frontiers

Supplementary Material

Metabolic adaptation, a specialized leaf organ structure and vascular responses to diurnal N_2 fixation by *Nostoc azollae* sustain the astonishing productivity of *Azolla* ferns without nitrogen fertilizer

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1 Supplementary Data

Supplementary Data files

- The Supplementary_Data_File.pdf:

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- Two excell Files:

Supplementary Table 2. RNA seq of diel N-responses in *Azolla filiculoides.*

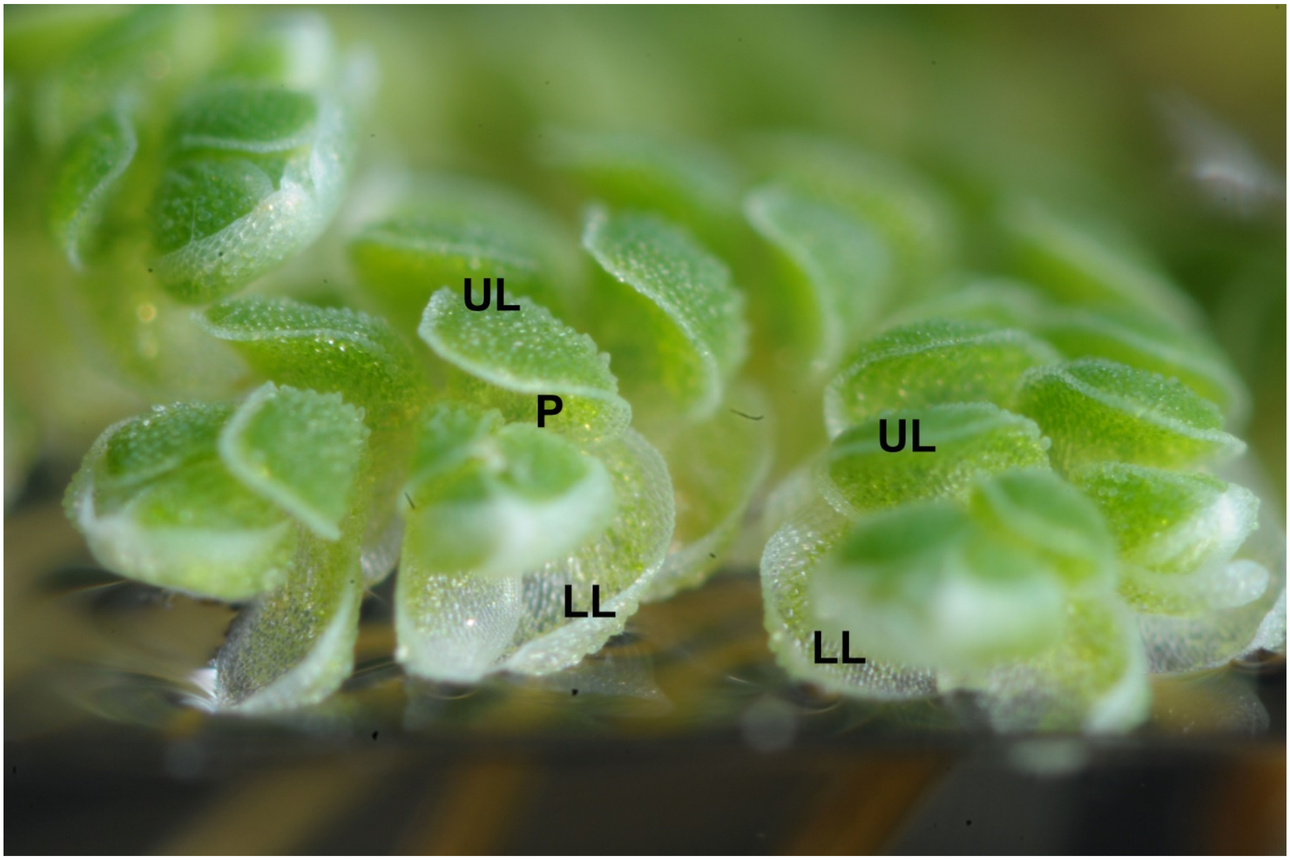
(including the following sheets: “alldata”; “summary”; “mapping_stats”; “clock genes”; “diel transcripts”; “clusters”; “cluster_ 10”; “N responsive”.)

Supplementary Table 4. Functional categories enriched when comparing *A. filculoides* grown without and with 2 mM NH_4NO_3 at the 4 time points during the diel cycle.

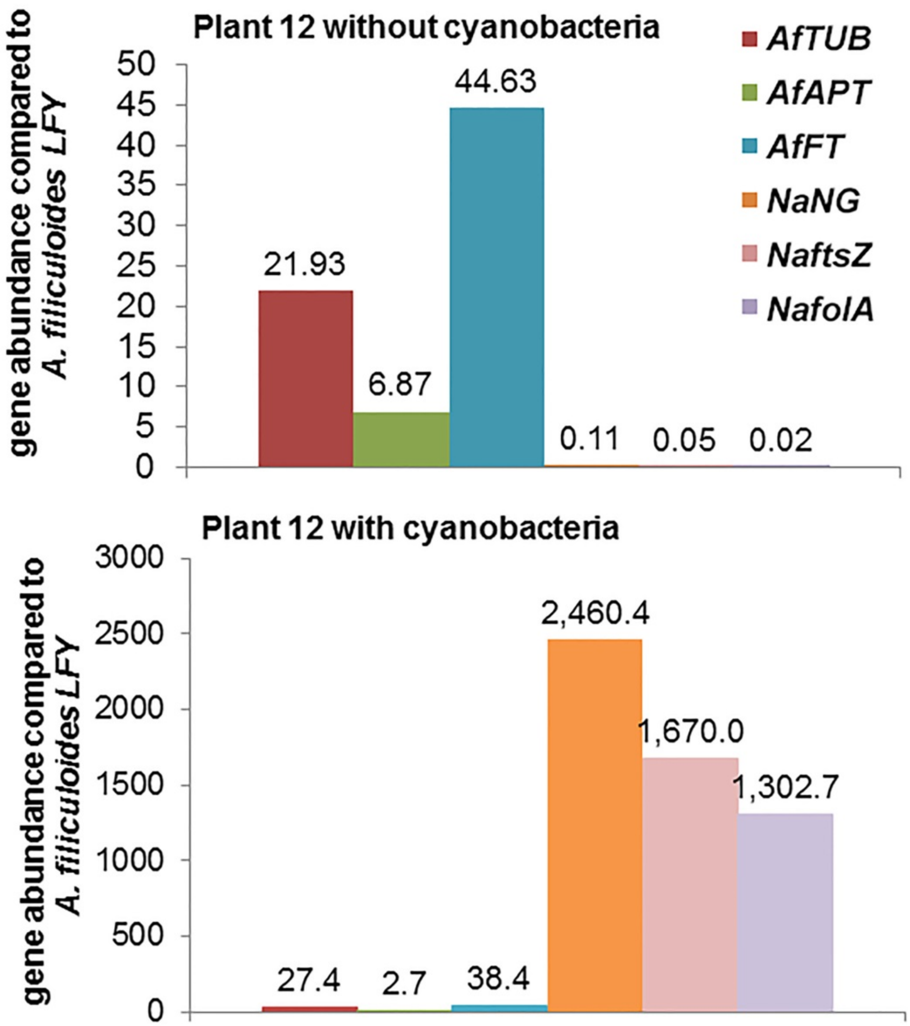
2 Supplementary Figures and Tables

2.1 Supplementary Figures

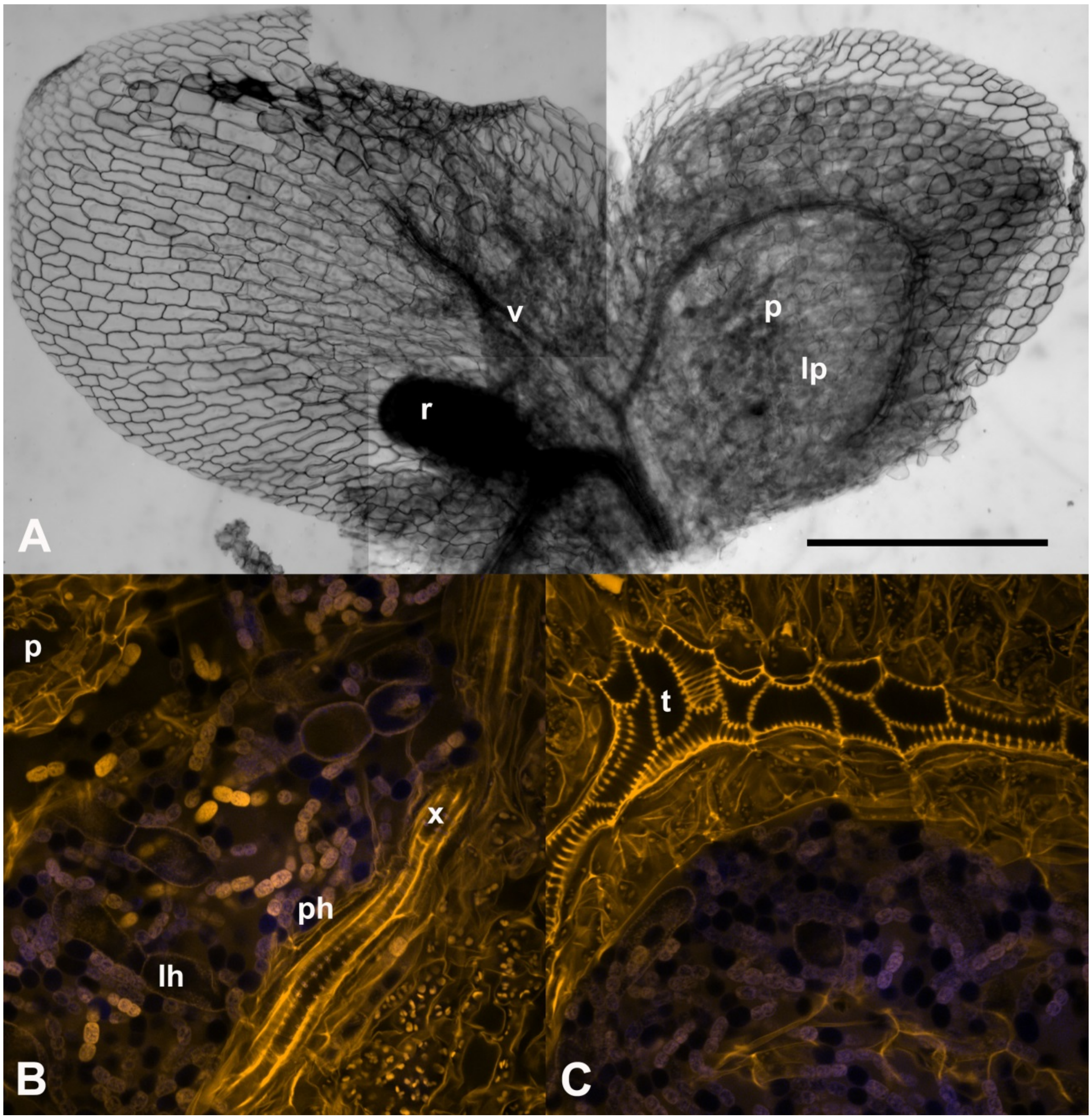
Supplementary Figure 1. Leaf-lobe arrangement in *Azolla filiculoides***.** Leaves are arranged into two rows**.** Each leaf is made of the lower (LL) and upper lobe (UL). The lower lobes resting on the water surface may hold the upper lobes in their aerial position with the dorsal ab-axial surface of the upper lobe facing light. The ventral ad-axial surface of the upper lobe bears the leaf pocket pore (P).



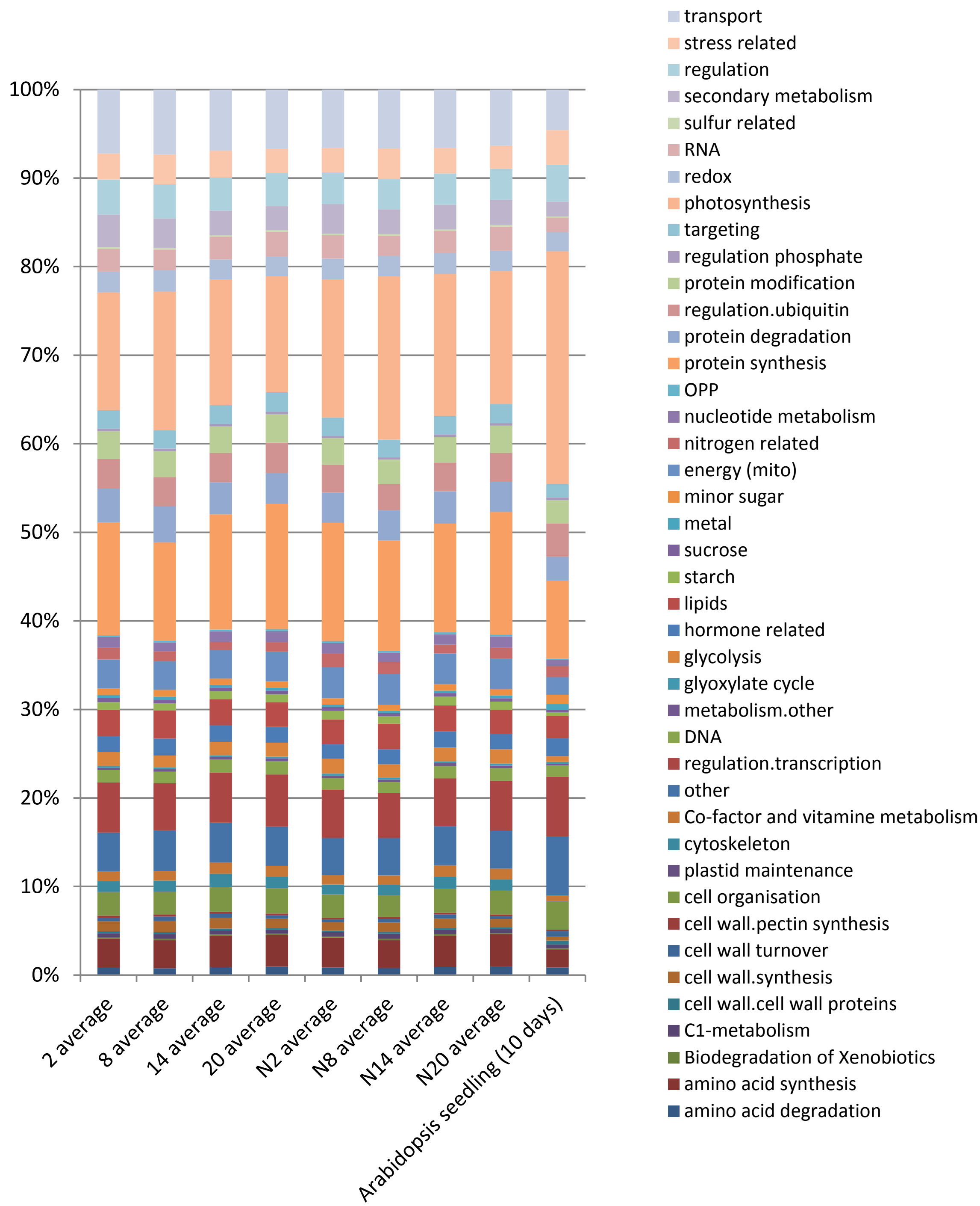
Supplementary Figure 2. Cyanobacterial DNA in *A. filiculoides* without and with cyanobacteria. DNA was extracted from clones of plant 12 without and with cyanobacteria. Subsequently, quantitative PCR was carried out to detect gene abundance from the fern (*TUBULIN* (*AfTUB*), *LEAFY* (*AfLFY*), *AfADENINE PHOSPHORIBOSYLTRANSFERASE* (*AfAPT*) and *FLOWERING LOCUS T* (*AfFT*)) and from the cyanobacteria (nitrogenase (*NaNG*); filamenting temperature-sensitive Z (*NaFtsZ*) and dihydrofolate reductase (*NafolA*). Shown are the average abundances relative to the average abundance of *LFY* from three replicate DNA extractions. Primer sequences for the amplifications are given in supplemental Table S1.



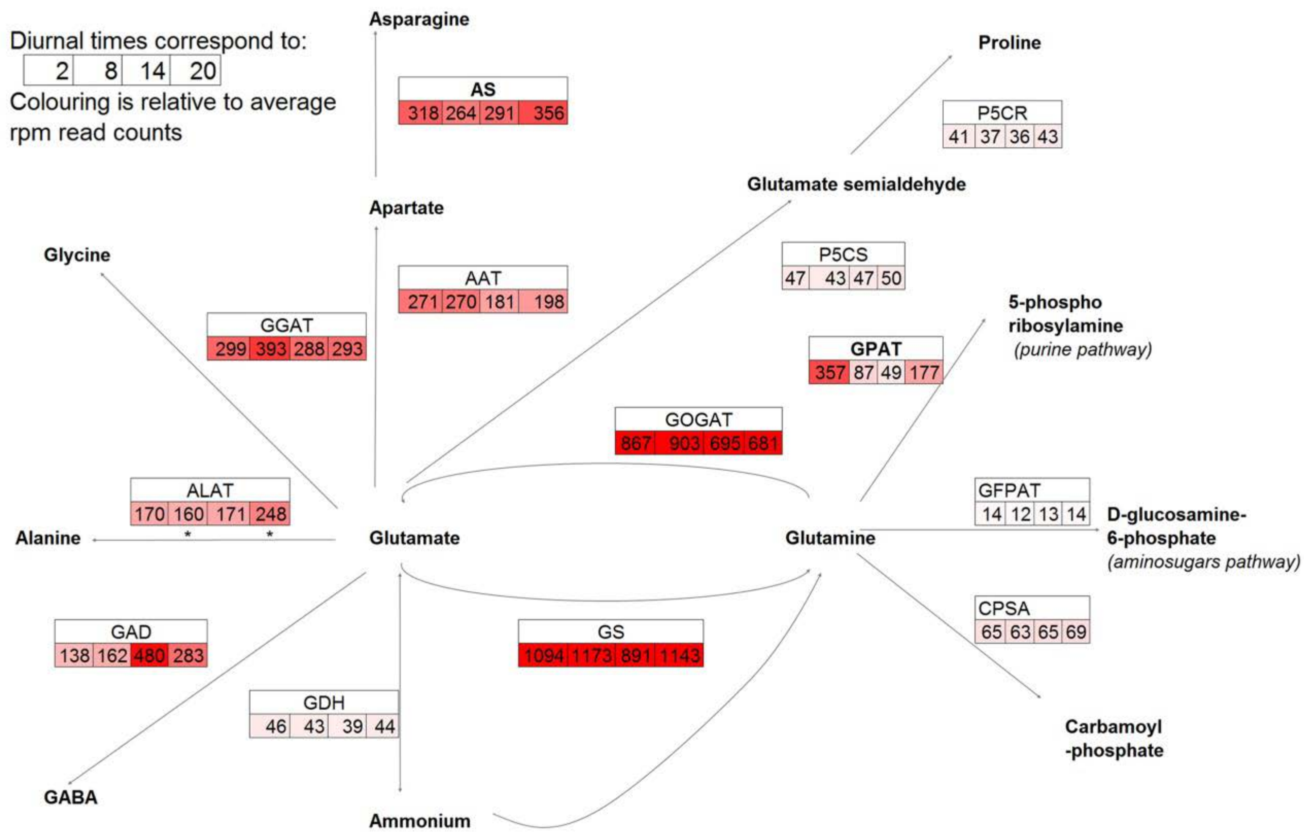
Supplementary Figure 3. Features of the *A. filiculoides* leaves. **(A)**, a single vasculature (v) branches away from the stem, then branches again to each one of the two leaf lobes: the lower leaf lobe without and the upper with leaf pocket. Only the leaf lobe with pocket has a prominent vasculature that curves around the leaf pocket (lp) with its pore (p); r, root; scale bar 200 µm. **(B)** and **(C)**, details of the interface between the leaf pocket and vasculature at the beginning and at the top curvature of the vasculature surrounding the leaf pocket respectively. lh, leaf hair; x, xylem; ph, phloem; t, tracheid cell.



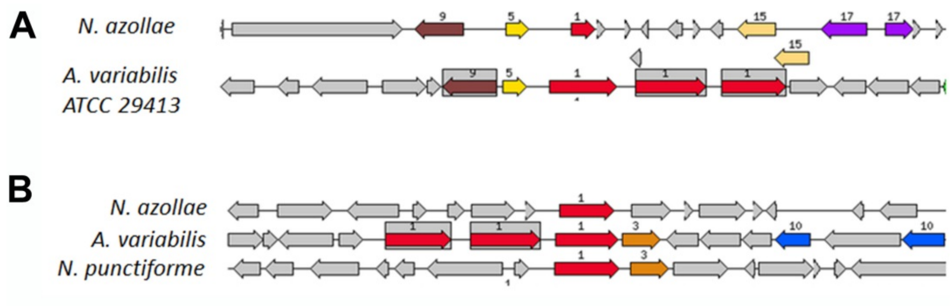
Supplementary Figure 4. Transcriptional investments in functional categories comparing *A. filiculoides* and *Arabidopsis thaliana* seedlings. Investment averages for *A. filiculoides* were for the 3 replicates at each of the time points 2, 8, 14 and 20 h either without or with N-fertilizer. Data of Arabidopsis seedlings was from (Scheible *et al.*, 2004)



Supplementary Figure 5. Diel transcript accumulation of major N-trafficking enzymes in *A. filiculoides*. Read counts are given as rpm for 2, 8, 14 and 20 h in each box; the numbers are averages from 3 biological replicates for the contig with most reads of ferns on medium without N. AAT, aspartate aminotransferase; ALAT, alanine aminotransferase; AS, asparagine synthase; CPSA, Carbamoyl phosphate synthetase; GAD, glutamic acid decarboxylase; GDH, glutamate dehydrogenase; GFPAT, glucosamine-phosphate n-acetyl transferase; GGAT, glutamate:glyoxylate aminotransferase; GOGAT, glutamate synthase; GPAT, glutamine phosphoribosylpyrophosphate amidotransferase; GS, glutamine synthase; P5CR, pyrroline-5-carboxylate reductase; P5CS, pyrroline-5-carboxylate synthetase. *marks the only transcripts with significantly changed steady states when ferns are grown with N compared to no N.



Supplementary Figure 6. The two loci encoding AmtB sequences in the *N. azollae* genome. Annotation of the *N. azollae* genome was visualized with SEED VIEWER (Overbeek *et al.* 2005 Nucleic Acids Res. 7;33(17):5691-702 DOI: 10.1093/nar/gki866). **(A)**, the locus of *N. azollae* similar to the AmtB locus in the closely related *Anabaena variabilis* ATCC 29413 strain contains truncated AmtB fragments flanked by mobile elements (15) ; 1, AmtB; 5, phosphoribosylaminoimidazole carboxylase (EC 4.1.1.21); 9, N-acetylglucosamine-6-phosphate deacetylase (EC 3.1.5.25). **(B)**, a locus entirely distinct from that of the AmtB loci of closely related bacteria contains the functional AmtB of *N. azollae*.



2.2 Supplementary Tables

Supplementary Table 1 Primers used for quantitative Reverse Transcription-PCR on RNA and for quantitative PCR on genomic DNA preparations.

Name	Azolla contig number	Forward primer	Reverse primer
Af_TUB-1	22933	CCTCCGAAAACTCTCCTTCC	GGGGGTGATCTAGCCAAAGT
Af_ACT7-5	3487	TGGGAGAACCACAGGTATTG	TCACGTCCAGCAAGATCAAG
Af_CAS-1	8998	TGGTGGATTCTCAGCAACAG	TCAGCAAACACAGATGACTGC
Af_ATPd-1	1569	AATGGTGGTAATGGCTCTCG	AGGCTTGGTGGTGGATATTG
Af_NR-4	36782	GAAGATCAACCCCTGCAAAG	ATCATCCATCCTCCTCCTTG
Af_APT-1		TAGAGATGCATGTGGGTGCAGT	AAAAGCGGTTTACCACCCAGTT
Af_FT-1		AAGAGATTTGGCAAGCTGGA	TAGCAACCACCAACAGCATC
Af_SOC1-1		ATGGGATCGTAAGGCTTCAAAA	AGCAGAGCACACAGGTCTCAAC
Af_LFY-1		GCGGCAAGAGGAAGAGATAGA	AGTGGATGTGCTCTTGCTGAA
Af_CAL_1		TTTGCATCTTTCGCTCTCTCA	CCAAGCTGCACAATGTAAGGA
Na_NifH-1*		TTCACTCCAAGGCTCAAACC	CGGAAACCGGTCAACATTAC
Na_SecA-1*		AGTATATGGCGCGGTTGAAG	AACAAAGCCTTGAGCACCAC
Na_folA-1*		ATCCTGTGATTCTCGGTCGTAAA	TGCTTCTGCAAAAATTTCTCCTC
Na_FtsZ-1*		TTCGCTATGCTGATGATGTCCTA	GCTCCTTCAATCGAGCATTCTAA

*From the *Nostoc* strain 0780 genome.

Supplementary Table 3. Pathways enriched in diel transcripts determined by Wilcoxon-Rank-Test.

Name	Elements	q-value*
cell wall.modification	24	5.99E-13
cell wall.pectin esterases	46	4.98E-08
major CHO metabolism.degradation.starch	53	3.83E-06
secondary metabolism.phenylpropanoids	74	4.47E-06
amino acid metabolism.synthesis.serine-glycine-cysteine group.glycine	8	7.88E-06
secondary metabolism.wax	36	1.11E-05
PS.lightreaction	115	1.73E-05
major CHO metabolism.synthesis.starch	45	2.48E-05
minor CHO metabolism.trehalose.TPS	12	7.69E-05
amino acid metabolism.synthesis.central amino acid metabolism.alanine	12	7.69E-05
lipid metabolism.Phospholipid synthesis	66	1.25E-04
N-metabolism.nitrate metabolism	3	1.56E-04
minor CHO metabolism.trehalose.TPP	5	1.62E-04
PS.calvin cycle	72	1.67E-04
lipid metabolism.lipid degradation.lysophospholipases	64	2.39E-04
S-assimilation	11	6.71E-04
minor CHO metabolism.raffinose family	9	0.00218605
PS.photorespiration	31	0.0027783
amino acid metabolism.synthesis.aspartate family.threonine	4	0.00281646
secondary metabolism.flavonoids	54	0.00292455
secondary metabolism.isoprenoids	112	0.00462668
lipid metabolism.glycolipid synthesis	10	0.00517521
major CHO metabolism.synthesis.sucrose	17	0.00545819
tetrapyrrole synthesis	57	0.00679658
C1-metabolism	48	0.0082183
N-metabolism.ammonia metabolism	26	0.00980327

***** q-value was corrected for multiple hypothesis testing by Benjamini Hochberg; only significantly enriched pathways are shown.

3 Supplementary list of AMT Protein sequences

>*Azolla_filiculoides*_9791_AMT2
MATSVPDWLDKGDNAWQLVAATIVGMQSMPGLVILYGSIVKKKWAVNSAFMALYAFAATLLCWVTWAYKMSFGEKLLPIWGKAGPALGI
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>*Dolichospermum_circinale*_AmtB
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GYSLSFAPGLPFIGGLQWLGLNGVGLETTGYLQGSAPAEVVSYAPTIPHQAFMIYQAMFAIITPALISGAIAERMSFRAYCLFVVLWSTFIYTPL
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>*Raphidiopsis_brookii*_AmtB
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>*Danio_rerio*_Rh_type_B
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>*Kryptolebias_marmoratus*_Rh_type_C
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