This is a repository copy of *Thylakoid localized bestrophin-like proteins are essential for the CO2 concentrating mechanism of Chlamydomonas reinhardtii*.

White Rose Research Online URL for this paper:
http://eprints.whiterose.ac.uk/148641/

Version: Published Version

**Article:**
Mukherjee, Ananya, Lau, Chun Sing, Walker, Charlotte Elizabeth et al. (8 more authors) (2019) Thylakoid localized bestrophin-like proteins are essential for the CO2 concentrating mechanism of Chlamydomonas reinhardtii. Proceedings of the National Academy of Sciences of the United States of America. ISSN 1091-6490

https://doi.org/10.1073/pnas.1909706116

**Reuse**
This article is distributed under the terms of the Creative Commons Attribution (CC BY) licence. This licence allows you to distribute, remix, tweak, and build upon the work, even commercially, as long as you credit the authors for the original work. More information and the full terms of the licence here:
https://creativecommons.org/licenses/

**Takedown**
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.
Thylakoid localized bestrophin-like proteins are essential for the CO₂ concentrating mechanism of Chlamydomonas reinhardtii

Ananya Mukherjee1, Chun Sing Lau2, Charlotte E. Walker3, Ashwani K. Rai4, Camille I. Prejean5, Gary Yates5, Thomas Emrich-Mills5, Spencer G. Lemoine5, David J. Vinyard5, Luke C. M. Mackinder5,6, and James V. Moroney1,7

1Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803; and 2Department of Biology, University of York, Heslington, York YO10 5DD, United Kingdom

Edited by Krishna K. Niyogi, Howard Hughes Medical Institute and University of California, Berkeley, CA, and approved July 15, 2019 (received for review June 7, 2019)

The green alga Chlamydomonas reinhardtii possesses a CO₂ concentrating mechanism (CCM) that helps in successful acclimation to low CO₂ conditions. Current models of the CCM postulate that a series of ion transporters bring HCO₃⁻ from outside the cell to the thylakoid lumen, where the carbonic anhydrase 3 (CAH3) dehydrates accumulated HCO₃⁻ to CO₂, raising the CO₂ concentration for Ribulose bisphosphate carboxylase/oxygenase (Rubisco). Previously, HCO₃⁻ transporters have been identified at both the plasma membrane and the chloroplast envelope, but the transporter thought to be on the thylakoid membrane has not been identified. Three paralogous genes (BST1, BST2, and BST3) belonging to the bestrophin family have been found to be up-regulated in low CO₂ conditions, and their expression is controlled by CIA5, a transcription factor that controls many CCM genes. YFP fusions demonstrate that all 3 proteins are located on the thylakoid membrane, and interactome studies indicate that they might associate with chloroplast CCM components. A single mutant defective in BST2 has near-normal growth on low CO₂, indicating that the 3 bestrophin-like proteins may have redundant functions. Therefore, an RNA interference (RNAi) approach was adopted to reduce the expression of all 3 genes at once. RNAi mutants with reduced expression of BST1–3 were unable to grow at low CO₂ concentrations, exhibited a reduced affinity to inorganic carbon (C₅) compared with the wild-type cells, and showed reduced C₅ uptake. We propose that these bestrophin-like proteins are essential components of the CCM that deliver HCO₃⁻ accumulated in the chloroplast stroma to CAH3 inside the thylakoid lumen.

Chlamydomonas | CO₂ concentrating mechanism | bicarbonate transport | photosynthesis | chloroplast thylakoid

Aquatic photosynthetic organisms, which account for close to 50% of the world’s carbon fixation (1), face several challenges in carrying out efficient photosynthesis. Limitations include the slow diffusive rate of gases in water, fluctuations in pH, and the slow interconversion of inorganic carbon (C₅) forms. Thus, most aquatic autotrophs have developed an adapted called the CO₂ concentrating mechanism (CCM) that increases the concentration of CO₂ around Ribulose bisphosphate carboxylase/oxygenase (Rubisco) to increase its carboxylase activity. Aside from Rubisco’s slow rate of catalysis, O₂ can compete with CO₂ for the active site of the enzyme, resulting in the wasteful process of photorespiration (2). Since CO₂ and O₂ are competitive substrates, the CCM reduces photorespiration and increases photosynthetic efficiency.

The CCM of the unicellular green alga Chlamydomonas reinhardtii (hereafter referred to as Chlamydomonas) has a number of bicarbonate (HCO₃⁻) transporters that help increase the HCO₃⁻ concentration in the chloroplast stroma relative to the external HCO₃⁻ concentration. These transporters are located on the plasma membrane (LCI1 and HLA3) as well as the chloroplast envelope (NAR1.2/LC1A). Loss of any one of these transporters reduces the ability of the cell to accumulate HCO₃⁻ at high external pH (3, 4). In addition, Rubisco is tightly packaged in a microcompartment of the chloroplast called the pyrenoid (5–7). Finally, carbonic anhydrase 3 (CAH3), located in the lumen of pyrenoid-traversing thylakoids, converts the accumulated HCO₃⁻ to CO₂ near the site of Rubisco (8, 9), increasing photosynthetic and growth rates at otherwise growth-limiting CO₂ levels.

Carbonic anhydrases play an essential role in the Chlamydomonas CCM (10). The loss of CAH3 results in cells that cannot grow on air levels of CO₂, even though these mutants tend to overaccumulate HCO₃⁻ (11). Chlamydomonas CCM models propose that mutants missing CAH3 accumulate the HCO₃⁻ brought into the chloroplast by the transport proteins but cannot convert that HCO₃⁻ to CO₂, the actual substrate of Rubisco (12, 13). These CCM models postulate that the pH gradient across the thylakoid membrane in the light helps drive the conversion of HCO₃⁻ to CO₂. The apparent acid dissociation constant (pKₐ) of the interconversion of HCO₃⁻ and CO₂ is about 6.4, with the chloroplast stoma having a pH close to 8 in the light and the thylakoid lumen having a pH close to 5.7 under low CO₂ concentrations (14). Therefore, as HCO₃⁻ is brought from the stroma to the thylakoid lumen, it goes from an environment favoring HCO₃⁻ to one favoring CO₂. Therefore, the

Significance

Models of the CO₂ concentrating mechanism (CCM) of green algae and diatoms postulate that chloroplast CO₂ is generated from HCO₃⁻ brought into the acidic thylakoid lumen and converted to CO₂ by specific thylakoid carbonic anhydrases. However, the identity of the transporter required for thylakoid HCO₃⁻ uptake has remained elusive. In this work, 3 bestrophin-like proteins, BST1–3, located on the thylakoid membrane have been found to be essential to the CCM of Chlamydomonas. Reduction in expression of BST1–3 markedly reduced the inorganic carbon affinity of the alga. These proteins are prime candidates to be thylakoid HCO₃⁻ transporters, a critical currently missing step of the CCM required for future engineering efforts of the Chlamydomonas CCM into plants to improve photosynthesis.

Author contributions: A.M., C.S.L., C.E.W., D.J.V., L.C.M.M., and J.V.M. designed research; A.M., C.S.L., C.E.W., D.J.V., L.C.M.M., and J.V.M. performed research; A.M., C.S.L., C.E.W., A.K.R., C.I.P., G.Y., T.E.-M., S.G.L., and J.V.M. analyzed data; and A.M., C.S.L., C.E.W., A.K.R., C.I.P., G.Y., T.E.-M., S.G.L., and J.V.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution License 4.0 (CC BY).

1To whom correspondence may be addressed. Email: luke.mackinder@york.ac.uk or btmoro@lsu.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1909706116/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1909706116
acidification of the thylakoid lumen is important to the functioning of the CCM.

The CCM models also proposed the presence of a thylakoid HCO$_3^-$ transporter that brings in HCO$_3^-$ from the stroma to the lumen for dehydration by CAH3 (12, 13). In a recent interactome study, the CCM complex LCIC/LCIC is shown to interact with the bestrophin-like proteins encoded by Cre16.g662600 and Cre16.g663450 (15). These proteins were also shown to interact with each other and another bestrophin-like protein encoded by Cre16.g663400 (15). All 3 genes were found to be up-regulated in low CO$_2$ conditions in a transcriptomic study showing they belonged to a cluster of genes that had increased expression in low CO$_2$ and were controlled by CIA5 (16). Bestrophins are typically chloride channels, including the Arabidopsis bestrophin-like protein on the 16th chromosome of Chlamydomonas. However, they have also been shown to transport a range of anions, with some showing high HCO$_3^-$ permeability (18). The interactome study also putatively localizes these bestrophin-like proteins to the thylakoid membrane, which makes them promising candidates to be the thylakoid HCO$_3^-$ transporter in the CCM of Chlamydomonas.

In the present study, we investigate the role of these 3 proteins using an RNA interference (RNAi) approach to knock down the expression of all 3 genes. This approach was feasible as the 3 genes are extremely similar at the DNA sequence level. Knockdown mutants were subsequently grown poorly under limiting CO$_2$ conditions, exhibit a poor affinity for external C$^-$, and have a severely reduced ability to accumulate HCO$_3^-$. This study sheds light on the intracellular location and function of these bestrophin-like proteins in the CCM of Chlamydomonas.

Results

Chlamydomonas Has 3 Very Similar Bestrophin-Like Proteins on the Thylakoid Membrane. BST1 (Cre16.g662600), BST2 (Cre16.g663400), and BST3 (Cre16.g663450) (collectively BST1–3) are paralogous bestrophin-like genes located within a 130-kilobase pair (kbp) region on the 16th chromosome of Chlamydomonas. Phylogenetic analyses revealed that bestrophin-like proteins are found in a diverse variety of photosynthetic organisms (Fig. 1), including vascular plants, nonvascular plants, and diatoms, with the homologs with the highest sequence identity to BST1–3 found in algae. The amino acid sequences encoded by these genes were analyzed in TMMHH, which predicted that BST1–3 are membrane proteins having 4 predicted transmembrane domains each. Further analysis using PredAlgo predicted that each BST protein had a chloroplast transit peptide and was likely to be a chloroplast membrane protein. BST1 was annotated as a bestrophin-like protein in Phytozome (version 12.1), and BST2 and BST3 were previously reported as LC111 by Fang et al. (16). An alignment between the 3 Chlamydomonas bestrophin-like proteins showed that the proteins are >80% identical to one another (SI Appendix, Fig. S1). There are 7 more genes annotated as encoding bestrophin-like proteins in the Chlamydomonas genome, but they share less than 50% identity to BST1–3. Sequence alignment of BST1–3 with human Bestrophin 1 (BEST1) showed low sequence identity between BEST1 and BST1–3 (21 to 23%; SI Appendix, Fig. S1). The most similar protein in terrestrial plants, the thylakoid localized AtVCCN1 protein of Arabidopsis (17), has approximatively a 30% sequence identity with BST1–3. To further explore the potential structure and function of BST1–3, we did homology modeling using SWISS-MODEL (19). Structural studies show that human and Klebsiella pneumoniae bestrophins are pentameric, and modeling of BST1 in a pentameric assembly is of high confidence (SI Appendix, Fig. S2A). The highest ranking template identified by SWISS-MODEL for BST1–3 was K. pneumoniae bestrophin. BST1–3 contain nonpolar residues along their selective pore that are conserved in proteins of the bestrophin family and are involved in anion transport (20) (SI Appendix, Fig. S2B). The electrostatic potential of BST1 has a predominantly neutral/negative electrostatic potential, and the selective pore is positively charged, suggesting the homology of BST1–3 transport negatively charged ions (19, 21) (SI Appendix, Fig. S2 C and D), as does AtVCCN1 in Arabidopsis (17).

Fig. 1. Phylogenetic analysis of Chlamydomonas bestrophin-like proteins BST1–3. The evolutionary history of Chlamydomonas bestrophin-like proteins BST1–3 was inferred by using the maximum likelihood method based on the Le and Gascuel (37) model with discrete Gamma distribution (3 categories) and 500 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Bootstrap value ≥ 50, **Bootstrap value ≥ 90.

| | Siliqua pseudocapsa | Theobroma cacao | Solanum tuberosum | Physcomitrella patens | Sphagnum fallax | Nicotiana tomentosiformis | Arabidopsis thaliana | Brassica rapa | Physcomitrella patens |
|---|---|---|---|---|---|---|---|---|---|
| BST1 | + | + | + | + | + | + | + | + | + |
| BST2 | + | + | + | + | + | + | + | + | + |
| BST3 | + | + | + | + | + | + | + | + | + |

| | Siliqua pseudocapsa | Theobroma cacao | Solanum tuberosum | Physcomitrella patens | Sphagnum fallax | Nicotiana tomentosiformis | Arabidopsis thaliana | Brassica rapa | Physcomitrella patens |
|---|---|---|---|---|---|---|---|---|---|
| BST1 | + | + | + | + | + | + | + | + | + |
| BST2 | + | + | + | + | + | + | + | + | + |
| BST3 | + | + | + | + | + | + | + | + | + |

BST1–3 Are Up-Regulated under Low CO$_2$ Growth Conditions and Localized to the Thylakoid. Semiquantitative RT-PCR (Fig. 2A) was performed using complementary DNA isolated from strains D66 and cia5 grown under high CO$_2$ or ambient CO$_2$ conditions. For this reason, we all used 5% CO$_2$ (vol/vol) air as high CO$_2$ 0.04% as ambient CO$_2$, and <0.02% as low CO$_2$. D66 is the wild-type strain for these studies, and cia5 is missing the CCM1 protein, which is required for the induction of the CCM in Chlamydomonas (22). This work demonstrated that all 3 BST genes were up-regulated under ambient CO$_2$ growth conditions in D66 and that this up-regulation was not observed in cia5 (SI Appendix, Fig. S3A). In addition, the cia5 mutant exhibited severely reduced expression of BST1 and BST3 under both CO$_2$ conditions, a transcriptional pattern observed with other CCM genes. BST2 transcript levels in cia5 cells showed reduced induction in ambient CO$_2$ when compared with D66 cells, where BST2 transcript levels increase in ambient CO$_2$ conditions. A time course study of the expression of these 3 genes during induction of the CCM was done by transferring high CO$_2$-grown cells to ambient CO$_2$ levels for 2 to 12 h (Fig. 2B and SI Appendix, Fig. S3B). All 3 genes had increased transcript levels within 2 h after the switch to low CO$_2$, and these elevated levels of expression continued until at least 12 h after induction. BST1 had a lower level of expression than BST2 or BST3 (Fig. 2). To determine the localization of these 3 BST-like proteins in Chlamydomonas, fluorescent protein fusions were constructed linking Venus to the C terminus of each BST protein. All 3 BST-like proteins localized to the thylakoid membranes of the chloroplast (Fig. 3A), and this localization visibly extended into the thylakoid tubules of the pyrenoid (Fig. 3B). The localization
we checked BST3 protein levels in the knockdown lines. All showed reduced levels relative to D66, although this was only significant for bsti-2 and bsti-3 (P < 0.05; Student’s t test; SI Appendix, Fig. S64). Thus, the BSTs are required for wild-type–like growth of *Chlamydomonas* under low CO$_2$ conditions.

**Reduction of BST1–3 Expression Also Results in Cells that Have a Reduced Capacity to Accumulate Inorganic Carbon.** Two characteristics of algal cells with a CCM are a very high affinity for C$_i$ and the ability to accumulate C$_i$ to levels higher than can be obtained by diffusion. The bsti-1, bsti-2, and bsti-3 acclimated to ambient CO$_2$ exhibited a lower affinity for C$_i$ as judged by their measured C$_i$ concentration needed for half-maximum oxygen evolution (K$_{1/2}$) (Fig. 5). When grown at high CO$_2$, bsti1–3 and D66 exhibited similar C$_i$ affinities (SI Appendix, Fig. S6B). These results indicate that the expression of BST1–3 is required for optimal C$_i$ affinity when cells are grown on ambient levels of CO$_2$. At pH 8.4, the K$_{1/2}$ values for bsti1–3 are elevated in sharp contrast to a low K$_{1/2}$ for D66 (Fig. 5 A and B). At the higher pH of 8.4, the predominant C$_i$ species in the medium would be HCO$_3^−$. Thus, the increased affinity of the cells for C$_i$ reflects their ability to actively take up and utilize HCO$_3^−$. For bsti-1, where the expression of all 3 BST genes is between 60 and 90% reduced, there is a reduced C$_i$ affinity at both pH 8.4 (Fig. 5 A and B) and pH 7.8 (Fig. 5 C and D). In contrast, bsti-3, the mutant missing only BST3, the difference in C$_i$ affinity with wild type (SI Appendix, Fig. S2B) is much smaller. Thus, we can conclude that BST1–3 are necessary components of the CCM of *Chlamydomonas*.

C$_i$ uptake activity was measured in D66, bsti-1, bsti-2, and bsti-3 to evaluate the importance of BST1–3 in accumulation and

**Reduction of BST1–3 Expression Also Results in Cells that Grow Slowly under Low CO$_2$ Conditions.** A BST3 knockout (bst3) was obtained from the *Chlamydomonas* Library Project (CLiP) mutant collection (23) with a paromomycin insert in the last exon of the bst3 gene (SI Appendix, Fig. S4A). The BST3 transcript was not detected in bst3 (SI Appendix, Fig. S4B), and the BST3 protein was absent (SI Appendix, Fig. S4C). We observed a weak growth difference for this strain as compared with wild-type cells under ambient CO$_2$ (SI Appendix, Fig. S5 A and B), but no clear phenotype on plates at pH 7 or pH 8.4 at 100 μmol of photons per m$^2$·s$^1$ at low CO$_2$ (SI Appendix, Fig. S5C). However, there was no significant difference in C$_i$ affinity between wild type and bst3 grown at ambient CO$_2$ (SI Appendix, Fig. S5D), and C$_i$ uptake by bst3 was only slightly lower than wild type (SI Appendix, Fig. S5 E and F). This led us to think that BST1 or BST2 function might be redundant with BST3 and that the expression of all 3 genes must be reduced to determine their physiological role(s). Therefore, to elucidate the function of BST1–3, RNAi constructs complementary to regions of identity among BST1–3 were designed (SI Appendix, Table S1). The D66 strain was transformed with these constructs, and colonies were kept at high CO$_2$. Colonies were then screened for growth on high CO$_2$ versus low CO$_2$, and BST1–3 expression was quantified using RT-qPCR. Three independent colonies from 2 different transformations were chosen for further study and designated as bsti-1, bsti-2, and bsti-3 (BST RNAi triple-knockdown lines 1, 2, and 3).

The growth of bsti-1, bsti-2, and bsti-3 on high and low CO$_2$ was compared with D66 and the CAH3 knockout mutant, cia5 (Fig. 4A). In low CO$_2$, bsti-1 showed severely reduced growth that was further exacerbated at high pH, resembling the growth of cia5 (Fig. 4A). The bsti-2 and bsti-3 also grew more slowly than wild-type cells, but better than bsti-1. However, at high CO$_2$, the growth of all 3 strains was comparable to wild type. RT-qPCR showed that bsti-1 had significantly reduced expression of BST1, BST2, and BST3 compared with D66 (Fig. 4B), and bsti-2 and bsti-3 had a more moderate knockdown of expression of the 3 genes. To see if reduced transcript levels resulted in decreased protein abundance,

**Reduction of BST1–3 Expression Also Results in Cells that Have a Reduced Capacity to Accumulate Inorganic Carbon.** Two characteristics of algal cells with a CCM are a very high affinity for C$_i$ and the ability to accumulate C$_i$ to levels higher than can be obtained by diffusion. The bsti-1, bsti-2, and bsti-3 acclimated to ambient CO$_2$ exhibited a lower affinity for C$_i$ as judged by their measured C$_i$ concentration needed for half-maximum oxygen evolution (K$_{1/2}$) (Fig. 5). When grown at high CO$_2$, bsti1–3 and D66 exhibited similar C$_i$ affinities (SI Appendix, Fig. S6B). These results indicate that the expression of BST1–3 is required for optimal C$_i$ affinity when cells are grown on ambient levels of CO$_2$. At pH 8.4, the K$_{1/2}$ values for bsti1–3 are elevated in sharp contrast to a low K$_{1/2}$ for D66 (Fig. 5 A and B). At the higher pH of 8.4, the predominant C$_i$ species in the medium would be HCO$_3^−$. Thus, the increased affinity of the cells for C$_i$ reflects their ability to actively take up and utilize HCO$_3^−$. For bsti-1, where the expression of all 3 BST genes is between 60 and 90% reduced, there is a reduced C$_i$ affinity at both pH 8.4 (Fig. 5 A and B) and pH 7.8 (Fig. 5 C and D). In contrast, bsti-3, the mutant missing only BST3, the difference in C$_i$ affinity with wild type (SI Appendix, Fig. S2B) is much smaller. Thus, we can conclude that BST1–3 are necessary components of the CCM of *Chlamydomonas*.

C$_i$ uptake activity was measured in D66, bsti-1, bsti-2, and bsti-3 to evaluate the importance of BST1–3 in accumulation and
fixation of C3. Ambient CO2-acclimated bsti-1 had a notably lower accumulation and fixation of 14C3 compared with D66 at pH 8.4 (Fig. 6), and bsti-2 and bsti-3 also had inhibited 14C3 uptake and fixation, although not as reduced as bsti-1 (Fig. 6). The most severely affected mutant, bsti-1, accumulated 14C3 to only 30 to 40% of the levels observed in D66 cells. These results indicate that BST1-3 play an important role in C3 uptake and fixation in low CO2 conditions in Chlamydomonas.

A bestrophin-like protein recently discovered in Arabidopsis, AtVCCN1, is a Cl− channel that helps regulate the proton motive force (pmf) in the Arabidopsis thylakoid (17). Elimination of AtVCCN1 results in plants that have an increased pmf, altering how the plant regulates nonphotochemical quenching and the ΔpH across the thylakoid membrane. It is possible that the reduction of these BST proteins in Chlamydomonas could render cells less able to regulate the membrane potential (Δψ) and ΔpH components of the pmf, leading to photodamage or to an adenine 5′-triphosphate (ATP)/NADPH imbalance. To investigate if BST1-3, in addition to being critical for C3 affinity and accumulation, have a role in regulating pmf similar to AtVCCN1, we measured electrophotographic shift to estimate the pmf in the knockdown lines under HCO3−-depleted conditions (SI Appendix, Fig. S7). We found a small reduction of the pmf in the bsti mutants (SI Appendix, Fig. S7 A and B), which is opposite to what is seen in Arabidopsis. In addition, the pmf decayed slightly faster in the bsti-1 and bsti-3 mutants than in wild type (SI Appendix, Fig. S7C). We also measured the yield of variable chlorophyll a fluorescence to estimate photosystem II function in the mutants and found that Fv/Fm was the same in mutant and wild-type cells (SI Appendix, Fig. S7D). The fact that the bsti-3 mutants grew normally at relatively high light levels (Fig. 4A) indicates that reducing BST1-3 does not cause severe photodamage.

In conclusion, the localization, the C3 affinity, and the C3 accumulation phenotypes of the bsti triple-knockdown mutants support an essential role for BST1-3 in the CCM.

Discussion

We present evidence here that BST1–3 are chloroplast thylakoid localized anion transporters that are important components of the Chlamydomonas CCM. Cells that have reduced BST1–3 transcript levels fail to grow on low CO2 (Fig. 4), have a lower affinity for C3 (Fig. 5), and have a reduced ability to accumulate added 14C2 (Fig. 6). A key aspect of current Chlamydomonas CCM models is that accumulated HCO3− is converted to CO2 by CAH3, a carbonic anhydrase located in the thylakoid lumen (11–13). This feature of algal CCMs may extend to other algal types, notably diatoms, where Kikutani et al. (24) recently discovered a 0-type carbonic anhydrase within the thylakoid of Phaeodactylum tricornutum that was required for CCM function. These CCM models predict that a thylakoid HCO3− transporter is required to deliver HCO3− from the chloroplast stroma to the thylakoid lumen. We propose that BST1–3 are the transporters that bring HCO3− to CAH3 inside the thylakoid.

Members of the human bestrophin family transport both HCO3− and Cl− ions (18). The homology modeling presented here supports the function of BST1–3 as anion transporters, with BST1–3 having predicted structural and conserved transport residue similarities to chicken and bacterial bestrophins (SI Appendix, Fig. S2).

The expression of all of the CCM transporters discovered previously is induced by ambient or lower CO2 conditions, and their expression is controlled by the transcription factor CIAS/CCM1 (22, 25). We have observed that all 3 BST genes are induced when Chlamydomonas is grown under ambient CO2 conditions (Fig. 2) and that this induction is absent in the cia3 mutant (Fig. 24). In addition, LCIB and LCIC, possible 0-carboxy anhydrases (24, 26) essential to the CCM (4) that interact with BST1–3 (15), have the same expression pattern.

Fig. 4. Growth of bsti-1–3 triple-knockdown RNAi lines and relative expression of BST1–3 in the triple-knockdown lines. (A) Spot tests showing growth of D66, cia3, and bsti-1–3. Cells were diluted to 6.6 x 106 cells per milliliter, followed by 1:10 serial dilution 3 times to compare growth in low CO2 (<0.02% CO2), ambient CO2 (0.04% CO2), and high CO2 (5% CO2 [vol/vol] in air) at pH 7 and pH 8.4. Cells were grown for 6 d. The CAH3 mutant, cia3, was included as a CCM-deficient control. (B) RT-qPCR shows that the expression of all 3 BST genes in the triple-knockdown lines is reduced when compared with their expression levels seen in D66. D66 and bsti-1, bsti-2, and bsti-3 were acclimated to air levels of CO2 for 12 h before harvesting the RNA. *P < 0.05 by Student t test.
Thus, the expression of the BST1–3 genes is consistent with these proteins playing a role in the uptake and accumulation of C\textsubscript{i} when Chlamydomonas is exposed to low CO\textsubscript{2} conditions.

An alternative hypothesis is that the 3 BST proteins have a function similar to AtVCCN1 (20) and are involved in C\textsuperscript{+} transport to regulate the pmf across the thylakoid. The presence of AtVCCN1 decreases pmf in Arabidopsis, but the presence of the 3 BST proteins increases pmf in Chlamydomonas. This result, in combination with our genetic and physiology data, suggests that the function of the BST proteins in Chlamydomonas is not the same as VCCN1 in Arabidopsis. A further understanding of this interconnection and the balancing/regulation of pmf within the context of the CCM is critical.

In Chlamydomonas, there seems to be a built-in redundancy of C\textsubscript{i} transporter functions. For example, both LCI1 and HLA3 are present on the plasma membrane, and loss of only one of the proteins fails to cause an extreme growth phenotype at low CO\textsubscript{2} (3). However, when more than 1 transporter is knocked down, a significant change in C\textsubscript{i} uptake and growth is observed (4, 5). BST1–3 also appear to have redundant or overlapping functions. This is demonstrated in this study, as knocking out BST3 by itself did not cause a drastic change in growth or reduction in C\textsubscript{i} affinity at ambient levels of CO\textsubscript{2} (SI Appendix, Figs. S4 and S5).

However, when the expression of all 3 genes is decreased in bst\textsubscript{i}-1, bst\textsubscript{i}-2, and bst\textsubscript{i}-3 simultaneously, the ability of the cell to uptake C\textsubscript{i} by itself is not completely lost. The pH of the chloroplast stroma, thought to be near 8.0, is well above the pK\textsubscript{a} of HCO\textsubscript{3}\textsuperscript{-}, while the pH of the thylakoid lumen is thought to be close to 5.7 (14), below the pK\textsubscript{a}. When HCO\textsubscript{3}\textsuperscript{-} moves from the chloroplast stroma to the thylakoid lumen, it moves from an environment that favors HCO\textsubscript{3}\textsuperscript{-} to one that favors CO\textsubscript{2}. This effectively allows the algal cells to increase the CO\textsubscript{2} concentration to levels higher than could be obtained by the action of carbonic anhydrase alone. Thus, a trans-thylakoid pH gradient is necessary for this proposed “CO\textsubscript{2} pump,” and this pH gradient is set up by the photosystems and requires light. To date, all experimental data available indicate that light and the activity of the photosystems are required for the Chlamydomonas CCM to function. In fact, some of the earliest work in the field indicated that electron transport inhibitors and mutations that disrupt electron transport also inhibited the Chlamydomonas CCM (31, 32). One potential problem with this CO\textsubscript{2} pump model is that it would partially reduce the pmf across the thylakoid membrane, thus reducing ATP biosynthesis. However, it should be pointed out that only a single H\textsuperscript{+} would be consumed per CO\textsubscript{2} generated, which is not fixed by Rubisco has the potential to simply diffuse out of the cell (4, 27–29). The LCIB/C complex is thought to help recapture this CO\textsubscript{2} by directionally driving CO\textsubscript{2} to HCO\textsubscript{3}\textsuperscript{-} or by acting as a tightly regulated carbonic anhydrase (30) at the pyrenoid periphery (28). This is interesting because there are data supporting the interaction of LCIB/C with BST1 and BST3 (15). Our model adds BST1–3 to this hypothesized recapture system (Fig. 7).

The discovery of CCM components on the thylakoid (BST1–3) and inside the thylakoid lumen (CAH3) also indicates how light energy may be used to energize the CCM. The apparent pK\textsubscript{a} of the interconversion of HCO\textsubscript{3}\textsuperscript{-} to CO\textsubscript{2} is about 6.4. The pH of the chloroplast stroma, thought to be near 8.0, is well above the pK\textsubscript{a}, while the pH of the thylakoid lumen is thought to be close to 5.7, below the pK\textsubscript{a}. When HCO\textsubscript{3}\textsuperscript{-} moves from the chloroplast stroma to the thylakoid lumen, it moves from an environment that favors HCO\textsubscript{3}\textsuperscript{-} to one that favors CO\textsubscript{2}. This effectively allows the algal cells to increase the CO\textsubscript{2} concentration to levels higher than could be obtained by the action of carbonic anhydrase alone. Thus, a trans-thylakoid pH gradient is necessary for this proposed “CO\textsubscript{2} pump,” and this pH gradient is set up by the photosystems and requires light. To date, all experimental data available indicate that light and the activity of the photosystems are required for the Chlamydomonas CCM to function. In fact, some of the earliest work in the field indicated that electron transport inhibitors and mutations that disrupt electron transport also inhibited the Chlamydomonas CCM (31, 32). One potential problem with this CO\textsubscript{2} pump model is that it would partially reduce the pmf across the thylakoid membrane, thus reducing ATP biosynthesis. However, it should be pointed out that only a single H\textsuperscript{+} would be consumed per CO\textsubscript{2} generated, which is the
This cost is far less than the 2 additional ATPs required for $C_4$ photosynthesis, and $C_4$ photosynthesis has been shown to be energetically competitive with $C_3$ photosynthesis once the costs of photosynthesis are considered (35). In conclusion, BST1–3 are bestrophin-like, thylakoid localized membrane proteins that are synthesized in coordination with other CCM components, and their predicted structures fit well with functionally characterized bestrophins. As such, these are excellent candidates to be the HCO$_3^-$ transporters that not only bring HCO$_3^-$ into the thylakoid lumen for $C_4$ generation but may also play a role in $C_4$ recapture as well.

Materials and Methods

Cell Cultures, Growth, and Photosynthetic Assays. $C$. reinhardtii culture conditions were set according to the conditions used previously (34). The D66 strain (mt$^-$, cwv15, mt$^+$) was obtained from Rogene Schnell (University of Arkansas, Fayetteville, AR), and CMU030 (CC-4533; cwv15, mt$^+$) and bst3 (BT3 knockout LM1.RY0402.089365) were obtained from the CLiP collection at the Chlamydomonas culture collection (23, 35). For acclimation experiments, Tris-acetate-phosphate-grown cells were switched to minimal media and bubbled with high CO$_2$ (5% [vol/vol] CO$_2$ in air) to reach an optical density at 730 nm of 0.5–1.0 (equivalent to 3–2×10$^5$ cells per milliliter). This was followed by CCM induction when the cells were transferred to ambient CO$_2$ (0.04% CO$_2$) bubbling. For photosynthetic assays, cells acclimated to 5% or 0.04% CO$_2$ were resuspended in C-mutant S. maxima at pH 7.8 or pH 8.4, and O$_2$ evolution was measured at different C concentrations. K$_{i/2}$ was calculated as the C concentration needed for the half-maximal rate of oxygen evolution.

Fluorescence Protein Tagging and Confocal Microscopy. The BST1–3 genes driven by the constitutive PSAD promoter were cloned as reported by Mackinder et al. (15). Briefly, the open reading frames of BST1–3 genes were PCR-amplified from genomic DNA and cloned into pLM005 with C-terminal Venus-3xFLAG and a PSAD promoter through Gibson assembly. BST3 driven by its native promoter was cloned using recombineering based on methods reported by Sarov et al. (36). Transformation of these genes into Chlamydomonas and selection of colonies are described in SI Appendix, SI Materials and Methods. Images were captured with a laser-scanning microscope (LSM880; Zeiss) equipped with an Airyscan module using a 63× objective with a 1.4 numerical aperture. Argon lasers at 514 nm and 561 nm were used for excitation of Venus and chlorophyll, respectively. Filters were set at 525 to 550 nm for the Venus emission and at 620 to 670 nm for chlorophyll emission.

Additional details of materials and methods are provided in SI Appendix, SI Materials and Methods.

ACKNOWLEDGMENTS. We thank the University of York Biosciences Technology Facility for confocal microscopy support. The project was funded by Biotechnology and Biological Sciences Research Council Grant BB/R001014/1 to L.C.M.M.; Leverhulme Trust Grant RP2-2017-402 (to L.C.M.M. and C.E.W.); a University of York Biology Pump Priming award (to L.C.M.M.); and a subaward from the University of Illinois as part of the Realizing Increased Photosynthetic Efficiency project (to J.V.M.) funded by the Bill & Melinda Gates Foundation, Foundation for Food and Agriculture Research, and United Kingdom Aid.