Characterization of five putative aspartate aminotransferase genes in the N₂-fixing heterocystous cyanobacterium *Anabaena* sp. strain PCC 7120

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Aspartate and glutamate are two key amino acids used in biosynthesis of many amino acids that play vital role in cellular metabolism. Aspartate aminotransferases (AspATs) are required for channelling nitrogen (N₂) between Glu and Asp in all life forms. Biochemical and genetic characterization of AspATs have been lacking in N₂-fixing cyanobacteria. In this report, five putative AspAT genes (*alr1039*, *alr2340*, *alr2765*, *alr4327* and *alr4853*) were identified in the N₂-fixing heterocystous cyanobacterium *Anabaena* sp. PCC 7120. Five recombinant C-terminal hexahistidine-tagged AspATs (AspAT-H₆) were overexpressed in *Escherichia coli* and purified to homogeneity. Biochemical analysis demonstrated that these five putative AspATs have authentic AspAT activity in vitro using aspartate as an amino donor. However, the enzymic activities of the five AspATs differed in vitro. *Alr4853*-H₆ showed the highest AspAT activity, while the enzymic activity for the other four AspATs ranged from 6.5 to 53.7 % activity compared to *Alr4853* (100 %). Genetic characterization of the five AspAT genes was also performed by inactivating each individual gene. All of the five AspAT knockout mutants exhibited reduced diazotrophic growth, and *alr4853* was further identified to be a Fox gene (requiring fixed N₂ for growth in the presence of oxygen). Four out of five P₅*aspAT-gfp* transcriptional fusions were constitutively expressed in both diazotrophic and nitrate-dependent growth conditions. Quantitative reverse transcriptase PCR showed that *alr4853* expression was increased by 2.3-fold after 24 h of N₂ deprivation. Taken together, these findings add to our understanding of the role of AspATs in N₂-fixing within heterocystous cyanobacteria.

INTRODUCTION

Aspartate aminotransferase (AspAT, EC 2.6.1.1), also known as glutamic oxaloacetic transaminase (GOT), catalyses a reversible transamination between Glu and oxaloacetate (OAA) to yield Asp and 2-oxoglutarate (OG) (Jansonius & Vincent, 1987) in a pyridoxal 5'-phosphate (PLP)-dependent manner. AspAT is ubiquitously found in animals, plants and microbes (Muriana et al., 1991) and plays the crucial role of channelling nitrogen (N₂) between Glu and Asp in all living organisms. In higher eukaryotes, AspATs exist as distinct isoforms with specific subcellular localization. For instance, the human cytosolic (GOT1) and mitochondrial (GOT2) isoenzymes are encoded by two different genes and share 49 % protein sequence identity to each other. Multiple distinct AspAT isoenzymes associated with different subcellular compartments such as the cytosol, mitochondria, peroxisome/glyoxysome and plastid/chloroplast have been reported in plants (de la Torre et al., 2014; Graindorge et al., 2010; Maeda et al., 2011; Siregar & Yunanto, 2010). Extensive studies in *Arabidopsis thaliana* identified six aspartate aminotransferase genes encoding AspATs that are targeted to different organelles: ASP1 (mitochondrial), ASP2 and ASP4 (cytosolic), ASP3 (peroxisomal), ASP5 and prokaryotic-type AspAT (plastidic). Further genetic analyses indicate that some of the AspAT isoenzymes in *Arabidopsis* may play overlapping roles in N₂ metabolism (Schultz & Coruzzi, 1995; Schultz et al., 1998). Interestingly, the prokaryotic-type AspAT (AT2G22250) shares little sequence homology with other eukaryotic AspATs from plants and animals, as well as with AspC from *Escherichia coli*, all of which belong to aminotransferase superfamily I subgroup I. (Jensen & Gu, 1996). The prokaryotic-type AspATs in

Abbreviations: AspAT, aspartate aminotransferase; GOGAT, glutamate synthase; GOT, glutamic oxaloacetic transaminase; GS, glutamine synthetase; OAA, oxaloacetate; OG, 2-oxoglutarate; PLP, pyridoxal 5'-phosphate
plants (de la Torre et al., 2006, 2007) are closely related to subgroup I; AspATs from Thermus thermophilus HB8 (TtAspAT) (Okamoto et al., 1996), Bacillus sp. YM-2 (Sung et al., 1991), Sinorhizobium meliloti (Alfano & Kahn, 1993) and Phormidium lapideum (PlAspAT) (Kim et al., 2003a, b) and the primary sequences share 42, 40, 45 and 44% identity with a prokaryotic-type AspAT (AT2G22250), respectively (E-value as 2e−95, 5e−102, 2e−106 and 5e−106, respectively).

Phor. lapideum is a non-$N_2$-fixing filamentous cyanobacterium bearing only one copy of AspAT gene encoding PIAspAT. Using L-aspartate as amino donor and OG as an amino acceptor, PIAspAT purified from cell extracts showed maximum catalytic activity at 80 °C. Further study by homology modelling using the TtAspAT structure (Protein Data Bank code: 1BJW) demonstrated that proline and salt-bridges in PIAspAT play important roles in its thermostable feature (Kim et al., 2003b). To our knowledge, PIAspAT is the only aspartate aminotransferase that has been studied in cyanobacteria. Little is known about the AspATs’ biological significance, particularly, in $N_2$-fixing cyanobacteria.

Anabaena sp. PCC 7120 (hereinafter referred to as Anabaena) is a filamentous cyanobacterium that simultaneously carries out plant-type $O_2$-producing photosynthesis and $O_2$-labile $N_2$-fixation using only air, mineralized water and sunlight. Anabaena mutants producing functional nitrogenase but unable to survive under oxic diazotrophic growth conditions have been redefined as Fox mutants, which require fixed $N_2$ for growth in the presence of oxygen. Approximately 85 Fox genes in Anabaena have been reported to date (Lechno-Yossef et al., 2011). In Anabaena, $N_2$ is first reduced to ammonia by nitrogenase in the heterocysts and is then incorporated into Glu to produce Gln by the action of glutamine synthetase (GS) (Thomas et al., 1977). Due to the lack of glutamate synthase (GOGAT) in the heterocysts, Gln must be imported from the adjacent vegetative cells into the heterocysts to sustain GS activity under diazotrophic growth conditions (Martin-Figueroa et al., 2000). Catalysing a reversible transamination between Gln/OAA and Asp/OG in both heterocysts and vegetative cells, AspAT plays an important role in maintaining metabolic homeostasis of free amino acids in Anabaena.

It has been reported that an alanine dehydrogenase gene (alr2355) mutation impairs the diazotrophic growth of Anabaena by 50% and that this gene was solely expressed in heterocysts (Pernil et al., 2010). It has been shown that alanine is also a metabolite transported from vegetative cells to heterocyst, in addition to Gln (Jüttner, 1983). Since AspAT is responsible for channeling $N_2$ between Gln and Asp, we hypothesize that the AspAT knockout mutants may also have defects on diazotrophic growth, similar to the alr2355 knockout mutant.

In this report, five putative AspATs in Anabaena were identified and characterized biochemically and genetically. Biochemical analysis demonstrated that these five putative AspATs all have authentic aminotransferase activity in vitro, although their activities varied. By inactivating each individual AspAT gene, we found that alr4853 was required for oxic diazotrophic growth in Anabaena. These findings add to our understanding of AspAT’s novel role in heterocystous filamentous cyanobacteria that are capable of simultaneous $N_2$ fixation and oxygenic photosynthesis.

**METHODS**

**Bacterial strains and growth conditions.** Bacterial strains used in this study are listed in Table 1. Escherichia coli strains TOP10 (Invitrogen) and NEB 10b (New England Biolabs) were used for routine maintenance and preparation of plasmids. BL21(DE3) (Novagen) was used for overexpression of recombinant protein. All E. coli strains were grown in lysogeny broth (LB) medium at 37 °C and 250 r.p.m., unless otherwise stated. Antibiotic selection was used when necessary at the following concentrations: 50 mg kanamycin ml$^{-1}$ or 100 mg ampicillin ml$^{-1}$ or 25 µg chloramphenicol ml$^{-1}$ (Chen et al., 2012). Anabaena and its mutant derivatives were grown in AA/8, eight times dilution of AA (Allen & Arnon, 1955) liquid medium, with (AA/8N) or without nitrate (AA/8; 212 mg NaNO$_3$ 1$^{-1}$ and 253 mg KNO$_3$ 1$^{-1}$), 10 µg spectinomycin ml$^{-1}$ and/or 10 µg erythromycin ml$^{-1}$ were used for transgenic Anabaena harbouring appropriate cargo plasmid(s). Anabaena cultures were grown under constant white light (≈ 50 µE m$^{-2}$ s$^{-1}$) at 30 °C and shaken at 100 r.p.m. in a temperature controlled Innova 44R lighted incubator (New Brunswick Scientific).

**Plasmid construction and genetic manipulations.** All plasmids used in this study are listed in Table 1. DNA used in this study was PCR amplified with Phusion high-fidelity DNA polymerase (New England Biolabs), using primers listed in Table 2. The resultant PCR products were purified with a Qiagen PCR purification kit and treated with Taq DNA polymerase to produce single 3′-A overhang products, which were cloned into pCR2.1-TOPO vector (Invitrogen). All cloned sequences were confirmed by DNA sequencing then excised and subcloned into an expression vector or integration vector using specific restriction endonucleases detailed in Table 1. The expression vector pZER618 (ampicillin resistant) (Chen et al., 2012) and its derivative pZER636 (kanamycin resistant) were used. These two vectors have same backbone with the exception of the antibiotic resistance gene. The resultant expression plasmids bearing the different target genes were transformed into BL21(DE3) for recombinant protein production. For target gene inactivation and promoter reporting in Anabaena, pZR606 (Chen et al., 2015) created by introducing multiple cloning sites into pRL2726 (Zhou & Wolk, 2002) was used. The pZR606-based cargo plasmid bearing the internal fragment of target gene was transferred into NEB 10b carrying the conjugal plasmid pRLA443 and helper plasmid pRL623 (Elhai et al., 1997). Then, fresh E. coli cells (OD$_{600}$ ~ 0.5) containing the above three plasmids were mated with wild-type Anabaena using a standard protocol (Elhai & Wolk, 1988). The correct integration loci and the complete segregation of single-crossover recombinants were verified by colony PCR. The resultant knockout mutants named SR1039, SR2340, SR2765, SR4327 and SR4853 were further examined for diazotrophic growth in air. The relative AspAT promoter activity was determined by GFP signals visualized under a fluorescent microscope. The complementation plasmid pZR1617 that bears the intact alr4853 ORF driven by the Anabaena glnK promoter in pRL2833a (Fan et al., 2005) was conjugally transferred into SR4853 for complementation testing.

**Recombinant protein expression, purification and in vitro AspAT activity assay.** BL21(DE3) cells transformed with the plasmid bearing each individual AspAT gene or Pro-α$^5$-(1-126) gene (Zhou & Kroos, 2004) were grown overnight in 2 ml LB containing appropriate concentrations of ampicillin and/or kanamycin. For recombinant protein production, these cells were further propagated in large-scale cultures in LB medium supplemented with appropriate antibiotic concentrations. The recombinant proteins were purified to near homogeneity by Ni$_2$-NTA agarose column (Qiagen) and concentrated to a final protein concentration of 1 mg ml$^{-1}$.
**Table 1.** Plasmids and bacterial strains used in this study

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<th>Plasmid or strain</th>
<th>Relevant characteristic(s)</th>
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<td>pET28b</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;; pET expression vector</td>
<td>Novagen</td>
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<td>Cm&lt;sup&gt;+&lt;/sup&gt;/Em&lt;sup&gt;+&lt;/sup&gt;; expression vector for <em>Anabaena</em></td>
<td>Fan <em>et al.</em> (2005)</td>
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<td>pZR12</td>
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Antibiotics, subcultured by adding 1 ml of overnight culture to 50 ml of fresh medium and incubated at 37 °C with rotation at 250 r.p.m. until it reached OD_{680} ~ 0.8. The recombinant protein expression was induced with 125 μM IPTG and incubated for an additional 3 h at 37 °C for production of Alr1039-H_s, Alr2765-H_s and Alr4853-H_s or at 30 °C for production of All2340-H_s and All4327-H_s. Following centrifugation, the cell pellets were washed once with PBS, resuspended in 10 ml equilibration buffer for CloneTEN TALON Metal Affinity Resins and disrupted by passage twice through a Nano DeBEE 30 electric homogenizer (BEE International) at 14 000 p.s.i. (96 600 kPa). The total cell extracts were then centrifuged at 12 000 g for 10 min at 4 °C to collect the supernatant. The His_s-tagged proteins were purified from the supernatant of the total cell extracts by a TALON Superflow Resin (Clontech) following the manufacturer’s instructions. The purified proteins were concentrated and desalted by Ultra Centrifugal Filters (Amicon 10 kDa cut-off) to achieve a final concentration of 60 mM NaCl and 40 mM imidazole in PBS. The total and purified proteins were separated on SDS-PAGE and stained with Coomassie brilliant blue R-250 as described previously (Chen et al., 2012).

The AspAT activity assay was performed based on the manufacturer’s instructions (Aspartate Aminotransferase Activity Assay kit MAK055; Sigma-Aldrich) with the following modifications. The protein concentration was determined by absorbance at 280 nm and approximately 0.5 μg desalted AspAT proteins (purified to homogeneity, Fig. 2a, b) were used for the AspAT assay. AspAT activity was determined by measuring the OD_{500} at 5 min intervals during 75 min incubation at 37 °C. Then, the OD_{500} values were converted to glutamate production (nanomoles) using a glutamate quantification standard curve. The AspAT enzymatic activity calculated was based on the most glutamate produced within 5 min (Fig. 2c). The experiments were performed in triplicate.

Growth phenotypic analysis of knockout mutants. To determine the growth phenotype upon N_{2} depletion, 1 ml of aspAT mutant or wild-type Anabaena cultures in AA/8N (OD_{700} ~ 0.2–0.3) were collected by centrifugation at 12 000 g, 25 °C for 1 min. The cell pellets were washed three times with AA/8 liquid medium and resuspended in 600 μl AA/8 medium. Seven microtitre cell suspensions were spotted onto nitrate-free AA or nitrate-containing AA agar-solidified plates and incubated at 30 °C under continuous white-light illumination (~ 50 μW m^{-2} s^{-1}). Colonies were recorded using an Epson GT-1500 scanner. To test the growth rate in the liquid medium, 100 μl of the suspended cells were mixed with 1.9 ml AA/8 or AA/8N medium, respectively, in a 12-well tissue culture plate (JET BIOFIL) with triplicates for each strain and incubated under Anabaena growth conditions. Optical density at 680 nm was determined every 24 h using Synergy 2 Multimode Microplate Reader (BioTek).

RNA extraction and quantitative reverse transcriptase (qRT)-PCR. To determine the gene expression levels of alr4853, alr4854 and 16S rRNA, 10 ml of SR4853, C4853 or wild-type Anabaena cultures grown in AA/8N or AA/8 for 24 h (OD_{700} 0.3–0.4) were collected by centrifugation at 4 750 g, 4 °C for 10 min. Total RNAs were extracted using an RNeasy Mini kit (Qiagen), according to the manufacturer’s instructions including an on-column DNase digestion step. The RT reaction (20 μl) was performed with ~ 700 ng total RNA preheated to 65 °C and specific reverse primers 4853R, 4854R, 16SR and rnpAR (Table 2) for mRNA of alr4853, alr4854, 16S rRNA (rrn16Sa) and rnpA (alr3413), respectively, using an OmniScript RT kit (Qiagen) and incubated at 37 °C for 1 h. Twenty microtitre PCR reactions containing 2 μl 50 × diluted cDNA, 1 × Q5 high-fidelity DNA polymerase buffer, 0.5 unit Q5 DNA polymerase (New England Biolabs), 0.2 mM dNTP, 1.2 μM primer pair, 1 × SYBR and 1 × ROX fluorescent dyes (Invitrogen) were run on an ABI 7900HT (Applied Biosystems); the PCR programme consisted of one cycle of 98 °C for 30 s and 40 cycles of 98 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s. The relative expression level was calculated as ratio = 2^{ΔCt(aim)/ΔCt(ref)}, where ‘aim’ is the experimental mRNA and ‘ref’ is the reference mRNA (Pfaffl, 2001) and presented as mean ± SD of the mean (n=5).
Fluorescence microscopic analysis of GFP expression upon N2 depletion. The relative promoter activity of target genes was determined by the GFP transcriptional marker. Nitrate depletion was performed as described above; GFP fluorescence signal was monitored 24 h post-inoculation. Bright-field images were captured with an exposure time of *300 ms using an Olympus AX70 upright microscope equipped with a digital camera and GFP-based fluorescence images were acquired with an exposure time of 2 s using the Olympus AX70 coupled with an SAP GFP filter (U-N31043; Olympus) to eliminate autofluorescence emitted by *Anabaena*.

Phylogenetic analysis and multiple sequence alignment. Putative AspATs in cyanobacteria were identified by BLAST search against the non-redundant protein databases at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using AT2G22250 from *Arabidopsis thaliana* as a template. Orthologous sequences from eukaryotic species were obtained by BLAST search using *E. coli* AspC as a query. Multiple sequence alignment was conducted by CLUSTAL Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). A phylogenetic tree was reconstructed using MEGA 6.06 and the evolution distance was calculated with the setting of exclude gap; bootstrap test was set 100 times (Dereeper et al., 2008).

### RESULTS

Identification of five putative AspAT genes in the *Anabaena* sp. PCC 7120 genome

Genome-wide comparative analysis of AspATs in *Anabaena* identified five genes (*alr1039*, *all2340*, *alr2765*, *all4327* and *alr4853*) encoding homologues of the aspartate

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**Table 2. Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5′→3′)*</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1039</td>
<td>TcataagACTAACCTCAGTTTCTCGGAGT</td>
<td>Primer pair amplifying <em>alr1039</em> ORF (1197 bp)</td>
</tr>
<tr>
<td>R1039</td>
<td>TcctagTTTT GCCGATCTACAAATTTTGT</td>
<td>Primer pair amplifying <em>all2340</em> ORF (1215 bp)</td>
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<tr>
<td>F2340</td>
<td>GGCATCTCAGTTTCTCGGAGT</td>
<td>Primer pair amplifying <em>alr2765</em> ORF (1212 bp)</td>
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<tr>
<td>R2340</td>
<td>TcctagTTTT TTCGAGGTTTCTCGGAGT</td>
<td>Primer pair amplifying <em>alr4327</em> ORF (1173 bp)</td>
</tr>
<tr>
<td>F2765</td>
<td>TcataagCTGTTTCTCAGTTTCTCGGAGT</td>
<td>Primer pair amplifying <em>alr4853</em> ORF (1167 bp)</td>
</tr>
<tr>
<td>R2765</td>
<td>TcctagTTTT GCCGATCTACAAATTTTGT</td>
<td>Primer pair amplifying <em>alr4853</em> ORF (1167 bp)</td>
</tr>
<tr>
<td>F4327</td>
<td>TcataagACTAACCTCAGTTTCTCGGAGT</td>
<td>Forward primer amplifying <em>alr4853</em> ORF (1167 bp) for complementation test</td>
</tr>
<tr>
<td>R4327</td>
<td>TcctagTTTT GCCGATCTACAAATTTTGT</td>
<td>Primer pair for site-directed mutagenesis of the NdeI site located in 5947–5952 nt in pRL2833a</td>
</tr>
</tbody>
</table>

*Lower case letters indicate a restriction site or mutation (SDM670F, SDM670R); underlined letters refer to the start codons of genes.*
aminotransferase family, using the prokaryotic-type AspAT from *A. thaliana* (AT2G22250) as a template and E-value 1e-20 as the cut-off.

To obtain structural and functional insights, the amino acid sequences of the five putative AspATs in *Anabaena* were compared with AspC from *E. coli* and the prokaryotic-type AspAT from *A. thaliana*. Multiple sequence alignment showed that the catalytic residue Lys236 (numbering of *E. coli* AspC), which forms a Schiff base (Malcolm & Kirsch, 1985) with the aldehyde group of PLP, is conserved among the seven aminotransferases. The residues that have been shown to interact with PLP (Tyr65, Gly102, Asn185, Asp211, Tyr214 and Arg254) or to be involved in substrate carboxyl binding (Arg37) (Inoue et al., 1991; Jäger et al., 1994) are also conserved (Fig. 1a). These results suggest that these five putative AspATs may also use PLP as a co-factor during AspAT activity.

To examine the evolutionary relationships among these enzymes from different organisms, an unrooted phylogenetic tree was reconstructed using the neighbour-joining method with 18 orthologues from *E. coli*. The phylogenetic tree reveals that these five putative AspATs may also use PLP as a co-factor during AspAT activity.

Aspartate aminotransferase activity varies among the five AspATs

To determine whether these five putative AspATs have aspartate aminotransferase activity, each of the five putative AspAT ORFs was cloned into an expression vector with a 6×-histidine tag fused to its C-terminus (Table 1). The recombinant proteins were purified to apparent homogeneity using His-tag affinity chromatography (Fig. 2a, b). The purified AspAT-H6 proteins have a calculated molecular mass about 44–46 kDa (arrowed in Fig. 2a, b), but somehow, they migrated more slowly than the 46 kDa protein marker. An *in vitro* AspAT activity assay was performed with the purified proteins.

Of the five AspATs, Alr4853-H6 showed the highest aspartate aminotransferase activity (Fig. 2c) with production of 2417 nmol glutamate min⁻¹ (mg protein)⁻¹. The enzymic activity of Alr1039-H6, All4327-H6, All2340-H6 and Alr2765-H6 was only 53.7, 30.0, 22.0 and 6.5%, in the order stated, as compared with the Alr4853-H6 (100%) (Fig. 2c). Purified Pro-α-K(1-126)-H6 from *E. coli* served as a negative control in the AspAT assay and did not exhibit any AspAT activity (data not shown). Thus, the five putative AspATs from *Anabaena* showed different degrees of aspartate aminotransferase activity *in vitro*.

Growth phenotypic analysis of the mutants reveals that *alr4853* is a Fox gene

Knockout mutants were studied to determine any possible biological function for the five AspATs. Disruption of *alr1039*, *all2340*, *alr2765*, *all4327* and *alr4853* genes was performed through single-crossover recombination (Fig. 3a). First, the internal fragment of each putative AspATs was PCR amplified and cloned into an integration vector, pZR606 (Chen et al., 2015), to produce cargo plasmids (Table 1). Following conjugal transfer of each cargo plasmid from *E. coli* into *Anabaena* and single-crossover recombination, the target gene was interrupted by producing 3' deleted and 5' deleted copies of that target gene (Fig. 3a). Then the correct integration loci and the complete segregation of the single-crossover recombinants (positive ex-conjugants) were verified by colony PCR (Fig. 3b). PCR products with expected sizes were obtained from the wild-type strain using the forward and reverse primers (Fig. 3a; Fx + Rx) outside the internal fragments of *alr1039*, *all2340*, *alr2765*, *all4327* and *alr4853* genes (Fig. 3a; bottom panel, lanes 2, 4, 6, 8 and 10, respectively), and no copies of the wild-type genes were detected in the mutant strains (Fig. 3a; top panel, lanes 2, 4, 6, 8 and 10, respectively). On the other hand, the expected PCR product sizes were observed (Fig. 3a; top panel, lanes 1, 3, 5, 7, and 9, respectively) using each gene specific forward primer Fx (Fig. 3, Table 2) and vector specific primer ZR90. Thus, the single-crossover recombinants at the target loci were successfully obtained and all the single-crossover recombinant mutants (*SR1039*, *SR2340*, *SR2765*, *SR4327* and *SR4853*) were confirmed to be completely segregated.

To test whether these mutants could grow diazotrophically, *SR1039*, *SR2340*, *SR2765*, *SR4327*, *SR4853* and wild-type *Anabaena* cultures were spotted onto AA(N) or nitrate-free (AA) agar-solidified plates and grown in air (Fig. 4a, b). No obvious growth defect was observed for *SR4327*. When compared to wild-type, *SR1039*, *SR2340* and *SR2765* mutant colonies turned yellow on day 3 (Fig. 4b; AA in middle panel) but reverted back to green on day 6 (Fig. 4b; bottom panel). Interestingly, *alr4853* knockout mutant showed severe growth defects during N₂ deprivation as colonies turned yellow and showed lack of protracted growth even at day 6 after N₂ deprivation (Fig. 4a; bottom panel). *SR1039*, *SR2340*, *SR2765*, *SR4327*, *SR4853* and wild-type *Anabaena* cultures grown in liquid medium were further tested. Little or no difference was observed among mutants and wild-type *Anabaena* grown in AA/8N (data not shown).
Fig. 1. Sequence alignment and phylogenetic tree analysis of putative AspAT proteins in *Anabaena* sp. PCC 7120.
(a) Sequence alignment of five AspAT proteins in *Anabaena* with *E. coli* and *Arabidopsis* prokaryotic-type AspATs. Consensus residues are shaded. Black, residue conserved in all the aligned sequences; pink, six out of seven sequences has the same residue; sky blue, four or five out of seven sequences has the same residue. The conserved catalytic lysine residue, the ligand and substrate binding sites and PLPs are denoted by arrows. (b) Phylogenetic tree of five putative AspATs in *Anabaena* and other homologues in *H. sapiens* [cytoplasmic (NP_002070.1, Hs C) and mitochondrial (NP_002071.2, Hs M)], *A. thaliana* [cytoplasmic (AT5G19550.1, At C), mitochondrial (AT2G30970.2, At M) and prokaryotic-type plastid (AT2G22250.2, At ptAspAT)], *Pinus pinaster* (Q5F4K8.1, Pp ptAspAT), *E. coli* (WP_001554866.1, EcAspAT), *T. thermophilus* HB8 (WP_014630167.1, TtAspAT), *Bacillus* sp. YM-2 (P23034.1, ByAspAT), *Sin. meliloti* (AAA71965.1, SmAspAT), *Phor. lapideum* (BAB86290.1, PlAspAT), *Proc. marinus* sp. AS9601 (A9601_07291, PmAspAT) and *Synechococcus* sp. WH8102 (SYNW1171). Bootstrap values (per 100 replicates) are marked on each node. Bar, evolution distance 0.1.
shown), however, all mutants grew more slowly than wild-type \textit{Anabaena} cultures in AA/8 (Fig. 4c). Consistent with
the growth phenotype on solid AA medium, SR4327 showed modest growth reduction in AA/8 liquid medium; SR1039, SR2340 and SR2765 mutants were impaired during diazotrophic growth, indicating that individual disruption of these genes caused lagged growth, but was not

essential for the survival and growth of \textit{Anabaena} under N$_2$
depleted conditions. We speculated that these four AspATs may have overlapping functions that can compensate for the loss of each other in \textit{Anabaena}. Notably, \textit{alr4853} knockout mutant showed little or no growth (Fig. 4a, c), which suggested that \textit{alr4853} is essential for the survival and growth of \textit{Anabaena} under N$_2$
depleted conditions.

The average number of vegetative cells between heterocysts was $11.81 \pm 3.76$, $11.99 \pm 3.89$ and $12.63 \pm 4.20$ for SR4853, C4853 and wild-type \textit{Anabaena} strains, respectively, based

Fig. 2. Aminotransferase activity assay of the five recombinant putative AspATs from \textit{Anabaena} sp. PCC 7120. (a) Expression and purification of \textit{Alr4853} and Pro-$\sigma^K$. M, ColourPlus Prestained Protein Marker in kDa (New England Biolabs); lane 1, cell extract (CE) of E. coli cells bearing empty vector pZR618 serving as a negative expression control; lane 2, CE of E4853 producing Alr4853-H$_6$; lane 3, purified Alr4853-H$_6$; lane 4, purified his-tagged protein, Pro-$\sigma^K$(1-126)-H$_6$, serves as a negative control in the assay for the AspATs’ activity. His-tagged proteins with the expected molecular mass are highlighted by arrows. (b) Expression and purification of \textit{Alr1039}, All2340, Alr2765, and All4327. M, protein marker (kDa); lanes 1, 2, 3 and 4 are total CE (top) and purified (bottom) All2340-H$_6$, Alr1039-H$_6$, Alr2765-H$_6$ and All4327-H$_6$, respectively. His-tagged proteins with the expected molecular mass are highlighted by arrows. (c) The amount of glutamate generated by AspAT in vitro assay varied among the five putative AspATs in \textit{Anabaena}. Approximately 0.5 mg purified AspAT protein was used for each assay and the results are the mean of three replicates, with error bars representing the SD.
by performing a complementation experiment. The \textit{alr4853} coding region was PCR amplified and cloned into an \textit{Anabaena} expression vector pZXR670 (Table 1), a derivative of pKL2833a (Fan et al., 2005), to produce pZRX670 (Table 1) that was then conjugally transferred into SR4853 to generate a complementation strain designated C4853. Unlike SR4853, C4853 showed appreciable growth in the oxic diazotrophic conditions (Fig. 4a, c), although growth was slower than the wild-type, which suggests that \textit{alr4853} cloned in pZXR670 was able to complement the mutant SR4853.

The qRT-PCR data combined with the complementation result support that the diazotrophic growth defect of SR4853 was unlikely due to a polar effect on \textit{alr4854}. Therefore, we conclude that \textit{alr4853} is a Fox gene coding for aspartate aminotransferase required for oxic diazotrophic growth.

\textbf{Individual aspAT promoter activity does not exhibit substantial change upon N\textsubscript{2} deprivation}

The relative promoter activity of target genes can be examined by the GFP transcriptional marker in SR1039, SR2340, SR2765, SR4327, SR4853 and C4853. GFP expression from P\textsubscript{alr1039}gfP, P\textsubscript{alr2340}gfP, P\textsubscript{alr2765}gfP, P\textsubscript{alr4327}gfP and P\textsubscript{alr4853}gfP was monitored under a fluorescence microscope after 24 h growth in medium containing nitrate. P\textsubscript{alr4327}gfP exhibited the strongest signal, P\textsubscript{alr1039}gfP, P\textsubscript{alr2765}gfP and P\textsubscript{alr4853}gfP showed similar modest levels and P\textsubscript{alr2340}gfP expression was undetectable in our experimental conditions.

To further address if the expression of \textit{alr4854} in diazotrophic growth conditions is affected in SR4853, additional qRT-PCR reactions using the constitutively expressed gene \textit{rnpA} (\textit{alr3413}) as an internal reference (Pinto et al., 2012) were performed. After 24 h of \textit{N\textsubscript{2}} deprivation, the expression levels of \textit{alr4854} in SR4853 and in C4853 were nearly identical; these were \textasciitilde{} 1.62-fold and 1.65-fold greater, respectively (data not shown) than in the wild-type, which indicated that plasmid insertional knockout of \textit{alr4853} did not inhibit \textit{alr4854} expression under non-diazotrophic conditions.

To address whether plasmid insertional inactivation of \textit{alr4853} causes a polar effect on the expression of a downstream gene \textit{alr4854}, qRT-PCR was performed with SR4853 mutants grown for 24 h, respectively, in diazotrophic conditions (AA/8) and non-diazotrophic conditions (AA/8N). The qRT-PCR data with 16S rRNA gene (\textit{rrn16Sa}) as reference revealed that \textit{alr4854} expression in SR4853 mutant grown in non-diazotrophic condition was \textasciitilde{} 3-fold greater (data not shown) than in the wild-type, which indicated that plasmid insertional knockout of \textit{alr4853} did not inhibit \textit{alr4854} expression under non-diazotrophic conditions.

To further address if the expression of \textit{alr4854} in diazotrophic growth conditions is affected in SR4853, additional qRT-PCR reactions using the constitutively expressed gene \textit{rnpA} (\textit{alr3413}) as an internal reference (Pinto et al., 2012) were performed. After 24 h of \textit{N\textsubscript{2}} deprivation, the expression levels of \textit{alr4854} in SR4853 and in C4853 were nearly identical; these were \textasciitilde{} 1.62-fold and 1.65-fold greater, respectively (data not shown) than in the wild-type, which indicated that plasmid insertional knockout of \textit{alr4853} did not inhibit \textit{alr4854} expression under non-diazotrophic conditions.

on counting 100 individual filaments for each sample after 24 h \textit{N\textsubscript{2}} deprivation. We concluded that inactivation of \textit{alr4853} has no effects on heterocyst frequency, although the \textit{alr4853} knockout mutant showed a severe growth defect during oxic diazotrophic growth (Fig. 4a, c).

Fig. 4. Characterization of the \textit{Anabaena} AspAT mutant phenotype under deprivation of fixed \textit{N\textsubscript{2}}. (a) Identification of \textit{alr4853} as a Fox gene and its complementation (AA, d6). Cultures from two independent isolates of \textit{alr4853} knockout mutant (SR4853), \textit{alr4853} complemented strain (C4853) and wild-type \textit{Anabaena} (WT7120) strain were spotted onto AA(N) (top) or AA (bottom) plates and grown in air for 0 and 6 days (d). (b) Growth phenotypes of SR1039, SR2340, SR2765 and SR4327 when deprived of fixed \textit{N\textsubscript{2}}. Cultures of mutant strains and WT7120 were spotted onto AA(N) (top) or AA (bottom) plates and grown in air for 0, 3 and 6 days. Note: the culture on day 6 AA(N) was overgrown and the blue colour was an artificial effect caused by the scanner. (c) Growth curve of wild-type \textit{Anabaena} (WT7120) and mutant strains grown in AA/8 without antibiotic. Chlorophyll content was determined by \textit{OD}_{680} with error bars representing SD of the mean (n=3).
(Fig. 5, left panel and data not shown). After combined N₂ deprivation for 24 and 48 h, GFP expression was observed in both heterocysts and vegetative cells from SR1039, SR2765, SR4327 and SR4853 (Fig. 5, right panel and data not shown). Nitrogen deprivation did not influence AspAT promoter activity. Notably, ectopic alr4853 expression in the complemented strain C4853 resulted in GFP (P<sub>alr4853-gfp</sub>) signal in all cell types, similar to SR4853; however, the heterocysts showed slightly stronger GFP signal than the vegetative cells upon N₂ starvation. Nonetheless, our data indicate that SR1039, SR2765, SR4327 and SR4853 genes were expressed in both heterocysts and vegetative cells and N₂ deprivation had no substantial influence on the expression level of each individual AspAT.

**DISCUSSION**

Of the five putative AspAT genes identified in the *Anabaena* genome, all of the knockout mutants had no detectable effect on the growth of *Anabaena* with a combined N₂ source, such as nitrate. However, each individual knockout mutant showed reduced diazotrophic growth. Except for alr4853, the other four AspATs (*alr1039, alr2340, alr2765* and *alr4327*) were not essential for oxic diazotrophic growth, indicating an overlapping function that could be compensated partially by other AspAT isoforms in *Anabaena*. Alr4853, an AspAT sharing 42% identity (E-value is 6 × 10⁻⁹⁹) with *Arabidopsis* prokaryotic-type AspAT (AT2G22520), exhibited the highest AspAT activity *in vitro* (Fig. 2c), which was ~2–15 fold higher than the activity of the other four AspATs.

The heterocysts in the complemented strain C4853 showed somewhat stronger GFP signal than the vegetative cells (Fig. 5), which might be the result of higher levels of *alr4853* expression in heterocysts compared to vegetative cells, although this will need to be confirmed by future work. Our qRT-PCR data showed that expression level of *alr4853* was 36.23 ± 8.7-fold higher than *alr4854* in the wild-type grown in AA/8N. The different expression pattern of these two genes was also found in previous RNaseq data, showing that the mRNA level of *alr4853* was 6.4-fold higher (at 0 h of N₂ deprivation) or 7.5-fold higher (after 24 h of N₂ deprivation) than that of *alr4854* (Flaherty et al., 2011). After 24 h of N₂ deprivation, we also observed 2.32 ± 0.53 and 0.92 ± 0.08 fold changes for *alr4853* and *alr4854* expressions, respectively. Unlike *alr4853*, the expression of *alr4854* had no response to 24 h N₂ deprivation. Taken together, these data indicate that expression of *alr4854* is not regulated by the *alr4853* promoter and the qRT-PCR data combined with the complementation result strongly support the conclusion that *alr4853* is a Fox gene required for *Anabaena*’s oxic diazotrophic growth.

Alr4853 is a major AspAT expressed in all cell types of *Anabaena*, but is especially important for oxic N₂ fixation, because it channels the two important N₂-containing metabolites Glu and Asp. Glu serves as a precursor for N₂ acquisition in the GS–GOGAT pathway and Asp, along with Arg, is the exclusive residue in cyanophycin [multi-L-arginyl-poly(L-aspartic acid)], the key N₂-storage molecule in the heterocyst (Picossi et al., 2004). Asp and Glu also serve as precursors for the biosynthesis of a series of amino acids, including Gln, Lys, Thr, Met, Ile and Arg (Lehninger et al., 2000; Miesak & Coruzzi, 2002). Thus, AspAT may play an important role in maintaining metabolic homeostasis of free amino acids in *Anabaena*. When a major AspAT, Alr4853, is disabled the Glu, Gln, Asp and Arg levels could all be affected, which in turn influences N₂ fixation, although such changes did not affect heterocyst patterning. The average number of vegetative cells between heterocysts of SR4853, C4853 and wild-type *Anabaena* strains was nearly identical. Thus, inactivation of *alr4853* did not affect heterocyst formation but impaired oxic diazotrophic growth.

Alr4853 shares 42 (E-value is 6 × 10⁻⁹⁹) and 40% identity (E-value is 2 × 10⁻⁹⁸) with prokaryotic-type AspATs in *Arabidopsis* and *Nicotiana benthamiana*, respectively. Both of these enzymes have recently been shown to be bifunctional enzymes, displaying both AspAT and prephenate
aminotransferase (PPA–AT) activity (de la Torre et al., 2014; Gräindorge et al., 2010). PPA–AT catalyses the reversible transamination between Glu or Asp and prephenate to yield 2-oxoglutarate or oxaloacetate and arogenate. Arogenate is the precursor for the biosynthesis of aromatic amino acids in the plants. Thus, Ahr4853 might also have a PPA–AT activity and be involved in aromatic amino acid metabolism in N2-fixing cyanobacteria. Future studies on aromatic amino acid metabolism are desirable to unveil the underlying mechanisms of diazotrophic growth defect in these AspAT mutants.

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REFERENCES


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