Functional characterisation of bicarbonate transporters from the cyanobacterial SbtA2 family and subsequent expression in tobacco

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Running title: SbtA2 transporter function and expression in tobacco

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Highlight

SbtA2 bicarbonate transporters from some marine α-cyanobacteria are high flux

transporters with an unusual chloride-dependence and show intermediate uptake

affinity when expressed in *E. coli*. Initial attempts failed to demonstrate enhanced

photosynthesis when directed to tobacco chloroplasts.

Abstract

Cyanobacteria rely on bicarbonate (HCO_3^-) as the primary inorganic carbon (Ci)

source for photosynthesis in aquatic environments. To use of this Ci source,

cyanobacteria employ CO_2 concentrating mechanisms (CCMs) that elevate

cytoplasmic HCO_3^- via plasma membrane transporters, enhancing carboxylation by

carboxysomal Rubisco. The sodium-dependent SbtA1 transporter family is well-

characterized in freshwater cyanobacteria, but the related SbtA2 family, prevalent in

marine α-cyanobacteria, remains uncharacterised. Here, we report functional

characterisation of SbtA2 homologues from marine *Synechococcus* spp., which

exhibit high Ci uptake flux with apparent chloride dependence and intermediate

HCO_3^- affinity (Km ≈ 150 µM), when assessed in *E. coli*. SbtA2 achieved internal Ci

accumulation up to 24 mM within 30 seconds. Co-expression with the putative

regulator SbtB2 reduced uptake activity, suggesting a regulatory role for this protein.

These findings indicate that SbtA2 transporters contribute significantly to carbon

acquisition in marine cyanobacteria. Given potential to enhance CO_2 supply to

Rubisco in C_3 plants, we targeted SbtA2 to the tobacco chloroplast inner envelope

membrane; however, this did not improve photosynthesis or growth. Our results

highlight the functional diversity of cyanobacterial Ci transporters and suggest that

additional components may be required for effective transfer of such systems into

plant chloroplasts.

Abbreviations

CA   carbonic anhydrase

CAfree   specialized *E. coli* strain that lacks CAs

CCM CO_2-concentrating mechanism

CO_2 carbon dioxide

Ci   inorganic carbon

34	C_{int}	intercellular CO_2
35	HCO_3^-	bicarbonate
36	IEM	inner envelope membrane
37	IPTG	isopropyl β-D-1-thiogalactopyranoside
38	LB	Luria-Bertani
39	SbtA	sodium-dependent bicarbonate transporter

Introduction

Cyanobacteria are single-celled, photosynthetic organisms of global ecological
importance due to their significant contribution to CO_2 fixation into the biosphere (Field
et al., 1998; Flombaum *et al.*, 2013). Their acquisition of carbon is primarily driven by
the enzyme Rubisco, which fixes CO_2 as part of the Calvin -Benson -Bassham (CBB)
cycle for use in cellular metabolism (Badger *et al.*, 2002; Nguyen *et al.*, 2025a). While
CO_2 is readily able to permeate cellular membranes, the most abundant form of
inorganic carbon (Ci; CO_2, CO_3^{2-}, and HCO_3^-) available for photosynthesis in aquatic
environments is the relatively membrane-impermeable bicarbonate ion (HCO_3^-;
Falkowski and Raven, 2007). Cyanobacteria and other aquatic photoautotrophs have
evolved CCMs to enable the use of this Ci source as a means to elevate CO_2 around
Rubisco and maximise carboxylation (Badger *et al.*, 2006; Price *et al.*, 2008).
Cyanobacterial CCMs are highly efficient bipartite systems, utilizing Ci transporters to
enable active accumulation of HCO_3^- in the cytoplasm, and HCO_3^--permeable micro-
compartments called carboxysomes in which Rubisco is encapsulated (Rae *et al.*,
2013a; Nguyen *et al.*, 2025b). Within carboxysomes, a specialized carboxysomal
carbonic anhydrase (CA) equilibrates HCO_3^- with CO_2 (Reinhold *et al.*, 1991; Pulsford
et al., 2024), thus increasing CO_2 available for carboxylation (Long *et al.*, 2021; Rae
et al., 2013b).

There are two main classifications of cyanobacteria based on the form of
Rubisco they contain (Badger and Bek, 2008). Using Form-IB Rubisco, β-
cyanobacteria are frequently found in smaller freshwater bodies and estuarine
habitats, whilst the more abundant α-cyanobacteria (containing Form-IA Rubisco) are
widespread in both oceanic and freshwater environments (Badger *et al.*, 2002;
Cabello-Yeves *et al.*, 2022). Some α-cyanobacteria are referred to as transition strains

and show evidence of genetic inheritance of CCM components by horizontal transfer,

often derived from β-cyanobacteria (Rae *et al.*, 2011).

The α-cyanobacteria are thought to have a significant impact on global CO_2

acquisition into the biosphere due to their relative abundance (Field *et al.*, 1998;

Cabello-Yeves *et al.*, 2022) and high rate of CO_2 fixation (Bar-On and Milo, 2019).

Estimates suggest as much as 25% of marine primary productivity can be attributed

to the predominant α-cyanobacterial genera, marine *Synechococcus* spp. and

Prochlorococcus spp. (Flombaum *et al.*, 2013).

Underpinning CCM function across these phylogenies is a suite of Ci transport

systems that support cellular HCO_3^- elevation. Currently, there are five Ci transport

systems involved in cyanobacterial CCMs that have been characterised: two CO_2-to-

HCO_3^- conversion complexes bound to thylakoid membranes, and three families of

plasma membrane HCO_3^- transporters (Price *et al.*, 2008; Long *et al.*, 2016). The

HCO_3^- transporters include the constitutively expressed, high affinity/low flux ABC-type

transporter BCT1 (Omata *et al.*, 1999; Rottet *et al.*, 2024), the inducible high-

affinity/low flux transporter SbtA1 (Shibata *et al.*, 2002; Du *et al.*, 2014), and the low

affinity/medium-high flux transporter BicA (Price *et al.*, 2004). Directional conversion

of CO_2 -to -HCO_3^- is performed by specialized Type 1 NDH complexes on the thylakoid

membranes that use the electron transport chain and specialised CupA/CupB (Walker

et al., 2024; Maeda *et al.*, 2002). Both HCO_3^- and CO_2 uptake systems are found in

most cyanobacterial species, with the exception of *Prochlorococcus* spp., which

generally possess only distant homologues of BicA and SbtA (Rae *et al.*, 2011).

Human nutrition relies heavily on C_3 crop plants (Salesse-Smith *et al.*, 2025).

However, under current atmospheric conditions, C_3 plants have inefficient

photosynthetic processes compared to most other photosynthetic organisms (for

instance C_4 plants and phytoplankton) due to a lack of a CCM. CCMs act to increase

the concentration of CO_2 at the site of Rubisco, the primary enzyme responsible for

carboxylation in photosynthetic organisms (Erb and Zarzycki, 2018). One approach to

improve the photosynthetic efficiency of these plants is to transplant a CCM from

cyanobacteria into the chloroplast in order to improve CO_2 delivery to Rubisco (Price

et al., 2011). However, there is a knowledge gap in the mechanistic and regulatory

understanding of cyanobacterial HCO_3^- transporters that needs to be filled before such

an ambitious goal can be achieved (Rottet *et al.*, 2021). Modelling indicates that

incorporating a carboxysome in the chloroplast stroma, and expression of HCO_3^-

transporters in the inner envelope membrane (IEM) of the chloroplast, could increase plant productivity by 36-60% (McGrath and Long, 2014). The expression of a HCO_3^- transporter in the chloroplast IEM alone is also likely to improve photosynthetic efficiency in plants by enhancing net CO_2 supply to Rubisco (Price *et al.*, 2011; McGrath and Long, 2014). HCO_3^- transport proteins have been expressed in the chloroplastic IEM, however there has been little evidence of active HCO_3^- uptake in the chloroplast so far (Rottet *et al.*, 2024; Rottet *et al.*, 2021; Forster *et al.*, 2023; Nölke *et al.*, 2019; Atkinson *et al.*, 2016; Pengelly *et al.*, 2014). While it is unknown as to why this is the case, it is possibly due to a lack of activation mechanisms for foreign transport proteins or incorrect conditions to drive transporter function (Rottet *et al.*, 2021). For instance, for Na^+-dependent HCO_3^- transporters such as SbtA1 and BicA, the Na^+ concentration in the cytoplasm (and therefore the cytoplasm:chloroplast stromal Na^+ gradient) may not be optimal for function *in planta*. This demonstrates the need to further understand the function of these HCO_3^- transporters and identify different classes of transporters that could be adapted for use in C_3 chloroplasts.

In this study, we focus on the previously undescribed HCO_3^- transporter gene family *sbtA2* (sodium-dependent bicarbonate transporter A2) which is largely found in the α-cyanobacteria (Figure 1; Cabello-Yeves *et al.*, 2022; Rae *et al.*, 2011) and has distant protein homology to the previously characterized sodium-dependent *sbtA1* family (Figure S1; Price *et al.*, 2008; Rae *et al.*, 2011; Du *et al.*, 2014).(Price *et al.*, 2008; Rae *et al.*, 2011; Du *et al.*, 2014). Similar to *sbtA1*, the *sbtA2* gene is typically co-located in cyanobacterial genomes with *sbtB2* (Figure S5A-D), coding for a putative regulatory protein SbtB2, homologous to the regulator SbtB1 (Du *et al.*, 2014; Förster *et al.*, 2023; Kaczmarski *et al.*, 2019).

Here we characterize the function of several members of the SbtA2 family from α-cyanobacteria in a heterologous bacterial expression system. Genes encoding SbtA2 and SbtB2 from marine α-cyanobacterial species were cloned individually, or in tandem, and expressed in a high-CO_2-requiring (HCR) *E. coli* strain, CAfree (Rottet *et al.*, 2024; Forster *et al.*, 2023; Desmarais *et al.*, 2019). SbtA2 homologues that rescued CAfree at ambient CO_2 were further characterized by measuring their HCO_3^- uptake rates, counter-ion dependency and affinity for HCO_3^-. We also assessed the hypothesis that their associated SbtB2 protein acts as a regulator of SbtA2 function in this system. Our results show that like SbtA1:B1, SbtB2 appears to be capable of limiting the activity of its SbtA2 counterpart in *E. coli*. We provide focused analysis of

135 SbtA2 function from *Synechococcus* WH5701 (hereafter SbtA2-WH5701), revealing a
136 potentially different function from its SbtA1 homologue. We then expressed SbtA2 in
137 the chloroplast IEM of *Nicotiana tabacum* to assess its capacity to improve the
138 photosynthetic efficiency of the plants by increasing CO_2 supply to Rubisco.
139

Materials and Methods

Molecular cloning of SbtA2 genes

Genes   encoding   *sbtAB2*   from   *Synechococcus*   WH5701   (accession
WP_006170362.1,   WP_006170363.1),   *Synechococcus*   RCC307   (NBQ36408.1,
CAK27720.1) and *Synechococcus* CB0205 (WP_010316543.1, WP_010316542.1),
Prochlorococcus   MIT0701   (WP_036914441.1,   WP_036914438.1)   and
Prochlorococcus marinus MIT9313 (WP_011130583.1, WP_011130582.1), were
synthesized by Genscript (USA) and cloned individually or in tandem under the control
of a lac inducible promoter in vectors pCk1 (Kanamycin resistance) or pCsA
(Spectinomycin resistance), using type-IIS restriction enzyme cloning that use key
features of Golden Gate cloning and Loop Assembly (Pollak *et al.*, 2019; Engler *et al.*,
2014). In the absence of a suitable antibody for SbtA2, despite numerous attempts to
make one, internal epitope tags were introduced to SbtA2 in the cytoplasmic loop
between membrane helices five and six of SbtA2 (Table S3) using the same cloning
methods.
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CAfree growth complementation assay

A double CA knockout strain of *E. coli* (CAfree; *Δcan ΔcynT*) described by
Desmarais et al. (2019), which is unable to grow at air levels of CO_2, was used to
screen for HCO_3^- transport. Cultures of CAfree expressing members of SbtA2 were
grown in Luria-Bertani (LB) broth at 37°C in air supplemented with 4% CO_2 for 18
hours, then diluted 1:5 and grown for an additional hour. The density of the cultures
was standardized based on their absorbance (OD_{600} = 0.2) and serially diluted before
spotting 10 µL onto LB containing 1.5% agar and appropriate antibiotics (kanamycin
– 50 µg/mL or spectinomycin – 100 µg/mL) and 100 µM isopropyl β-D-1-
thiogalactopyranoside (IPTG). Plates were incubated overnight at 37°C in air levels
(0.04%) and elevated (4%) CO_2 to test for complementation.

CAfree growth assay

Cultures of CAfree expressing each form of SbtA2 were incubated in LB
supplemented with antibiotics (as for the complementation assay) with shaking for 18
h at 37 °C in CO_2-supplemented air (4% CO_2). These were diluted 1:5 and grown for
approximately 60-90 minutes to achieve log phase growth. Cultures were diluted 1:10
into final culture volumes of 200 µL and were grown at 37 °C in 96 well plates in a
plate reader (BMG Labtech) under air and CO_2-supplemented-air (4% CO_2) conditions
in LB supplemented with antibiotics and IPTG. Growth was measured by taking
automated absorbance (OD_{600}) readings at 10-minute intervals for 20 h.

Inorganic carbon uptake assay

Inorganic carbon uptake was examined in *E. coli* (DH5α strain) expressing
sbtAB2 gene constructs in pCk1 or pCsA (and their derivatives) along with appropriate
controls. Starter cultures were grown overnight from a standard glycerol stock in LB,
this culture was then diluted 1:5 in LB and grown at 37°C for approximately one hour
until absorbance readings (OD_{600}) reached 0.6. Expression was induced by the
addition of IPTG to a final concentration of 1 mM, and cultures were grown for an
additional 2.5 h at 37°C with shaking. Culture density was measured, and cell volumes
adjusted to a standard cell density for each experiment with an approximate OD_{600}
1.0-1.2. Cells (~2 mL) were depleted of Ci by washing twice with 1 mL of CO_2-free
buffer (20 mM BisTrisPropane-H_2SO_4, pH 7.5, salts as required; sparged with N_2 gas)
after centrifugation (20,238 × *g* for 30 s). $NaH^{14}CO_3$ (~60 MBq/mmol) was added to
the cells to a final concentration of 0.5 mM and 200 µL of this suspension was
overlayed onto 50 µL silicone oil mix AR200:20 3.5:4, above a 20 µL kill solution (3 M
sodium hydroxide, 50% methanol) in a 400 µL polypropylene tube (Beckman Coulter).
After 30 second incubation the $NaH^{14}CO_3$-cell mix was immediately centrifuged for 20
seconds to separate the cells with incorporated ^{14}C from free $NaH^{14}CO_3$ which remains
in the supernatant. Tubes were immediately placed in an aluminium block on dry ice,
before clipping off the base of the tube containing the cell pellet into scintillation vials
containing 400 µL 2 M NaOH. Vials were vortexed well and 2 mL UltimaGold
Scintillation cocktail (Perkin Elmer) added before being counted using a Hidex
Scintillation counter for 2 minutes. Variations of assay buffer pH, including the
concentration of Ci, acid used to pH the buffer, ions added to the uptake buffer and
how the $NaH^{14}CO_3$ stock was diluted were also used.

To characterise the kinetics of SbtA2 transporters, Ci uptake rates were
measured over a range of Ci concentrations to cells expressing each SbtA2. The

resulting data were analysed using Michalis-Menten non-linear regression with the
statistical fit to assess if the transport kinetics of SbtA2 meets the criteria for active
transport.

Determination of SbtA2 cellular localization in *E. coli*

Overnight *E. coli* (DH5α) cultures expressing SbtA2 forms carrying an internal
His×6 tag were grown and induced as per Ci uptake assay. Cells were harvested at
4,800 × g for 5 minutes, washed in lysis buffer (50 mM HEPES-KOH pH 8.0, 100 mM
NaCl, 5% glycerol), and left to incubate with ~30 kU of rLysozyme (Merck Millipore)
for 30 minutes at 37°C with gentle shaking. Cells were broken using approximately
200 µL silica beads using a mini beadbeater-16 homogeniser (BioSpec Products) for
5 minutes. Cell debris and beads were removed by centrifugation (20,238 × g, 1 minute
at room temperature). Supernatants were transferred to clean tubes and membrane
enriched fractions collected by centrifugation at 16,700 × g in an Eppendorf bench top
centrifuge for 30 minutes at 4 °C. Pellets were resuspended in a buffer containing 50
mM HEPES pH 8.0, 100 mM NaCl, 25 mM imidazole, and 1% *n*-Dodecyl-*β-D*-
maltoside (DDM) for 30 minutes on ice to solubilise membrane proteins. Insoluble
debris was removed by centrifugation, 16,700 × g at 4 °C for 20 minutes, and the
supernatant taken was used as the membrane enriched fraction.

SDS-PAGE and western blot

Proteins were separated by SDS PAGE (NuPAGE, 4-12% Bis-Tris mini protein
gel, Thermo Fisher Scientific) and subsequently transferred on to PVDF membrane
for 45 minutes (BioRad Transblot Turbo). The membrane was blocked with 5% skim
milk powder and probed for His×6 or HA tagged proteins using mouse-anti-His×6 or
mouse-anti-HA antibodies (and goat-anti-mouse AP-conjugated secondary antibody;
Table S5), and developed with Attophos (Promega, USA) prior to imaging on a BioRad
Chemidoc MP system as previously detailed (Long *et al.*, 2018).

Plant methods

Nuclear transformation

Agrobacterium transformation: *Agrobacterium tumefaciens* (strain GV3101)
was transformed with 1µg of pDNA by electroporation, and recovered with 0.5 mL LB
at 28°C for 2 h before plating on LB-agar supplemented with 30µg/mL kanamycin and
50 µg/mL rifampicin and incubated for ~48 h at 28°C.

N. tabacum transformation: _Agrobacterium_ harbouring plasmid of interest was
cultured in LB supplemented with 30 μg/mL kanamycin and 50 μg/mL rifampicin at
28°C for 48 h. Cells (~20 mL) were harvested by centrifugation for 10 minutes at 1650
× _g_. Cells were suspended in resuspension medium, and left at room temperature for
1 h. Sterile tobacco leaves were cut from plantlets growing in tissue culture into 0.4
cm^2 pieces and placed adaxial side down on co-cultivation plates with 5 mL of
Agrobacterium in resuspension medium and left for 5 minutes, before transferring
plant pieces to fresh co-cultivation plates. Plates were sealed and left in a controlled
temperature growth room at 25°C with 16:8 h photoperiod.

After 48 h leaf pieces were transferred to regeneration plates. When shoots
appeared, callus with shoots were transferred to fresh regeneration plates and
returned to the growth room. When the shoots became big enough to excise they were
removed from the leaf pieces and transferred to rooting plates. Once roots had formed,
plantlets were transferred to soil (6L of commercial seed raising mix with 2-3 cups of
vermiculite and ~25-30 mL of Osmocote) in 8×8 cm^2 punnets in growth chamber.

Growth chamber conditions: Temperature was controlled at 25°C daytime/
20°C night time, relative humidity 60%, light intensity of 400 μmol s^{-1} m^{-2}, daylength
0700-2300 h. After 1-2 weeks, leaf samples (1 cm^2 discs) were taken for determination
of the hygromycin resistance gene insert number using droplet digital PCR (iDNA
Genetics Ltd, Norwich, UK). Only plants with one insert were continued to the next
stages. Leaves were also sampled at this time for immunoblot analysis to assess the
expression levels of the transgene. When necessary, plants were transferred to 4.5 L
pots filled with steam-sterilised Martins Mix supplemented with 7 g/L Osmocote and
moved to the glasshouse to reach maturity prior to seed harvest.

For plant growth in glasshouses, pots were placed on benches ~ 1 m off the
ground and temperature was controlled to 25°C daytime/20°C night by commercial air-
conditioning.

Plant height, fresh and dry weight determination

The height of plants was measured each day from the base of the stem to the
base of the apical bud. At 63 days post cotyledon emergence (pce), the above-ground
tissue of plants was harvested by cutting at the base of the stem and the entire plant
was placed into a paper bag and weighed. The weight of the paper bag was subtracted
to determine fresh weight. Plants were then placed in 80°C oven for seven days to dry

the plant tissue. The weight of the tissue and bag was recorded, and the weight of the

bag subtracted to obtain the dry biomass weight.

$\Delta^{13}C$ determination of plant biomass

Leaf samples (0.5 cm diam discs) were harvested following gas exchange

measurements (30 ± 5 cm in height). Samples were placed in 1.5 mL tubes and dried

at 80°C for 24 h. The tubes were capped and $\Delta^{13}C$ determination was performed by

mass spectrometer at RSB, ANU (Förster *et al.*, 2023)

Leaf gas exchange measurements

Using LI-6800 (LICOR) with a 6 cm^2 aperture, fully expanded leaves of plants

with similar size (30 cm ± 5cm) and leaf number were equilibrated to the room for ~20

minutes before gas exchange measurements. Leaves were equilibrated at 400 µmol

mol^{-1} CO_2 for the reference for 10-20 minutes before starting CO_2 response curves

over a series of CO_2 concentrations at two-minute intervals (400, 200, 100, 50,150,

250, 400, 600, 800, 1200, 400 µmol mol^{-1} for reference) at a leaf temperature of 25°C,

55% relative humidity, and illumination of 1500 µmol s^{-1} m^{-2}.

A/C_{int} curves were generated from the data by plotting Assimilation rate (A)

against intercellular CO_2 concentration (C_{int}) as calculated in the LI-6800 output.

Estimation of CO_2 compensation point

The CO_2 compensation point (the intercellular CO_2 concentration where net

CO_2 assimilation rate is 0 µmol m^{-2} s^{-1}) was determined from the A/C_{int} curves. The

linear part of the curve (0-300 µmol mol^{-1} intercellular C_{int}) was used to determine the

compensation point.

Chloroplast membrane enrichment

Chloroplast membrane-enriched protein fractions were isolated from two leaf

discs (12 mm^2) that were snap frozen when sampled and stored at -80°C. Samples

were ground using Dounce homogenisers with 1 mL of lysis buffer (50 mM EPPS pH

8, 5 mM $MgCl_2$, 2 mM DTT, 1 mM Na-diethyl-dithiocarbamate, 0.01g/mL

polyvinylpyrrolidone [PVPP]) and 10 µL plant protease inhibitor cocktail (Cat # P9599,

Merck Life Science, USA). The suspension was transferred to a 1.5 mL tube, on ice

and centrifuged for 30 s at 20,238 × *g* to remove large debris and PVPP. The

supernatant was transferred to a new 1.5mL tube and centrifuged for 15 minutes at

20,238 × *g* at 4°C. The aqueous supernatant was discarded before resuspending the

pellet in wash buffer (50 mM EPPS pH 8, 5 mM $MgCl_2$, 2 mM DTT, 1 mM Na-diethyl-

dithiocarbamate, 200 mM NaCl). Centrifugation and washing were repeated twice. All

traces of supernatant were removed, and the pellet was resuspended in 50 µL of 2 ×

resuspension buffer (4% SDS, 125 mM tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and left

at 4°C overnight. The membrane-enriched suspension was centrifuged for 1 minute at

20,238 × g at 4°C and the soluble fraction was transferred to a new tube.

Total protein in the samples was determined using the detergent compatible

Bio-Rad DC Protein Assay kit, by diluting the samples 1:10 in 2 × resuspension buffer.

Sample (5 µL) or BSA standard was added in triplicate in a 96-well plate, and the kit

used as per instruction protocol. Samples were standardised based on total protein

values of the samples before being loaded onto SDS PAGE for immunoblot analysis.

Results

Based on phylogenetic analysis of multiple protein sequence alignments of

SbtA transporter proteins, the well characterised SbtA1 HCO_3^- transporters (Du et $al.$,

2014), largely present in freshwater and estuarine β-cyanobacteria, are distinct from

homologues found in α-cyanobacteria (Figure 1; Cabello-Yeves et $al.$, 2022; Rae et

$al.$, 2011; Du et $al.$, 2014). On this basis, we refer here to the former group as SbtA1,

and the latter as SbtA2 (Rae et $al.$, 2011). As an example of the phylogenetic distance

between SbtA1 and SbtA2 members, sequence identity of SbtA1 and SbtA2 from

$Synechococcus$ WH5701 is approximately 25% (Figure S1A).

Heterologous expression and bicarbonate transport function of SbtA2 homologues

To evaluate the HCO_3^- transport function of SbtA2 homologues, genes from

members of the SbtA2 family (from marine α-cyanobacterial species $Synechococcus$

WH5701, RCC307, CB0205, and $Prochlorococcus$ MIT0701, and MIT9313) were

expressed in the $E.$ $coli$ strain CAfree (Desmarais et $al.$, 2019) under the control of the

IPTG-inducible $lacI^q$-trc hybrid promoter (de Lorenzo et $al.$, 1993).

The CAfree strain of $E.$ $coli$ lacks both CA genes $cynT$ and can (Desmarais et

$al.$, 2019) and is therefore incapable of enzymatic HCO_3^- to-CO_2 interconversion within

the cytoplasm. This renders CAfree reliant on exogenous CO_2 entry and slow

spontaneous chemical conversion to HCO_3^- as a carboxylation substrate to supply

anaplerotic pathways in the cell (Merlin et $al.$, 2003). CAfree is unable to grow under

atmospheric CO_2 concentrations (~0.04% CO_2) as the rate of HCO_3^- supply is too slow

to support these pathways. The subsequent phenotype is a dependency on either

high-CO_2 for growth, due to high relative rates of spontaneous HCO_3^- production within

the cell, or heterologous expression of either a functional CA (Pulsford *et al.*, 2024) or HCO$_3^-$ transporter (Rottet *et al.*, 2024; Forster *et al.*, 2023) to grow at air levels of CO$_2$. In our hands we have seen no reversion of this cell line under standard experimental conditions.

Of the five SbtA2 family members assessed (chosen as genes with moderate GC content that therefore did not require codon optimisation), three supported growth of CAfree at ambient CO$_2$ (Figure 2A, S2A). These were from *Synechococcus* spp. WH5701, RCC307 and CB0205; referred to hereafter as SbtA2-WH5701, SbtA2-RCC307 and SbtA2-CB0205 respectively. Neither of the SbtA2 homologues from *Prochlorococcus* spp. (MIT0701 and MIT9313) showed function in CAfree at ambient CO$_2$ under the complementation conditions assessed (Figure 2A). Functional SbtA2 proteins were expressed in *E. coli* strain DH5α and characterised by radiolabelled NaH^{14}CO$_3$ uptake assays using the silicone oil centrifugation-filtration method to determine their HCO$_3^-$ transport activity (Rottet *et al.*, 2024; Du *et al.*, 2014; Forster *et al.*, 2023).

Previous analysis of members of the SbtA1 family revealed they were Na$^+$-dependent (Du *et al.*, 2014). Due to their similarity to the SbtA1 family, SbtA2 function was initially assessed under similar assay conditions used for SbtA1, namely in the presence of 20 mM NaCl (Du *et al.*, 2014). Under these conditions, we observed high rates of HCO$_3^-$ transport by SbtA2 (Figure 2B, C), particularly for SbtA2-WH5701 which had significantly higher flux than SbtA1 from *Synechococcus* PCC7942 (SbtA1-7942) when compared on a cell density basis in the presence of 20 mM NaCl (Figure 2B). Typical HCO$_3^-$ uptake rates for SbtA1-7942 were less than 0.5 µmol Ci·OD$_{600}^{-1}$·h^{-1} (Figure 2B), whereas SbtA2-WH5701 achieved 1-1.5 µmol Ci·OD$_{600}^{-1}$·h^{-1} (Figure 2B, C).

Since SbtA2 was active in the presence of 20 mM NaCl, we hypothesised that SbtA2 might also be Na$^+$-dependent. Notably, when SbtA1-7942 was assessed for HCO$_3^-$ uptake in response to Na$^+$, this Na$^+$-dependent transporter showed very little uptake in buffers with no added NaCl (Figure 3A). However, SbtA2-WH5701 demonstrated elevated rates of HCO$_3^-$ uptake across the range of 0 – 20 mM NaCl (Figure 3A, B), and in the presence of 200 mM NaCl the HCO$_3^-$ uptake rate of SbtA2-WH5701 was as high as 2.5 - 3 µmol Ci·OD$_{600}^{-1}$·h^{-1} (Figure 3C).

Based on this result, we then assessed which ion, Na$^+$ or Cl$^-$, was contributing to the HCO$_3^-$ uptake function by SbtA2, by performing assays in the presence or

absence of Na^+ or Cl^- in the uptake assay buffers. SbtA2 HCO_3^- uptake rates were

elevated in the presence of NaCl, KCl and LiCl above a control with no added salt, but

not in the presence of $NaNO_3$ (Figure 3D), revealing that SbtA2 HCO_3^- uptake activity

increased in the presence of Cl^- and not Na^+. This observation was further assessed

by measuring the HCO_3^- uptake activity of SbtA2-WH5701 in the presence of NaCl,

$MgCl_2$, Na_2SO_4 or $MgSO_4$, revealing the possibility of both Cl^- or SO_4^{2-}-dependency

(Figure 3E).

SbtA2 effect on *E. coli* Ci pool sizes

Using cell volumes measurements for *E. coli* previously estimated by our group

(Du *et al.*, 2014), we calculated the internal Ci pool accumulation for a 30 second

period from the addition of 0.5 mM Ci to be 5.5 - 8 mM for SbtA2-WH5701, 2 – 3 mM

for SbtA1-7942 and 0.2 mM for the empty vector in the presence of 20 mM NaCl.

However, in the presence of 200 mM NaCl, the pool size increased to 15 - 23.5 mM

for cells carrying SbtA2-WH5701 and 0.3 mM for cells carrying the empty vector (Table

2).

SbtA2 affinity for HCO_3^-

HCO_3^- affinity estimates (K_m) were calculated to be approximately 150 - 200 µM

for all three SbtA2 homologues from *Synechococcus* in the presence of 20 mM NaCl

(Figure 4, S4, Table 1 and Table S4). Maximum HCO_3^- uptake rates for SbtA2-

WH5701 in the presence of 100 mM NaCl were approximately double that observed

at 20 mM NaCl, but the K_m for HCO_3^- was unaffected by the increased NaCl

concentration (Figure 4 and Table 1).

The effect of SbtB2 on SbtA2 HCO_3^- uptake activity

Like the *sbtA1* family, *sbtA2* is commonly co-located with *sbtB2* in the genome

(Du *et al.*, 2014) (Figure S5). SbtB1 acts to down regulate SbtA1 function when

expressed in *E. coli* and cyanobacteria preventing SbtA1 activity (Du *et al.*, 2014;

Förster *et al.*, 2023; Fang *et al.*, 2021). We therefore assessed whether the SbtB2

protein would affect SbtA2 function in CAfree. Dicistronic constructs of *Synechococcus*

SbtA2:B2 with a C-terminal HA-tag on SbtB2 (referred to as SbtA2:B2-HA) were

assembled similar to SbtA2 alone, under the control of the *lacI^q-trc* promoter.

Complementation of CAfree with SbtA2:B2-HA exhibited similar growth to
CAfree expressing SbtA2 alone at ambient CO_2 (Figure 5A, S2B). This suggested that
SbtB2-HA does not suppress SbtA2 function in this system. However, $NaH^{14}CO_3$
uptake assays using the DH5α strain of *E. coli* co-expressing SbtA2-WH5701 and
SbtB2-HA-WH5701 (SbtA2:B2-HA-WH5701) exhibited a ~30% reduction in HCO_3^-
uptake activity in comparison to SbtA2 alone (Figure 5B). This suggests SbtB2 has a
regulatory role for SbtA2, but not sufficient to completely prevent SbtA2 activity in *E.*
coli. To determine if the HA tag on SbtB2 was interfering with the likely capacity for
SbtB2 to completely inactivate SbtA2, the HCO_3^- uptake activity of SbtA2:B2-WH5701
devoid of epitope-tags was also assessed. SbtA2:B2-WH5701 showed similar HCO_3^-
uptake activity to SbtA2:B2-HA-WH5701 (Figure 5B). Assessment of alternative
expression constructs with protein expression under alternative promoters indicated
that the relative abundance of SbtA2 and SbtB2 protein influences the activity of SbtA2
(Figure 5C and S5G). Higher expression levels of SbtB2 led to a suppression of SbtA2
activity by up to 90% (Figure 5C and S5G).
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Functional characterisation of SbtA2 in *N. tabacum* directed to the chloroplast IEM
In order to assess the potential for SbtA2 to improve net CO_2 delivery to
Rubisco *in planta*, we expressed SbtA2 in *Nicotiana tabacum* cv Samsun using genetic
constructs designed to direct the protein to the chloroplast IEM (Rolland *et al.*, 2016).
Two SbtA2 expression constructs with different promoters (*viz*. the tomato Rubisco
small subunit promoter; *Sl*RbcS2, and the Arabidopsis ubiquitin promoter; *At*Ub10i)
were generated to express an HAH6-tagged form of SbtA2-WH5701 (SbtA2-HAH6-
WH5701) in *N. tabacum* cv Samsun.
Due to a lack of specific antibody for SbtA2, it was imperative to add an epitope
tag to SbtA2-WH5701 in order to detect protein expression. Interestingly, adding a tag
at the C- or N-terminus resulted in transporter non-function. However, we
demonstrated that SbtA2-WH5701 carrying a tag located in the flexible cytoplasmic
loop between transmembrane helices five and six (hereafter the 5-6 loop) had similar
HCO_3^- uptake activity as untagged SbtA2-WH5701 (Figure S3, S4). Using this epitope,
we were able to determine the relative level of SbtA2-WH5701 expression in
transgenic T_2 *N. tabacum* (Tob[SbtA2]) plants. SbtA2-HAH6 protein expression in Tob[SbtA2]
plants differed across their developmental age, dependent upon the promoter used.
SbtA2 protein was more abundant in younger leaves of Tob[SbtA2] plant lines using the

*Sl*RbcS2 promoter, while those lines using the *At*ubi10i promoter had higher SbtA2

abundance in older leaves (Figure S6).

Tob[SbtA2] plants appeared similar to wild type (Tob[WT]) with little to no bleaching

of leaves throughout their lifespan (Figure 6H), which has been observed previously

in *N. tabacum* expressing SbtA1 proteins (Figure S9). Compared to Tob[WT], no

differences were observed in relation to photosynthetic performance (CO_2 assimilation

rates and derived CO_2 compensation point), final height, or whole plant fresh weight

for Tob[SbtA2] plants (Figure 6). While one *At*ubi10i line (Tob[SbtA_ubi-1.1.3]) demonstrated

an elevated dry weight compared to Tob[WT] (Figure 6C), and two Tob[SbtA2] lines had

significantly lower $\Delta^{13}C$ values than Tob[WT] (Figure 6G), these traits were not

significantly different across all lines tested.

Discussion

The open-ocean α-cyanobacteria of the *Prochlorococcus* and *Synechococcus* (SynPro) clade is attributed with significant contributions to global CO_2 fixation (Flombaum *et al.*, 2013). Based on the enzyme kinetics of Form-IA Rubisco found in these organisms, cells require the accumulation of at least 10 mM HCO_3^- to approach saturation of CO_2 fixation (>200 µM CO_2) within their α-carboxysomes (Whitehead *et al.*, 2014; Shih *et al.*, 2016). In the oceans, available CO_2 levels are less than 15 µM (Young *et al.*, 2016), although HCO_3^- is stably available at around 2 mM (Falkowski and Raven, 2007). This highlights that HCO_3^- uptake has evolved as a feature of biophysical CCMs in many oceanic single-celled algae and cyanobacteria, otherwise conditions would be severely limiting if dependent on diffusive CO_2 uptake alone (Ritchie *et al.*, 1996; Beardall and Raven, 2017). To date, most reports on cyanobacterial HCO_3^- transporters have been on those originating from β-cyanobacteria, and relatively little is known about the Ci uptake systems that power the CCMs of the ecologically important α-cyanobacterial SynPro clade.

The type and number HCO_3^- transporters differ in α- and β-cyanobacteria (Cabello-Yeves *et al.*, 2022; Rae *et al.*, 2011). Of the functional cyanobacterial HCO_3^- transporters known (BCT1, SbtA1, BicA), generally the α-cyanobacterial *Synechococcus* clade has genes for one BicA HCO_3^- transporter, while *Prochlorococcus* species have no genes for these HCO_3^- transporters (Cabello-Yeves *et al.*, 2022; Rae *et al.*, 2011). Only distant homologues of SbtA1 and BicA, SbtA2 and BicA2, are apparent in SynPro (Rae *et al.*, 2011). SbtA2 protein sequence identity is only approximately 25% compared to SbtA1 (Figure S1), however, its predicted structure (AlphaFold 3; Abramson *et al.*, 2024) maps with high confidence to that of SbtA1 (Synechocystis PCC6803; PDB: 7egl; Fang *et al.*, 2021 ; Figure S3A-C). This led us to hypothesise that SbtA2 likely functions as a HCO_3^- transporter in species belonging to the SynPro clade.

SbtA2 functional characteristics

In the present study we show for the first time that several members of the SbtA2 family from *Synechococcus* spp*.* are indeed functional HCO_3^- transporters and displayed an 'always-active' HCO_3^- transport function when expressed in *E. coli* (Figure 2). Compared to SbtA1-7942, SbtA2-WH5701 has a high HCO_3^- transport flux (Figure 2B, 3A, 3C), in fact the highest we have recorded. In the presence of 200 mM

NaCl, SbtA2-WH5701 had greater than six times the flux of SbtA1-7942 (Figure 3C).

The maximum uptake flux by SbtA2 of 2.5 - 3 $\mu mol \cdot Ci \, OD_{600}^{-1} \cdot h^{-1}$ generates an internal

Ci pool size of up to 23.5 mM in *E. coli* over a 30 second period, and an accumulation

factor of up to 70-fold compared to *E. coli* empty vector controls (Table 2). This extent

of accumulation, if translated to a cyanobacterium or a C_3 chloroplast, would be more

than enough to reach the levels required to optimally operate the cyanobacterial CCM

(Whitehead *et al.*, 2014).

Kinetic characterisation revealed that SbtA2 is a medium affinity transporter

compared with those previously described (Rottet *et al.*, 2021) exhibiting K_m values of

approximately 150 - 200 μM (Table 1). When measured in *E. coli*, SbtA1 from

Cyanobium PCC7001 and *Picosynechococcus* PCC7002 have affinities for HCO_3^- of

189 μM and <100 μM respectively (Du *et al.*, 2014). However, it is likely that more

accurate affinities can be measured in model cyanobacteria in future studies since

respiratory CO_2 production in heterotrophic *E. coli* can lead to errors in affinity

estimates (Du *et al.*, 2014). For instance, the estimates of SbtA1 affinity for HCO_3^-

when expressed in cyanobacteria has been measured to be ~2 μM (Price *et al.*, 2004)

which is much lower than when measured in *E. coli*. In comparison to the high affinity

SbtA1 family, members of the low affinity transporter BicA, have K_m for HCO_3^-

estimated to be 75-353 μM when measured in cyanobacteria (BicA from

Synechococcus WH8102, *Picosynechococcus* PCC7002 and *Synechocystis*

PCC6803; Price *et al.*, 2004).

Recognizing that the K_m of SbtA2 is well below the Ci concentration present in

seawater (Falkowski and Raven, 2007), and the widespread nature of this gene family

in α-cyanobacteria, leads us to believe that SbtA2 members could play a critical role

in CCMs in this globally important cyanobacterial group. Furthermore, assuming

sufficient expression, this indicates that SbtA2 could readily achieve the cytoplasmic

HCO_3^- accumulation known to be required in cyanobacteria for efficient CCM activity

(Whitehead *et al.*, 2014).

Activation/Energisation of SbtA2 HCO_3^- transport

From microbes to animals, HCO_3^- transporters have been shown to function in

a multitude of modes, including; HCO_3^-/SO_4^{2-} and HCO_3^-/Cl^- exchangers (some of

which are Na^+-dependent; Cordat and Reithmeier, 2014), Na^+/HCO_3^- symporters, K^+-

dependent transporters (Parker and Boron, 2013), and ABC-type transporters that rely

on ATP for HCO_3^- transport (Omata *et al.*, 1999; Gao *et al.*, 2015). This highlights that,
mechanistically, several drivers for HCO_3^- uptake or exchange are entirely feasible
and offer potential conceptual frameworks for SbtA2 function.

SbtA1 has a distinct requirement for Na^+ for function, typically requiring 10 – 50
mM Na^+ for maximum activity when expressed in *E. coli*, and likely a Na^+/HCO_3^-
symporter (Shibata *et al.*, 2002; Du *et al.*, 2014). Despite SbtA2 having a predicted
structure similar to SbtA1 (Figure S3A-C), evidence here suggests that Na^+ does not
drive SbtA2 activity. Instead, both Cl^- or SO_4^{2-} might potentially drive SbtA2 function
(Figure 3D, E), noting that Cl^- is substantially more abundant in seawater than SO_4^{2-}
(Nessim *et al.*, 2015). SbtA2 demonstrated a high basal rate of HCO_3^- transport when
assayed in *E. coli*, which was not improved with additional Na^+ (Figure 3). Moreover,
the addition of 100 mM Cl^- or SO_4^{2-} to the assay buffer (in the form of NaCl, KCl, LiCl,
$MgCl_2$, $MgSO_4$) resulted in an increase in the HCO_3^- transport flux, approximately
doubling the HCO_3^- uptake activity compared to when no additional Cl^- or SO_4^{2-} was
added to the uptake buffer (Figure 3).

In the heterologous system used here, it is difficult to identify the exact
mechanism enabling SbtA2 to transport HCO_3^-. Clearly further work in alterative
systems is required to confirm the driving ion flux. Below we propose scenarios as to
how Cl^- or SO_4^{2-} may act on SbtA2 function.

HCO_3^-/Cl^- antiport

The results presented in here are consistent with the possibility that SbtA2 may
act to exchange HCO_3^- with Cl^- or SO_4^{2-}, moving Cl^- or SO_4^{2-} out of the cell as HCO_3^-
is moved in. There are multiple examples of HCO_3^-/Cl^- and HCO_3^-/SO_4^{2-} exchangers
(Zhang *et al.*, 2023; Reithmeier, 2001; Reithmeier *et al.*, 2016; Jennings, 2021).
Evidence suggests that Cl^- can accumulate in *E. coli* during growth (Schultz *et al.*,
1962), likely via H^+/Cl^- exchangers that can actively transport Cl^- (eClC, Garcia-Celma
et al., 2013), meaning that cells would have a Cl^- pool capable of driving HCO_3^- uptake
via HCO_3^-/Cl^- exchange. This could explain the high basal rate of HCO_3^- transport of
SbtA2 in *E. coli*, which is further stimulated with addition of Cl^- (Figure 3), if such an
antiport mechanism were in operation. For this mode of action to be observed in *E.*
coli there would need to be membrane transport systems enabling rapid import of Cl^-
to increase HCO_3^- uptake activity by SbtA2. Blocking these Cl^- transporters or
measuring their net flux during HCO_3^- uptake may provide evidence that this type of
mechanism is in action. For such a mechanism to occur in oceanic cyanobacteria,

however, cells would need to hyperaccumulate Cl^- from an external environment that
already contains ~500 – 600 mM Cl^- (Nessim *et al.*, 2015) in order to enable gradient-
driven HCO_3^-/Cl^- antiport. While Cl^- uptake has been observed (Ritchie, 1992), a
mechanism to accumulate and maintain such high internal Cl^- would be required, and
seems unlikely (Hagemann, 2011).

HCO_3^-/Cl^- symport

In contrast, it may be possible that SbtA2 acts as a HCO_3^-/Cl^- symporter,
transporting Cl^- and HCO_3^- into the cell simultaneously. This seems less likely due to
the resulting hyperpolarising effect of the ion influx on the membrane potential of the
SbtA2-expressing organism, especially within a marine context. However, this may be
possible if there are also active efflux mechanisms involved in the system to regulate
cellular Cl^- concentration, preventing hyperpolarization and mitigating the ionic effects
of SbtA2 function. Observed rates of HCO_3^- uptake increased when exogenous Cl^-
was experimentally elevated in *E. coli* expressing SbtA2 (Figure 3C-E), possibly
supporting HCO_3^-/Cl^- symport as a potential mode of action. However, evidence
against this mechanism is supported by the elevated base HCO_3^- uptake rate of SbtA2
in the absence of SO_4^{2-} or Cl^- (when Hepes buffered solution with pH adjusted to 7.5
with KOH was used as the assay buffer; Figure S7). In addition, existing literature does
not clearly define HCO_3^-/Cl^- symport as a likely scenario.

Allosteric control by Cl^-/SO_4^{2-}

It is possible that allosteric control by Cl^-/SO_4^{2-} leads to SbtA2 activation.
Evidence exists for this mechanism in alternate organisms (Davis-Kaplan *et al.*, 1998;
Tulloch *et al.*, 2008; Perutz *et al.*, 1994) and therefore sets a precedence for this type
of control. In this scenario, the presence of Cl^-/SO_4^{2-} would alter protein conformation,
oligomerisation, or charge around the active site to activate SbtA2, thus enabling
transport function. Since SbtA2 exhibits Michaelis-Menten kinetics in Ci response
assays (Figure 4 and S4), it is unlikely that SbtA2 is a passive channel and that after
allosteric activation by Cl^- a secondary counter-ion (K^+, Na^+, Mg^{2+}) must be required
for HCO_3^- transport by SbtA2. If this is the mode of activation for SbtA2, the site of Cl^-
/SO_4^{2-} elevation (intracellular or extracellular), the mechanism of Cl^-/SO_4^{2-} action on
SbtA2, and the secondary counter-ion would need to be determined to optimise
function in non-native environments.

These speculative ideas need further investigation to clarify the mode of action
of SbtA2, perhaps using $^{36}Cl^-$ to assess Cl^- flux in *E. coli*, or by expressing SbtA2 in

Xenopus oocytes, or in liposomes where other membrane transport systems may be
less likely to confound results. Without further analysis we cannot yet conclude what
is the energisation mode for SbtA2. Additionally, expression of SbtA2 in the *Δ5* mutant
of *Synechocystis* sp. PCC6803, which has all five Ci uptake mechanisms deleted (Xu
et al., 2008), may provide functional information in a cyanobacterial context.

***Prochlorococcus* SbtA2**

Curiously, the two SbtA2 members from *Prochlorococcus* (Figure 2A) did not
function in *E. coli* despite the high sequence similarity of all five homologues (Figure
S1). There may be three things to consider here. Firstly, SbtA2's from *Prochlorococcus*
may be pseudo-genes, though this seems unlikely since there are so few genes coding
for putative HCO_3^- transporters in *Prochlorococcus* genomes (Rae *et al.*, 2011).
Secondly, there may be another level of allosteric control that cannot be satisfied in
the heterologous *E. coli* expression system. If so, this will require further work to
resolve the functional activation of SbtA2 from *Prochlorococcus*. Using an approach
such as laboratory evolution to generate functional HCO_3^- transporters has been
shown to be useful to resolve important sequence elements contributing to both HCO_3^-
transporter and CA function (Pulsford *et al.*, 2024; Rottet *et al.*, 2024). This was
attempted on the non-functional members; however, no functional forms have been
generated to date in our hands. Thirdly, *Prochlorococcus* species appear to use
sulpholipids bilayers (Van Mooy *et al.*, 2006) rather than phospholipid bilayers found
in *E. coli*, so there is uncertainty whether *Prochlorococcus* membrane proteins will
insert or interact correctly within bacterial membranes. The importance of
Prochlorococcus as an oceanic contributor to carbon acquisition, and the lack of
evidence of any HCO_3^- transport in this genus is intriguing and led us to question how
these organisms can be achieving efficient CO_2 fixation in low CO_2 environments
without possessing any characterised HCO_3^- transporters. On balance, the most likely
scenario is that SbtA2, and/or the distant BicA homologue, are functional HCO_3^-
transporters in *Prochlorococcus* spp. or there are alternative HCO_3^- transporters or
regulators present that are still undiscovered.

SbtA2:B2 interaction

The SbtB1 protein has been shown to regulate the SbtA1 transporter as a
"curfew protein" that inhibits the action of the pump (Förster *et al.*, 2023; Kaczmarski

et al., 2019; Fang *et al.*, 2021). Additionally, SbtB1 appears to act as a nucleotide- and
redox-sensitive regulator that controls SbtA1 by switching between binding states and
promoting AMP-dependent complex formation, possibly modulating HCO_3^- transport
in response to cellular energy and light/dark conditions (Selim *et al.*, 2023). In either
case, the system is regulated by cellular ATP, or the relative adenylate charge, such
that high ATP concentrations lead to the unbinding of the SbtB1 trimer from the SbtA1
transport trimer, thereby activating SbtA1 (Förster *et al.*, 2023). Previously, Du *et al.*
(2014) showed that SbtA1 activity could be eliminated through co-expression of SbtB1
in *E. coli* (DH5α and EDM636 strains). This implies that ATP concentrations inside
growing *E. coli* cells must be low enough for SbtB1 to bind SbtA1, resulting in the
prevention of SbtA1 function and no growth of *E. coli* (EDCM636 strain) at atmospheric
CO_2. Here we found that SbtB2 can inhibit its cognate SbtA2 function by around 30-
90% (Figure 5 and S5), depending on protein expression levels, and that the
expression level of SbtB2 is important in controlling SbtA2 function. Co-expression of
SbtA2:B2 in CAfree did not prevent growth of CAfree *E. coli* at atmospheric CO_2
(Figure 5A and S2B). However, HCO_3^- uptake assays in *E. coli* DH5α demonstrated
reduced levels of uptake (Figure 5, and S5), and highlights that this is likely sufficient
to overcome the CAfree phenotype in growth studies.
636
The effect of epitope tagging on SbtA2 function
Efforts to generate antibodies to SbtA2 for use in immunoblot identification of
heterologously expressed protein was unsuccessful. We initially attempted to
introduce epitope tags to the N- or C-terminus of SbtA2 but found these resulted in
non-functional transporters (Figure S8). Using the predicted structure of SbtA2
(Abramson *et al.*, 2024) and the crystal structure of SbtA1 (Fang *et al.*, 2021; Liu *et*
al., 2021) as guides (Figure S3), we were able to conclude that the cytoplasmic 5-6
loop is a disordered flexible region that may be amenable to epitope tagging.
Functional data from tagging studies of SbtA1-7942 suggested this region was not
functionally critical in that form of SbtA1 (Du *et al.*, 2014). Here, all assessments of
SbtA2 forms carrying an internal epitope-tag within this flexible cytoplasmic 5-6 loop
region indicated that these proteins retained function comparable to their untagged
counterparts (Figure S3). This understanding was critical to using these SbtA2 forms
to assess protein expression in both *E. coli* and tobacco without loss of HCO_3^-
transport activity.

Expression of SbtA2 in *N. tabacum*

654   Introduction of CCMs to chloroplasts of C_3 plants has been modelled to boost Rubisco carboxylation, increasing photosynthetic efficiency, thereby improving crop productivity (Price *et al.*, 2011; Rottet *et al.*, 2021; McGrath and Long, 2014) and a possible strategy to circumvent the looming food shortages expected as global population increases (Ray *et al.*, 2013). The expression of functional HCO_3^- transporters in the chloroplast IEM could support a 7-16% increase in crop productivity resulting from increased CO_2 supply to Rubisco (Price *et al.*, 2011; McGrath and Long, 2014). Previously several HCO_3^- transporters have been expressed in the chloroplast IEM of model plants, although with mixed results with respect to improvement in photosynthetic parameters. For the most part, there has been no evidence of active chloroplast HCO_3^- accumulation, and where there have been improvements in photosynthetic parameters, this has not been clearly linked to increased CO_2 supply to Rubisco (Rottet *et al.*, 2024; Forster *et al.*, 2023; Nölke *et al.*, 2019; Atkinson *et al.*, 2016; Pengelly *et al.*, 2014).

668   Here we successfully directed the epitope-tagged SbtA2-HAH6-WH5701 to the chloroplast IEM of *N. tabacum* after nuclear expression (Figure S6). T_2 plants were assessed for phenotypic and photosynthetic changes to determine the impact of the expressed SbtA2 protein. Although one Tob^{SbtA2} expression line had increased dry matter and two with lower $\Delta^{13}C$ (Figure 6G), there was no consistent improvement of photosynthetic parameters or phenotypic traits of plants expressing SbtA2 compared to wild type (Figure 6). Notably, however, transgenic plant phenotypes appeared similar to wild type plants, and no detrimental effects on plant growth was observed. This contrasts with previous attempts to express SbtA1 in *N. tabacum* which were slow growing and demonstrated a chlorotic phenotype (Figure S9). We conclude from this that SbtA2 may either be non-functional *in planta*, or that the level of function is too low to provide any observable benefit in the lines analysed. It is possible that an alternate SbtA2 homologue may be a better candidate for expression *in planta* (for example SbtA2-CB0205 which appears to have higher activity per unit protein than SbtA2-WH5701 [Figure S3E, F]), or that plant lines showing higher expression across all stages of plant growth might provide a better indication of expected photosynthetic improvements.

It still needs to be established with greater certainty if SbtA2 members are in

fact Cl^-/HCO_3^- (and/or SO_4^{2-}/HCO_3^-) antiporters, or indeed if they are under Cl^-/SO_4^-

allosteric control. If SbtA2 does rely on Cl^- (or SO_4^{2-}) for HCO_3^- transport function, it is

important to understand the existing requirement, magnitude, and maintenance of a

Cl^- (or SO_4^{2-}) gradient between the cytoplasm and the chloroplast stroma. This also

highlights the pressing need to understand the Cl^- and SO_4^{2-} concentration gradients

and movement in leaf tissue sub-compartments and the endogenous transporters

involved. This will determine whether the appropriate activation or energisation

conditions exist for SbtA2 function in the chloroplast IEM.

It is understood that Cl^- is the most abundant anion in the chloroplast

(Colmenero-Flores et $al.$, 2019) and serves an important role in photosynthesis as an

essential cofactor in photosystem II function (Colmenero-Flores et $al.$, 2019). Cl^-

concentrations have been measured at 36-60 mM in chloroplasts of the glaucophyte

spinach (Demmig and Gimmler, 1983). Interestingly, similar Cl^- concentrations have

been measured in chloroplasts of other important C_3 plants, barley and wheat (39 and

48 mM Cl^- respectively; Robinson and Downton, 1984). Additionally, tobacco can

accumulate Cl^- in macronutrient quantities without apparent detrimental effects on the

plants when grown in elevated Cl^- as long as particular cations are not in excess

(Franco-Navarro et $al.$, 2019). Furthermore, growth in elevated NaCl can increase

chloroplastic Cl^- concentrations in halophytic plants (Demmig and Winter, 1986). This

is an example of a factor that could be optimised in future plant expression studies, if

SbtA2 is indeed a Cl^-/HCO_3^- antiporter, or in some way linked to Cl^- availability.

However, there are a number of other issues that need optimisation, such as;

achieving sustained expression and insertion of SbtA2 targeted to the chloroplast

envelope inner membrane, studies to ascertain if a sufficient electrochemical gradient

exists between the cytosol and chloroplast stroma, and whether this can be optimised

by expression of CLC-type Cl^- importers.

Conclusion

This study involved characterisation of a previously undescribed family of

HCO_3^- transporters, SbtA2, chiefly found in α-cyanobacteria (Figure 1). Although

SbtA2 appears to be a distant relative of the Na^+-dependent SbtA1, the mode of action

of SbtA2 is quite different, with an apparent dependency on Cl^- or SO_4^{2-}. SbtA2 also

displays high HCO_3^- transport rates, and an affinity for HCO_3^- that can be described

as intermediate with respect to other known HCO_3^- transporters. The functional
characterisation of SbtA2 in *E. coli* presents as an 'always active' phenotype, which
lends itself as a potential candidate for insertion into the IEM of the chloroplast with
the aim of improving photosynthetic efficiency in C_3 crop plants. However, *N. tabacum*
expressing SbtA2 showed no consistent improvement in photosynthetic parameters
compared to wild type plants. Nonetheless, this work highlights that a broader
selection of HCO_3^- transporters could have the potential to enhance photosynthetic
performance in C_3 plants by delivering Ci to the chloroplast. In order for successful
function of SbtA2 *in planta* it is important to understand the apparent Cl^-/SO_4^{2-}
dependency of SbtA2 and the concentrations of these ions in sub-compartments in
plant leaf tissue, and the consequences of over-expressing SbtA2 and any related ion
imbalances this may cause.

Supplementary Data

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Tables

Table 1: The affinity for HCO_3^- of SbtA2 homologues

	WH5701	WH5701 Cl^{-} [a]	RCC307	CB0205
V_{max}	1.34 ± 0.07	2.19	0.38 ± 0.06	0.34 ± 0.02
K_m (µM)	146 ± 33	140	147 ± 16	197 ± 21

The K_m (µM) and V_{max} (µmol $Ci \cdot OD_{600}^{-1} \cdot h^{-1}$) of the transporters in the presence of 20 mM NaCl in the assay buffer, mean from 3 assays. [a] Results for WH5701 Cl^{-} are from a single HCO_3^- uptake experiment assessing the K_m in of SbtA2-WH5701 in the presence of 100 mM NaCl in the assay buffer.

Table 2: The estimated Ci pool sizes of *E. coli* cells expressing SbtA2 homologues

	WH5701 [c]	WH5701 [d]	SbtA1-7942 [c]	EV [c]	EV [d]
Ci pool (mM) [a]	5.5 - 8	15 - 24	2 - 3	0.2	0.3
Fold increase [b]	30 - 36	57 - 69	12-14	-	-

[a] Ci pool sizes are estimated based on cell volumes from our previous study (Du et al., 2014) and from uptake rates over a 30 sec period during silicone-oil centrifugation assays using 0.5 mM $NaH^{14}CO_3$. [b] Fold increase in pool size is compared with estimated pool sizes in empty vector (EV) controls in 30 sec uptake assays. [c] assessed in the presence of 20 mM NaCl. [d] assessed in the presence of 200 mM Cl^{-}.

Figure Legends

Figure 1: Phylogenetic tree showing the relative protein relationships between members of the SbtA family across cyanobacteria.

The primary sequence alignment, and derived tree, were prepared using Geneious Prime software and iTol (Letunic and Bork, 2024). The SbtA2 family is highlighted in blue and the SbtA1 family in pink. The internal size marker indicates 0.1 substitutions per residue. Red branches indicate α-cyanobacteria and purple branches indicate β-cyanobacteria. Coloured bold text indicates that HCO_3^- uptake activity has been assessed with those and in black bold text demonstrating no function in E. coli (this study and Du *et al.*, 2014). The outgroup SbtA1 from *Labrenzia* DFL-11, an α-proteobacterium found in close association with dinoflagellates of the *Alexandrium* genus (Biebl *et al.*, 2007), was previously assessed for function by (Du *et al.*, 2014).

Figure 2: **The HCO_3^- transport function of SbtA2 was assessed using complementation assays and HCO_3^- uptake assays.**

(A) Cultures of CAfree E. coli expressing SbtA2 homologues were adjusted to the same density prior to being spotted onto LB-agar containing 100 µM IPTG and grown at ambient (0.04%) or 4% CO_2 for ~20 h. The growth of SbtA2 members (Synechococcus WH5701, RCC307, CB0205 and Prochlorococcus MIT0701 and MIT9313) were compared to a negative control (empty vector; EV) and a positive control (SbtA1 from Synechococcus elongatus PCC7942; SbtA1-7942). **(B)** The HCO_3^- uptake activity of SbtA2-WH5701 (A2), SbtA1 from Synechococcus PCC7942 (SbtA1-7942; A1) and empty vector (EV). n=12 from 3 biological replicates. **(C)** HCO_3^- uptake activity of SbtA2 from Synechococcus WH5701 (WH), RCC307 (RCC) and CB0205 (CB). n=12 from 3 biological replicates. HCO_3^- uptake assay buffer composition: BTP - H_2SO_4 pH 7.5 + 20 mM NaCl. Different letters indicate significantly different means when analysed by one-way ANOVA (P<0.05).

Figure 3: Assessment of the counter-ion driving the HCO_3^- uptake function of SbtA2.

Analysis of ion dependency was determined using HCO_3^- uptake assays in DH5α strain of E. coli. Values presented have empty vector rates for each condition subtracted. Different letters indicate significantly different means when analysed by one-way ANOVA (P<0.05). **(A)** The HCO_3^- uptake activity of SbtA2-WH5701 (SbtA2) and SbtA1-7942 (SbtA1) was assessed in the presence of 0, 1, 5 or 20 mM NaCl. Assay buffer composition: 20 mM BTP-H_2SO_4 pH 7.5 + (0, 1, 5, or 20 mM NaCl). Na^+ provided by $NaH^{14}CO_3$/$KHCO_3$ was ~20 µM. The connecting line represents the mean; n= 3-4 from 1 biological replicate. **(B)** The HCO_3^- uptake rates of SbtA2-WH5701 in the presence of 0 and 20 mM NaCl in the assay buffer were compared. Assay buffer composition: 20 mM BTP-H_2SO_4 pH 7.5 + (0 or 20 mM NaCl). Data presented as mean ± SD; n=27 from 8 biological replicates. **(C)** The HCO_3^- uptake activity of SbtA2-WH5701 was measured in response to an increase in NaCl concentration in the assay buffer. Uptake assay buffer composition: 20 mM BTP-H_2SO_4 pH 7.5 + 10 mM K_2HPO_4 + (0, 50,

100 or 200 mM NaCl). n=11-16 from 3-4 biological replicates. **(D)** The HCO_3^- uptake activity of SbtA2-WH5701 was assessed in the presence of various salts; no additional NaCl, 100 mM NaCl, 100 mM KCl, 100 mM LiCl and 100 mM $NaNO_3$ added to the assay buffer of 20 mM BTP-H_2SO_4 pH 7.5. Salt concentrations added to the uptake buffer are in mM. 20 µM Na^+ was supplied by $NaH^{14}CO_3$. Data presented as mean ± SD; n=16-20 from 4-5 biological replicates. **(E)** The HCO_3^- uptake activity of SbtA2-WH5701 was assessed in response to different salts; no additional NaCl, 100 mM NaCl, 50 mM $MgCl_2$, 50 mM $MgSO_4$ and 50 mM $NaSO_4$, 200 mM NaCl or 100 mM $MgCl_2$ added to the assay buffer of 20 mM BTP-H_2SO_4 pH 7.5. 20 µM Na^+ was supplied by $NaH^{14}CO_3$. Data presented as mean ± SD; n=8-12 from 2-3 biological replicates.

Figure 4: The affinity (K_m) for HCO_3^- was determined for SbtA2 from *Synechococcus* WH5701.
The affinity for HCO_3^- was estimated using Ci uptake assays performed in response to increasing exogenous HCO_3^- concentrations in E. coli DH5α. The HCO_3^- affinity of SbtA2-WH5701 was assessed in the presence of 20 mM NaCl or 100 mM NaCl. Assay buffer composition: BTP - H_2SO_4 pH 7.5 + (20 mM NaCl or 100 mM NaCl). HCO_3^- uptake rates at each HCO_3^- concentration had empty vector rates specific for that condition subtracted. Similar trends were observed across experiments although the activity values varied, as a result representative data from single experiments are shown. Individual data values are shown along with their fitted Michaelis-Menten curve which is presented as a solid line. Curve fitting protocols are stated in the materials and methods section.

Figure 5: Assessment of the effect of SbtB2 on SbtA2 HCO_3^- transport function.
(A) Cultures of CAfree were adjusted to the same density prior to spotting onto LB-agar containing antibiotics and 100 µM IPTG and grown at ambient (0.04%) or 4% CO_2 for ~20 h. The cultures assessed included positive control SbtA1 from *Synechococcus* PCC7942, (SbtA1-7942) SbtA2:B2-HA from *Synechococcus* WH5701, RCC307, and CB0205, a negative control (empty vector; EV) and positive controls (SbtA2-WH5701, SbtA2-RCC307, SbtA2-CB0205). **(B)** The relative HCO_3^- uptake activity of DH5α *E. coli* carrying SbtA2:B2-HA-WH5701 and SbtA2:B2-WH5701 compared to SbtA2-WH5701 (A2). Assay buffer composition: 20 mM BTP-H_2SO_4 pH 7.5. Data presented as the mean with empty vector control subtracted ± SD; n=8 from 2 biological replicates. **(C)** The relative HCO_3^- uptake activity of DH5α *E. coli* carrying *lacI*[q]-SbtA2-H6:B2-HA-WH5701 (*l*AB2), *lacI*[q]-SbtA2-H6:*tet*-B2-HA-WH5701 (*l*A2*t*B2) and *lacI*[q]-SbtB2-HA:A2-H6-WH5701 (*l*BA2) compared to *lacI*[q]-SbtA2-H6-WH5701 (*l*A2). Assay buffer composition: 20 mM BTP-H_2SO_4 pH 7.5 + 200 mM NaCl. Data presented as the mean ± SD with empty vector controls subtracted; n= 12 from 3 biological replicates.

Figure 6 : Phenotypic measurements of *N. tabacum* expressing SbtA-HAH6-WH5701 (Tob[SbtA]) compared to wild-type (Tob[WT]) plants.
(A) The heights of plants were measured 63 days post cotyledon emergence (pce) from the base of the plants to the base of the apical meristem. The data is presented as means ± SD; n=6. **(B)** Each

plant was harvested at the base of the stem 63 days pce and the fresh weight was measured. **(C)** The harvested plants were dried for 7 days at 80°C and the weight were measured. Data presented as mean ± SD; n=6. **(D-F)** A/C_{int} curves were measured on plants 30 ± 5 cm in height on young fully expanded leaves of similar size using a Licor 6800 with the following parameters; photon flux density 1500 µmol·m^{-2}·s^{-1}, at 25°C and photosynthetic measurements taken at 400, 200, 100, 50,150, 250, 400, 600, 800, 1200, 400 µmol·mol^{-1} CO_2. Data presented as mean ± SD. n= 3-5 for Tob^{SbtA2} and n=9 for Tob^{WT}. **(G)** $\Delta^{13}C$ was determined from dry matter samples taken when the plants were 30 ± 5 cm in height. Asterisk (*) indicates significant difference between Tob^{SbtA} and Tob^{WT} plants as determined using unpaired *t*-test (P>0.05). Data presented as mean ± SD; n=4-6. **(H)** Comparison of Tob$^{SbtA-ubi}$ and Tob^{WT} plants 63 days post cotyledon emergence.

Figure 1: Phylogenetic tree showing the relative protein relationships between members of the SbtA family across cyanobacteria.

The primary sequence alignment, and derived tree, were prepared using Geneious Prime software and iTol (Letunic and Bork, 2024). The SbtA2 family is highlighted in blue and the SbtA1 family in pink. The internal size marker indicates 0.1 substitutions per residue. Red branches indicate α-cyanobacteria and purple branches indicate β-cyanobacteria. Coloured bold text indicates that HCO_3^- uptake activity has been assessed with those and in black bold text demonstrating no function in E. coli (this study and Du *et al.*, 2014). The outgroup SbtA1 from *Labrenzia* DFL-11, an α-proteobacterium found in close association with dinoflagellates of the *Alexandrium* genus (Biebl *et al.*, 2007), was previously assessed for function by (Du *et al.*, 2014).

Figure 2: **The HCO$_3^-$ transport function of SbtA2 was assessed using complementation assays and HCO$_3^-$ uptake assays.**

(A) Cultures of CAfree E. coli expressing SbtA2 homologues were adjusted to the same density prior to being spotted onto LB-agar containing 100 µM IPTG and grown at ambient (0.04%) or 4% CO$_2$ for ~20 h. The growth of SbtA2 members (Synechococcus WH5701, RCC307, CB0205 and Prochlorococcus MIT0701 and MIT9313) were compared to a negative control (empty vector; EV) and a positive control (SbtA1 from Synechococcus elongatus PCC7942; SbtA1-7942). **(B)** The HCO$_3^-$ uptake activity of SbtA2-WH5701 (A2), SbtA1 from Synechococcus PCC7942 (SbtA1-7942; A1) and empty vector (EV). n=12 from 3 biological replicates. **(C)** HCO$_3^-$ uptake activity of SbtA2 from Synechococcus WH5701 (WH), RCC307 (RCC) and CB0205 (CB). n=12 from 3 biological replicates. HCO$_3^-$ uptake assay buffer composition: BTP - H$_2$SO$_4$ pH 7.5 + 20 mM NaCl. Different letters indicate significantly different means when analysed by one-way ANOVA (P<0.05).

Figure 3: Assessment of the counter-ion driving the HCO₃⁻ uptake function of SbtA2.

Analysis of ion dependency was determined using HCO_3^- uptake assays in DH5α strain of E. coli. Values presented have empty vector rates for each condition subtracted. Different letters indicate significantly different means when analysed by one-way ANOVA ($P<0.05$). **(A)** The HCO_3^- uptake activity of SbtA2-WH5701 (SbtA2) and SbtA1-7942 (SbtA1) was assessed in the presence of 0, 1, 5 or 20 mM NaCl. Assay buffer composition: 20 mM $BTP-H_2SO_4$ pH 7.5 + (0, 1, 5, or 20 mM NaCl). Na^+ provided by $NaH^{14}CO_3$/$KHCO_3$ was ~20 µM. The connecting line represents the mean; n= 3-4 from 1 biological replicate. **(B)** The HCO_3^- uptake rates of SbtA2-WH5701 in the presence of 0 and 20 mM NaCl in the assay buffer were compared. Assay buffer composition: 20 mM $BTP-H_2SO_4$ pH 7.5 + (0 or 20 mM NaCl). Data presented as mean ± SD; n=27 from 8 biological replicates. **(C)** The HCO_3^- uptake activity of SbtA2-WH5701 was measured in response to an increase in NaCl concentration in the assay buffer. Uptake assay buffer composition: 20 mM $BTP-H_2SO_4$ pH 7.5 + 10 mM K_2HPO_4 + (0, 50, 100 or 200 mM NaCl). n=11-16 from 3-4 biological replicates. **(D)** The HCO_3^- uptake activity of SbtA2-WH5701 was assessed in the presence of various salts; no additional NaCl, 100 mM NaCl, 100 mM KCl, 100 mM LiCl and 100 mM $NaNO_3$ added to the assay buffer of 20 mM $BTP-H_2SO_4$ pH 7.5. Salt concentrations added to the uptake buffer are in mM. 20 µM Na^+ was supplied by $NaH^{14}CO_3$. Data presented as mean ± SD; n=16-20 from 4-5 biological replicates. **(E)** The HCO_3^- uptake activity of SbtA2-WH5701 was assessed in response to different salts; no additional NaCl, 100 mM NaCl, 50 mM $MgCl_2$, 50 mM $MgSO_4$ and 50 mM $NaSO_4$, 200 mM NaCl or 100 mM $MgCl_2$ added to the assay buffer of 20 mM $BTP-H_2SO_4$ pH 7.5. 20 µM Na^+ was supplied by $NaH^{14}CO_3$. Data presented as mean ± SD; n=8-12 from 2-3 biological replicates.

Figure 4: The affinity (K_m) for HCO$_3^-$ was determined for SbtA2 from *Synechococcus* WH5701. The affinity for HCO$_3^-$ was estimated using Ci uptake assays performed in response to increasing exogenous HCO$_3^-$ concentrations in E. coli DH5α. The HCO$_3^-$ affinity of SbtA2-WH5701 was assessed in the presence of 20 mM NaCl or 100 mM NaCl. Assay buffer composition: BTP - H$_2$SO$_4$ pH 7.5 + (20 mM NaCl or 100 mM NaCl). HCO$_3^-$ uptake rates at each HCO$_3^-$ concentration had empty vector rates specific for that condition subtracted. Similar trends were observed across experiments although the activity values varied, as a result representative data from single experiments are shown. Individual data values are shown along with their fitted Michaelis-Menten curve which is presented as a solid line. Curve fitting protocols are stated in the materials and methods section.

Figure 5: Assessment of the effect of SbtB2 on SbtA2 HCO$_3^-$ transport function.

(A) Cultures of CAfree were adjusted to the same density prior to spotting onto LB-agar containing antibiotics and 100 μM IPTG and grown at ambient (0.04%) or 4% CO$_2$ for ~20 h. The cultures assessed included positive control SbtA1 from *Synechococcus* PCC7942, (SbtA1-7942) SbtA2:B2-HA from *Synechococcus* WH5701, RCC307, and CB0205, a negative control (empty vector; EV) and positive controls (SbtA2-WH5701, SbtA2-RCC307, SbtA2-CB0205). **(B)** The relative HCO$_3^-$ uptake activity of DH5α *E. coli* carrying SbtA2:B2-HA-WH5701 and SbtA2:B2-WH5701 compared to SbtA2-WH5701 (A2). Assay buffer composition: 20 mM BTP-H$_2$SO$_4$ pH 7.5. Data presented as the mean with empty vector control subtracted ± SD; n=8 from 2 biological replicates. **(C)** The relative HCO$_3^-$ uptake activity of DH5α *E. coli* carrying *lacl^q*-SbtA2-H6:B2-HA-WH5701 (*l*AB2), *lacl^q*-SbtA2-H6:*tet*-B2-HA-WH5701 (*l*A2*t*B2) and *lacl^q*-SbtB2-HA:A2-H6-WH5701 (*l*BA2) compared to *lacl^q*-SbtA2-H6-WH5701 (*l*A2). Assay buffer composition: 20 mM BTP-H$_2$SO$_4$ pH 7.5 + 200 mM NaCl. Data presented as the mean ± SD with empty vector controls subtracted; n= 12 from 3 biological replicates.

Figure 6 : Phenotypic measurements of *N. tabacum* expressing SbtA-HAH6-WH5701 (Tob^SbtA) compared to wild-type (Tob^WT) plants.

(A) The heights of plants were measured 63 days post cotyledon emergence (pce) from the base of the plants to the base of the apical meristem. The data is presented as means ± SD; n=6. **(B)** Each plant was harvested at the base of the stem 63 days pce and the fresh weight was measured. **(C)** The

harvested plants were dried for 7 days at 80°C and the weight were measured. Data presented as mean ± SD; n=6. **(D-F)** A/C_{int} curves were measured on plants 30 ± 5 cm in height on young fully expanded leaves of similar size using a Licor 6800 with the following parameters; photon flux density 1500 µmol·m^{-2}·s^{-1}, at 25°C and photosynthetic measurements taken at 400, 200, 100, 50,150, 250, 400, 600, 800, 1200, 400 µmol·mol^{-1} CO_2. Data presented as mean ± SD. n= 3-5 for Tob^{SbtA2} and n=9 for Tob^{WT}. **(G)** $\Delta^{13}C$ was determined from dry matter samples taken when the plants were 30 ± 5 cm in height. Asterisk (*) indicates significant difference between Tob^{SbtA} and Tob^{WT} plants as determined using unpaired t-test (P>0.05). Data presented as mean ± SD; n=4-6. **(H)** Comparison of Tob$^{SbtA-ubi}$ and Tob^{WT} plants 63 days post cotyledon emergence.

Tree scale: 0.1

100 mM NaCl
20 mM NaCl