis of closely related C₃ and C₄ species for which gene expression is altered, and these groups identified genes associated with the C₄ pathway [35,36]. Up to 603 and 3582 transcripts differed in abundance between C₃ and C₄ leaves in the genera *Cleome* and *Flaveria*, respectively. While these two experiments were designed to identify C₄-related transcriptomic gene expression changes, it is difficult to tell if the observed variation in transcript abundance was associated with differences between the species or C₄ photosynthesis. However, the comparative transcriptomics of C₃ cotyledons and C₄ assimilating shoots in this study will identify more C₄-specific genes than before because there is no genetic variation between these different species; more importantly, the natural developmental C₃-to-C₄ transition may give clues concerning its master switch.

**Materials and Methods**

**Plant Growth and Harvesting**

Seeds of *Haloxylon ammodendron* were provided by the Turpan Eremophyte Botanic Garden, Chinese Academy of Sciences in Turpan, Xinjiang, China (http://english.egi.cas.cn/rs/sr/tdbg/). The seeds were incubated and germinated on moist filter paper in Petri dishes. After germination, seedlings were planted in sand in a greenhouse maintained at 30/20°C day/night, 70% relative humidity and a photoperiod of 12 h light/12 h dark under a light intensity of 1000 µE m⁻² s⁻¹. Cotyledons were fully expanded after 2 days growing in sand and sampled for all analyses. Assimilating shoots were collected from plants at 10 days of age. For mRNA-Seq, samples were taken from 10–15 individual plants during the middle of the light period, immediately frozen in liquid nitrogen, and stored at —80°C until use.

**Light Microscopy**

Samples of fully expanded cotyledons and assimilating shoots were fixed in Formalin–acetic acid–alcohol (FAA) for 24 hours, dehydrated through an alcohol series, cleared with xylene, and embedded in paraffin wax. Cross-sections were obtained using a microtome. For light microscopy, semi-thin sections were stained with safranin O solution and studied under DIC microscope (BX51, Olympus, Japan) equipped with an LM Digital Camera (DP70, Olympus).

**Stable Carbon Isotope Analysis**

Stable carbon isotope ratios (¹³C/¹²C) were quantified in cotyledons and dried assimilating shoots from plants grown in the greenhouse. Then, 1–2 cm segments of the middle regions of fully expanded cotyledons or assimilating shoots were collected. All samples were oven-dried at 65°C for 48 h to a constant weight.

The measurements of stable carbon isotope ratios were carried out at the Chinese Academy of Forestry’s Stable Isotope Laboratory (Beijing, China) using a Flash EA1112 HT elemental analyzer (Thermo Scientific) coupled with a Delta V advantage isotope ratio mass spectrometer (Thermo Scientific). Stable carbon isotope ratios were expressed as δ¹³C (‰).
calculated as follows:

$$\delta^{13}C(%) = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right) \times 1000$$

where $R_{\text{sample}}$ and $R_{\text{standard}}$ are the $^{13}$C/$^{12}$C ratios for an individual sample and the reference standard (Pee Dee Belemnite), respectively.

RNA Preparation and Sequencing

Total RNA was prepared with TRIzol according to the manufacturer’s instructions (Invitrogen Life Technologies, Shanghai, China). Following extraction, total RNA was purified using the RNeasy Mini Kit from Qiagen (Shanghai, China), including on-column DNase digestion (Qiagen, Shanghai, China). Purified RNA was checked for integrity and quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The cDNA library was constructed for sequencing as described in the Illumina TruSeq RNA sample preparation v2 guide (Catalog # RS-930–1021). Sequencing was performed on an Illumina HiSeq 2000.

Mapping and Quantification of the Sequence Reads

We filtered and examined the quality of the raw sequence reads as described by Xu et al. [37]. The first 10 bases in each read were trimmed off before the mapping process.

**Mapping and Quantification of the Sequence Reads from Cotyledons and Leaves of Arabidopsis.** Clean reads were mapped onto the latest Arabidopsis thaliana genome assembly (http://www.phytozome.net/arabidopsis.php) using the Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) [38]. The best hit of each read with a maximum of three nucleotide mismatches was used. For each Arabidopsis Genome Initiative (AGI) code, the number of matching reads was counted, and the raw digital gene expression counts were normalized using the RPKM (Reads Per Kilobase per Million mapped reads) method [39,40].

**Mapping and Quantification of the Sequence Reads from the Cotyledons and Assimilating Shoots of H. ammodendron.** Clean reads were mapped onto the coding sequences of the latest A. thaliana genome assembly (http://www.phytozome.net/arabidopsis.php) using BLAT [41]. Alignments were performed in the protein space, and the best hit for each read was retained. For each Arabidopsis Genome Initiative (AGI) code, the number of matching reads was counted, and the raw digital gene expression counts were normalized using the RPKM method [39,40].

The differential expression between samples was statistically accessed by the R/Bioconductor package edgeR [42]. Genes with FDR $\leq$ 0.001 and $|\log_2(\text{Fold change})| \geq 1$ were considered significant.

Overrepresentation Analysis

To identify functional categories with significant differences between cotyledons and assimilating shoots, we performed an overrepresentation analysis using the GO Term Enrichment of AmiGO (http://amigo.geneontology.org/amigo) [43]. We used all detected transcripts in cotyledons and assimilating shoots as the background set and TAIR as the filter.

Results

Photosynthetic Features of Assimilating Shoots and Cotyledons

*Haloxylon* species have an unusual photosynthetic apparatus. The true leaves are reduced, and the young annual cylindrical shoots (assimilating shoots) are the main photosynthetic tissues. Fifteen years ago, Pyankov et al. [29] discovered that two *Haloxylon* species, *H. aphyllum* and
H. persicum, have a C₄ type of photosynthesis in assimilating shoots with Kranz anatomy, whereas leaf-like cotyledons lack Kranz-anatomy and incorporate CO₂ via C₃ photosynthesis, as observed through analyses of stable carbon isotope ratios, anatomy, primary photosynthetic products, and activities of carbon metabolism enzymes. We examined the anatomy assimilating shoots and cotyledons in H. ammodendron and confirmed that H. ammodendron, similarly to the other two Haloxylon species, uses different types of photosynthesis in assimilating shoots and cotyledons, as shown in Fig. 1. The cotyledons have no Kranz-type anatomy and several layers of mesophyll cells around only a few vascular bundles (Fig. 1A). The assimilating shoots have Salsoloid-type Kranz anatomy with two continuous layers of chlorenchyma (a layer of palisade mesophyll cells and an inner layer of bundle sheath cells) on the periphery and water-storage parenchyma in the center (Fig. 1B). The main vascular bundle occupies the central position, and only the small, peripheral vascular bundles are in contact with bundle sheath cells (Fig. 1B).

Stable carbon isotope ratios are used to distinguish the photosynthetic CO₂-fixing pathways of plants [44–46]. To further confirm the photosynthetic types of cotyledons and assimilating shoots, we analyzed the stable carbon isotope ratios by measuring δ¹³C values. The δ¹³C value for H. ammodendron cotyledons was -15.58±0.72 ‰ (mean ± SE, n = 3), similar to that of H. aphylhum and H. persicum reported by Pyankov et al. (-17.5 ‰) [29]. Unexpectedly, the assimilating shoots exhibited a more negative δ¹³C value of -21.89±1.05 ‰ (mean ± SE, n = 3).

Major Transcriptional Changes
To identify differences in transcript abundance related to C₄ syndrome, the transcriptomes of H. ammodendron assimilating shoots and cotyledons were compared. cDNA libraries of assimilating shoots and cotyledons were constructed and sequenced using the Illumina HiSeq 2000 platform, resulting in 30,287,044 and 36,971,687 reads, respectively, with a mean read length of 101 nucleotides. After trimming adapters and filtering out low-quality reads, 29,558,368 reads from assimilating shoots and 36,070,605 reads from cotyledons were retained for further analysis. Clean reads were mapped onto the coding sequences of the latest A. thaliana genome assembly (http://www.phytozome.net/arabidopsis.php) using BLAT [41]. 27037741 reads (~75.0%) from cotyledons and 23188916 reads (~78.5%) from assimilating shoots could be mapped onto the Arabidopsis transcriptome.

mRNA-Seq analysis comparing the transcriptomes of H. ammodendron assimilating shoots and cotyledons yielded 2959 differentially expressed genes [FDR≤0.001 and abs (|log₂(Fold change)|)≥1], with 1852 and 1107 more abundant transcripts in assimilating shoots and cotyledons, respectively (see Table A in S1 File). To test whether these differentially expressed transcripts are enriched in functional categories, we performed overrepresentation analysis using the GO Term Enrichment of AmiGO. The significantly up-regulated transcripts in assimilating shoots were enriched in several fundamental biological process categories, including methylation, cytokinesis, DNA replication, cell wall organization or biogenesis, biosynthetic process, anatomical structure morphogenesis, signaling pathway and developmental process (see Table B in S1 File). Down-regulated GO categories are less abundant than up-regulated ones, mainly in response to endogenous stimulus (see Table C in S1 File). Because we used TAIR as a filter and Arabidopsis is a typical C₃ plant, no functional C₄ class was detected.

C₄ Cycle Genes Were Up-regulated in Assimilating Shoots
Transcript analysis of known C₄ genes showed that all genes necessary for the core C₄ cycle of NADP-ME type plants were significantly up-regulated in assimilating shoots compared with cotyledons (Table 1). Among these genes, NADP-ME was most significantly different, with a
Figure 1. Transverse sections of a cotyledon (A) and an assimilating shoot (B) of *Haloxylon ammodendron*. MC, mesophyll cell; BSC, bundle sheath cell; WS, water storage tissue; V, vascular tissue. Scale bars represent 100 μm.

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12.9- (At5g11670), 12.7- (At2g19900) or 9.3- (At1g79750) fold higher transcript abundance in assimilating shoots. The second biggest difference came from PEPC, with a 12.1- (At2g42600) or 10.5- (At1g53310) fold up-regulation. Additionally, transcripts encoding AspAT (At4g31990) and the chloroplastidic MDH (At5g58330) were up-regulated 8.5- and 2.1-fold, respectively. Transcripts encoding PPDK (At4g15530) were increased 2.7-fold.

All genes required for the NAD-ME type of C4 photosynthesis were also up-regulated in assimilating shoots compared with cotyledons (Table 1). The transcripts encoding NAD-ME (At4g00570) were up-regulated 1.5-fold, and those encoding AlaAT (At1g17290) and mtNAD-MDH (At1g53240) were up-regulated 3.8- and 1.5-fold, respectively. Transcripts encoding PPDK (At4g15530) were increased 2.7-fold.

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Transcripts encoding the regulatory factors PEPC kinase (PEPC-K) increased 11-fold, which was significant (Table 1). Additionally, the transport proteins required for C4 photosynthesis were significantly up-regulated (see Table 1), such as pyruvate sodium symporter (BASS2), phosphoenolpyruvate/phosphate translocators (PPT1 and PPT2) and chloroplast dicarboxylate transporters (DiT1 and DiT2).

Table 1. Transcript abundance of C4 cycle genes and C4-related transporters.

| Gene ID     | Protein                  | Ha-AS(RPKM) | Ha-C(RPKM) | Fold Change | \(\log_2(\text{Ha-AS/Ha-C})\) |
|-------------|--------------------------|-------------|------------|-------------|-------------------------------|
| At4g15530   | PPDK                     | 5768.71     | 2133.50    | 2.70        | 1.44                          |
| At1g53310   | PEPC                     | 1081.51     | 102.90     | 10.51       | 3.39                          |
| At2g42600   | PEPC                     | 1201.30     | 99.31      | 12.10       | 3.60                          |
| At1g68750   | PEPC                     | 2.84        | 6.96       | 0.41        | -1.29                         |
| At5g58330   | cpNADP-MDH               | 1287.07     | 601.46     | 2.14        | 1.10                          |
| At1g79750   | NADP-ME                  | 814.71      | 87.96      | 9.26        | 3.21                          |
| At2g19900   | NADP-ME                  | 2480.24     | 194.89     | 12.73       | 3.67                          |
| At5g11670   | NADP-ME                  | 1161.41     | 90.10      | 12.89       | 3.69                          |
| At1g17290   | AlaAT                    | 393.42      | 103.14     | 3.81        | 1.93                          |
| At4g31990   | AspAT                    | 748.92      | 87.97      | 8.51        | 3.09                          |
| At5g19550   | AspAT                    | 59.18       | 80.65      | 0.73        | -0.45                         |
| At5g11520   | AspAT                    | 79.59       | 112.71     | 0.71        | -0.50                         |
| At2g22250   | AspAT                    | 37.61       | 26.50      | 1.32        | 0.40                          |
| At1g53324   | mtNAD-MDH                | 190.23      | 129.15     | 1.47        | 0.56                          |
| At4g00570   | NAD-ME                   | 42.94       | 27.82      | 1.54        | 0.63                          |
| At4g37870   | PEP-CK                   | 125.01      | 168.60     | 0.74        | -0.43                         |
| At3g47520   | cpNAD-MDH                | 90.47       | 34.47      | 2.62        | 1.39                          |
| At1g08650   | PEPC-K                   | 17.44       | 1.59       | 10.98       | 3.46                          |
| At5g47840   | AMK2                     | 489.96      | 230.02     | 2.13        | 1.09                          |
| At5g35170   | adenylate kinase family  | 141.21      | 216.67     | 0.65        | -0.62                         |
| At5g09650   | PPA6                     | 1042.96     | 739.28     | 1.41        | 0.50                          |
| At2g26900   | BASS 2                   | 1083.92     | 249.28     | 4.35        | 2.12                          |
| At3g56160   | BASS 4                   | 39.89       | 6.88       | 5.80        | 2.54                          |
| At5g33320   | PPT1                     | 225.80      | 39.88      | 5.66        | 2.50                          |
| At3g01550   | PPT2                     | 25.57       | 4.82       | 5.30        | 2.41                          |
| At5g46110   | TPT                      | 1386.73     | 2111.57    | 0.66        | -0.61                         |
| At5g12860   | Dit1                     | 390.82      | 259.62     | 1.51        | 0.59                          |
| At5g64280   | Dit2                     | 91.71       | 24.54      | 3.74        | 1.90                          |

Ha-AS = *Haloxylon ammodendron* assimilating shoots, Ha-C = *Haloxylon ammodendron* cotyledons.

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Photorespiratory Genes Were Down-regulated in Assimilating Shoots

A major advantage of the C₄ pathway is the reduction in photorespiration because high CO₂ concentration around Rubisco in bundle sheath cells effectively suppresses photorespiration. Detailed analysis of gene expression of all photorespiration genes showed that the transcripts of nearly all genes related to photorespiration were lower in abundance in assimilating shoots than in cotyledons (Table 2). The genes AtAGT1 (At2g13360), AtGGT1 (At1g23310), AtGLDT1 (At1g11860), AtSHM1 (At4g37930) and AtGLYK (At1g80380), all of which play major roles in photorespiration, were significantly down-regulated (log₂ (Fold change) ≤ -1).

Genes Controlling Vein Density Were Up-regulated in Assimilating Shoots

Kranz anatomy is accompanied by high vein density [16], so we assessed the transcript abundance of known genes controlling vein density. All of these genes were up-regulated in assimilating shoots (Table 3). Among them, the transcription factors MONOPTEROS/AUXIN RESPONSE FACTOR5 (MP/ARF5) MP and ATHB8, which are involved in the auxin signal transduction pathway controlling leaf vascular development, were up-regulated 3.9- and 1.9-fold, respectively.

### Table 2. Transcript abundance of photorespiration genes.

| Enzyme                        | Gene name | Gene ID    | Ha-AS (RPKM) | Ha-C (RPKM) | log₂(Ha-AS/Ha-C) |
|-------------------------------|-----------|------------|--------------|-------------|-----------------|
| 2PG phosphatase               | AtPGLP1   | At5g36790* | 243.47       | 228.19      | 0.09            |
| Glycolate oxidase             | AtGOX1    | At3g14420  | 911.87       | 3690.36     | -2.02           |
|                              | AtGOX2    | At3g14415  | 330.80       | 1326.77     | -2.00           |
|                              | AtGOX3    | At4g18360  | 151.29       | 679.40      | -2.17           |
|                              | AtHAOX1   | At3g14130  | 8.32         | 12.51       | -0.59           |
|                              | AtHAOX2   | At3g14150  | 9.20         | 16.56       | -0.85           |
| Ser:glyoxylate aminotransferase | AtAGT1   | At2g13360  | 362.28       | 2052.76     | -2.50           |
| Glu:glyoxylate aminotransferase | AtGGT1  | At1g23310  | 670.03       | 1607.05     | -1.26           |
| Gly decarboxylase P-protein   | AtGLDP1   | At4g33010  | 503.01       | 1461.69     | -1.54           |
|                              | AtGLDP2   | At2g26080  | 266.08       | 770.75      | -1.53           |
| Gly decarboxylase H-protein   | AtGLDH1   | At2g35370  | 153.54       | 644.93      | -2.07           |
|                              | AtGLDH2   | At2g35120  | 84.97        | 54.76       | 0.63            |
|                              | AtGLDH3   | At1g32470  | 168.58       | 721.63      | -2.10           |
| Gly decarboxylase T-protein   | AtGLDT1   | At1g11860  | 546.86       | 1701.11     | -1.64           |
| Gly decarboxylase L-protein   | AtLPD1    | At3g17240  | 164.91       | 271.07      | -0.72           |
| Ser hydroxymethyltransferase  | AtSHM1    | At4g37930  | 696.67       | 1457.01     | -1.06           |
|                              | AtSHM2    | At5g26780  | 378.13       | 774.57      | -1.03           |
| Hydroxypyruvate reductases    | AtHPR1    | At1g68010  | 647.42       | 1269.02     | -0.97           |
|                               | AtHPR2    | At1g79870* | 115.77       | 104.57      | 0.15            |
| Glycerate kinase              | AtGLYK    | At1g80380  | 46.14        | 115.70      | -1.33           |

Ha-AS = *Haloxylon ammodendron* assimilating shoots, Ha-C = *Haloxylon ammodendron* cotyledons.

*no significant differences in the expression of these genes between cotyledons and assimilating shoots (p > 0.01).*

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Differentially Expressed Transcription Factors Encoding Genes

A total of 248 transcription factor-encoding genes were identified, showing differential expression [FDR ≤ 0.001 and abs (|log2(Fold change)|) ≥ 1] in assimilating shoots compared with cotyledons (Table 4). In total, 107 genes were up-regulated, and 141 genes were repressed in assimilating shoots. To avoid capturing variation in transcript abundance associated with differences between the different developmental stages that do not relate to C4 photosynthesis, we also sequenced the transcriptomes of Arabidopsis cotyledons and leaves in parallel to minimize the influence of developmental stage-specific effects. Excluding the genes that had the same developmental expression pattern in *Hammodendron* and *Arabidopsis*, there were 96 putative positive regulators and 130 putative negative regulators. We tested the likelihood that SCARECROW/SHORTROOT components [47] of the C4 regulatory network were among these identified genes (see Table 5). Scarecrow plays a role in establishing Kranz anatomy in maize leaves, and its mutation results in abnormal Kranz anatomy [48]. SHR was included among the identified genes, and SCR was up-regulated by 1.3-fold in assimilating shoots, although it was not identified.

**Discussion**

The *Haloxylon* genus comprises three closely related species, including *H. ammodendron* (saxaul), *H. aphyllum* (black saxaul) and *H. persicum* (white saxaul). *H. aphyllum* and *H. persicum* were reported to have different types of photosynthesis in assimilating shoots and cotyledons. Similarly, *H. ammodendron* assimilating shoots had Salsoloid-type Kranz anatomy (Fig. 1B), indicative of C4 photosynthesis, while the structure of cotyledons was of the non-Kranz type (Fig. 1A). The developmental transition from a C3 pathway to a two-celled C4 pathway in *Haloxylon* species in nature is a good system with which to study the genetic regulatory network of C4 syndrome.

Stable carbon isotope analysis is used as a screening method to determine the photosynthetic pathway when it is unknown in a species [46]. We measured the δ13C values of cotyledons and assimilating shoots. The *H. ammodendron* cotyledons had a δ13C value of -15.58±0.72‰ (mean ± SE, n = 3), falling into the range of typical values for C4 plants of -6 to -19‰ [49]. Although this finding is contrary to the anatomic results, it is consistent with a previous report.
Table 4. Differentially expressed transcription factor-encoding genes.

| Putative positive regulators | Putative negative regulators |
|------------------------------|------------------------------|
| At1g01250                    | At1g01030                     |
| AP2-EREBP family            | ABI3VP1 family               |
| At3g57670                    | At5g50915                     |
| C2H2 family                 | bHLH family                  |
| At2g34710                    | At2g46680                     |
| Homeobox family             | Homeobox family              |
| At1g68550*                  | At1g25560                     |
| AP2-EREBP family            | ABI3VP1 family               |
| At4g27240                    | At5g52610                     |
| C2H2 family                 | bHLH family                  |
| At3g18010                    | At3g01220                     |
| Homeobox family             | Homeobox family              |
| At1g79700                    | At1g03800                     |
| AP2-EREBP family            | AP2-EREBP family             |
| At5g03740*                  | At5g567060                    |
| C2H2 family                 | bHLH family                  |
| At4g08150                    | At3g61890                     |
| Homeobox family             | Homeobox family              |
| At4g16750                    | At1g12610*                    |
| AP2-EREBP family            | AP2-EREBP family             |
| At5g39550                    | At1g08320                     |
| C2H2 family                 | bZIP family                  |
| At4g32880                    | At5g15150                     |
| Homeobox family             | Homeobox family              |
| At4g23750*                  | At3g10210                     |
| AP2-EREBP family            | AP2-EREBP family             |
| At5g54630                    | At3g08100                     |
| C2H2 family                 | bZIP family                  |
| At5g46880                    | At4g18350                     |
| Homeobox family             | Homeobox family              |
| At4g37750                    | At1g50040                     |
| AP2-EREBP family            | C2C2-CO-like family          |
| At5g57520                    | At5g19200                     |
| C2H2 family                 | G2-like family               |
| At5g60690                    | At2g62460                     |
| Homeobox family             | REM family                   |
| At5g11190                    | At1g08000                     |
| AP2-EREBP family            | C2C2-Gata family             |
| At1g68200                    | At5g42630                     |
| C3H family                  | G2-like family               |
| At1g24260                    | At1g02065                     |
| MADS family                 | SBP family                   |
| At5g17430                    | At3g06740                     |
| AP2-EREBP family            | C2C3-Gata family             |
| At2g44580                    | At1g50420*                    |
| C3H family                  | GRAS family                  |
| At2g03026                    | At1g69170                     |
| MADS family                 | SBP family                   |
| At5g57390                    | At4g16141                     |
| AP2-EREBP family            | C2C4-Gata family             |
| At3g01330                    | At1g63100                     |
| bZIP family                 | GRAS family                  |
| At1g62700                    | At5g43270                     |
| NAC family                  | SBP family                   |
| At2g28050                    | At2g36930                     |
| C2C2-CO-like family         | GRAS family                  |
| At3g48160                    | At1g52890                     |
| E2F-DP family               | NAC family                   |
| At3g57150                    | At1g57100                     |
| NAC family                  | TUB family                   |
| At2g33500                    | At5g49300                     |
| C2C3-CO-like family         | C2C6-Gata family             |
| At3g10760                    | At1g16070                     |
| G2-like family              | TUB family                   |
| At4g28500                    | At2g22840                     |
| GRF family                  | C2C2-YABBY family            |
| At4g39410                     | At1g30490                     |
| REM family                  | C2C2-YABBY family            |
| At1g75240                    | Homeobox family              |
| ZF-HD family                | At1g07520                     |
| At1g46768                    | At1g25560                     |
| AP2-EREBP family            | ABI3VP1 family               |
| At1g25440                    | At5g50915                     |
| C2C2-CO-like family         | bHLH family                  |
| At4g26170                     | At2g46680                     |
| HRT family                  | Homeobox family              |
| At1g50640                    | At1g75710                     |
| C2H2 family                 | C2H2 family                  |
| At2g29660                    | At1g46480                     |
| Homeobox family             | Homeobox family              |
| At2g18350                    | At5g57150                     |
| ZF-HD family                | ZF-HD family                 |
| At3g12270                    | At1g62990                     |
| C2H2 family                 | Homeobox family              |
| At4g24660                    | At5g65410                     |
| ZF-HD family                | ZF-HD family                 |
| At3g14740                    | At1g79840                     |
| C2H2 family                 | Homeobox family              |
| At5g65410                    | Homeobox family              |
| ZF-HD family                | Homeobox family              |
| At3g47450*                  | C2H2 family                  |
| C2H2 family                 | At2g29790                     |
| Putative negative regulators | Putative negative regulators |
|------------------------------|------------------------------|
| At1g01250                    | At1g01030                     |
| ABI3VP1 family               | ABI3VP1 family               |
| At5g50915                    | At5g52610                     |
| bHLH family                 | bHLH family                  |
| At2g46680                    | At3g01220                     |
| Homeobox family             | Homeobox family              |
| At1g25560                    | At1g03800                     |
| ABI3VP1 family               | AP2-EREBP family             |
| At5g52610                    | At5g567060                    |
| bHLH family                 | bHLH family                  |
| At3g61890                    | Homeobox family              |
| Homeobox family             | Homeobox family              |
| At1g12610*                  | At1g08320                     |
| AP2-EREBP family            | bZIP family                  |
| At3g10210                     | At5g15150                     |
| Homeobox family             | Homeobox family              |
| At1g9210                     | At3g10800                     |
| AP2-EREBP family            | bZIP family                  |
| At5g47370                    | Homeobox family              |
| (Continued)
of *Haloxylon* that proposed that these C₄-type values are a result of old C₄ assimilates stored in the cotyledons during seed formation [29]. Interestingly, the assimilating shoots exhibited a δ¹³C value of -21.89±1.05‰ (mean ± SE, n = 3) and were closer to that of C₃ plants (-23‰ to -32‰). Because *Haloxylon* seeds have no endosperm, cotyledon photosynthesis provides C₃ assimilates to support early plant development [29]. *H. ammodendron* has two large and long-lived cotyledons; therefore, the δ¹³C values of young assimilating shoots (10 days of age) are

Table 4. (Continued)

| Gene Symbol | Functional Family | Gene Symbol | Functional Family | Gene Symbol | Functional Family |
|-------------|------------------|-------------|------------------|-------------|------------------|
| At2g40340   | AP2-EREBP family | At3g02380   | C2C2-CO-like family | At1g01720  | NAC family |
| At3g11020   | AP2-EREBP family | At4g39070   | C2C2-CO-like family | At1g52880  | NAC family |
| At3g15210   | AP2-EREBP family | At5g15840   | C2C2-CO-like family | At1g96490  | NAC family |
| At3g20310   | AP2-EREBP family | At5g15850   | C2C2-CO-like family | At1g77450  | NAC family |
| At3g54990   | AP2-EREBP family | At5g57660   | C2C2-CO-like family | At2g17040  | NAC family |
| At4g17490   | AP2-EREBP family | At1g29160   | C2C2-Dof family | At4g17980  | NAC family |
| At4g25470   | AP2-EREBP family | At1g51700   | C2C2-Dof family | At4g27410  | NAC family |
| At4g25490   | AP2-EREBP family | At1g64620   | C2C2-Dof family | At4g28530  | NAC family |
| At4g34410   | AP2-EREBP family | At1g69570   | C2C2-Dof family | At5g08790  | NAC family |
| At4g36900   | AP2-EREBP family | At3g21270   | C2C2-Dof family | At5g18270  | NAC family |
| At5g05410   | AP2-EREBP family | At1g27730   | C2H2 family | At5g39610*  | NAC family |
| At5g07310   | AP2-EREBP family | At3g19580   | C2H2 family | At5g61430  | NAC family |
| At5g13330   | AP2-EREBP family | At3g49930   | C2H2 family | At5g83790  | NAC family |
| At5g25190   | AP2-EREBP family | At5g12850   | C2H2 family | At1g64530  | NLP family |
| At5g44210   | AP2-EREBP family | At5g04340   | C2H2 family | At1g13260  | RAV family |
| At5g47230   | AP2-EREBP family | At5g67450   | C2H2 family | At1g68840*  | RAV family |
| At5g50080   | AP2-EREBP family | At1g26800   | C3H family | At2g36080  | RAV family |
| At5g51190   | AP2-EREBP family | At2g15580   | C3H family | At2g46870*  | RAV family |
| At5g51990   | AP2-EREBP family | At3g10910   | C3H family | At3g11580  | RAV family |
| At5g61000   | AP2-EREBP family | At3g58720   | C3H family | At3g25730  | RAV family |
| At5g61890   | AP2-EREBP family | At4g13100   | C3H family | At5g06250  | RAV family |
| At5g64750   | AP2-EREBP family | At1g67910   | CAMTA family | At1g53230  | TCP family |
| At5g20730   | ARF family       | At2g13570   | CCAAT-HAP3 family | At2g38250  | Trihelix family |
| At1g76110   | ARID family      | At1g68670   | G2-like family | At5g01380  | Trihelix family |
| At1g48880   | ARID family      | At2g05300   | G2-like family | At1g29860  | WRKY family |
| At3g48100   | ARR-B family     | At3g08030   | G2-like family | At1g62300  | WRKY family |
| At1g09530   | bHLH family      | At5g18240   | G2-like family | At1g69310  | WRKY family |
| At1g18400   | bHLH family      | At1g07520   | GRAS family | At1g80840*  | WRKY family |
| At1g22380   | bHLH family      | At1g07530   | GRAS family | At2g23320  | WRKY family |
| At1g26260   | bHLH family      | At2g29060   | GRAS family | At2g38470  | WRKY family |
| At1g73830   | bHLH family      | At2g37650   | GRAS family | At2g47260  | WRKY family |
| At2g18300   | bHLH family      | At4g17230   | GRAS family | At4g04450  | WRKY family |
| At2g20180   | bHLH family      | At4g24150   | GRF family | At4g18170  | WRKY family |
| At3g07340   | bHLH family      | At5g36660   | GRF family | At4g22070  | WRKY family |
| At3g21330   | bHLH family      | At1g26960   | Homeobox family | At4g31800  | WRKY family |
| At4g34530   | bHLH family      | At1g69780   | Homeobox family | At5g26170  | WRKY family |
| At4g36540   | bHLH family      | At2g44910   | Homeobox family | At5g46350* | WRKY family |

Ha-AS = *Haloxylon ammodendron* assimilating shoots, Ha-C = *Haloxylon ammodendron* cotyledons.

*these genes had the same developmental expression pattern in *H. ammodendron* and *Arabidopsis*.

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more negative and closer to that of C₃ due to a mixture of assimilates from C₃ (cotyledons) and C₄ photosynthesis (assimilating shoots).

In this study, we performed deep mRNA-Seq using the Illumina HiSeq 2000 platform to analyze the transcriptomes of *H. ammodendron* assimilating shoots and cotyledons, which exhibit different types of photosynthesis. Using *Arabidopsis* as the reference genome, 2959 differentially expressed genes [FDR ≤ 0.001 and abs (|log₂(Fold change)|) ≥ 1] were identified, with 1852 and 1107 transcripts being more abundant in assimilating shoots and cotyledons, respectively (see Table A in S1 File).

It was reported that the assimilating shoots of *H. aphyllum* and *H. persicum* mainly perform NADP-ME-type C₄ shuttling and a small fraction of NAD-ME-type C₄ shuttling, as shown by photosynthetic enzyme activity analysis and immunoblot analysis [29]. The mRNA-Seq analysis presented here confirmed this discovery and further showed that up-regulation occurs at the level of transcript abundance. All genes necessary for the core C₄ cycle of NADP-ME type plants were significantly up-regulated, and all genes required for the NAD-ME type of C₄ photosynthesis were also up-regulated, but to a lesser extent, in assimilating shoots compared with cotyledons (Table 1). This suggests that NADP-ME-type C₄ photosynthesis is predominant, and NAD-ME-type C₄ photosynthesis makes a small contribution to photosynthetic CO₂ fixation. Likewise, nearly all genes encoding photorespiratory proteins had lower steady-state transcriptional levels in assimilating shoots (Table 2).

We identified a list of positive and negative regulators of C₄ syndrome (Table 4). Although not all of these transcription factors are known to be components of the C₄ regulatory network, our observations suggest that at least a subset of these factors are very likely involved in vein density and the development of Kranz anatomy. Within the list, the transcription factor ATHB8 (Table 4) and the upstream gene MP (Table 3) in the auxin signal transduction pathway controlling leaf vascular development were both up-regulated. This evidence is supportive of a role for at least a subset cohort in vein density. Very recently, Wang et al. (2013) performed a genome-wide comparative analysis of developmental trajectories in Kranz (foliar leaf blade) and non-Kranz (husk leaf sheath) leaves of the C₄ plant maize to look for regulators of Kranz anatomy and identified 48 putative positive regulators, of which, 40 genes were assigned to 38 *Arabidopsis* orthologous genes [47]. Although maize has a Panicoid-type (classical NADP-ME type) Kranz anatomy and *Haloxylon* species have a Salsoloid-type Kranz anatomy, there remains high overlap between the list of positive regulators in each study. Within our list of putative positive regulators of C₄ syndrome, 11 of 38 *Arabidopsis* transcription factor-encoding genes were also significantly up-regulated in *H. ammodendron* assimilating shoots. Another two genes, AT3G54220 and AT3G13960, were increased by 1.3- and 1.9-fold (Table 6). This high overlap ratio confirms that the methods we used identified Kranz anatomy regulators.

### Table 5. SCARECROW/SHORTROOT regulatory network.

| Maize Gene ID       | Arabidopsis Gene ID | Arabidopsis ortholog | Hal-AS (RPKM) | Hal-C (RPKM) | log₂(Ha-AS/Ha-C) |
|---------------------|---------------------|----------------------|---------------|--------------|-----------------|
| GRMZM2G131516       | AT3G54220            | SCR                  | 27.26         | 20.31        | 0.42            |
| GRMZM2G132794       | AT4G37650            | SHR                  | 12.63         | 0.90         | 3.81            |
| GRMZM2G172657       | AT1G13290            | DOT5                 | 3.16          | 1.86         | 0.77            |

Ha-AS = *Haloxylon ammodendron* assimilating shoots, Ha-C = *Haloxylon ammodendron* cotyledons.

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Previous studies suggested C₄ cycle genes, trans-factors and even cis-elements were recruited from ancestral C₃ plants [50–53], but the mechanism behind is not clear. With the same genomic context of the cotyledons and assimilating shoots, this natural and developmental transition from C₃ to C₄ would be an ideal model system to study the molecular mechanism of recruitment, if whole genome sequence and genetic transformation are available.

Supporting Information

S1 Fig. *Haloxylon ammodendron* grown in its natural habitat. (TIF)

S1 File. Contains Tables A-C. Table A. 2959 differentially expressed genes [FDR ≤ 0.001 and abs ([log2(Fold change)] ≥ 1)] Table B. Up-regulated GO categories. Table C. Down-regulated GO categories. (XLSX)

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Author Contributions

Conceived and designed the experiments: YL XGZ YZ HZ. Performed the experiments: XM JZ JS. Analyzed the data: JX YL. Wrote the paper: YL HZ.
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