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# Thylakoid localized bestrophin-like proteins are essential for the CO<sub>2</sub> concentrating mechanism of *Chlamydomonas reinhardtii*

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The green alga *Chlamydomonas reinhardtii* possesses a CO<sub>2</sub> concentrating mechanism (CCM) that helps in successful acclimation to low CO<sub>2</sub> conditions. Current models of the CCM postulate that a series of ion transporters bring HCO<sub>3</sub><sup>-</sup> from outside the cell to the thylakoid lumen, where the carbonic anhydrase 3 (CAH3) dehydrates accumulated HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub>, raising the CO<sub>2</sub> concentration for Ribulose biphosphate carboxylase/oxygenase (Rubisco). Previously, HCO<sub>3</sub><sup>-</sup> 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 CO<sub>2</sub> 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 *BST3* has near-normal growth on low CO<sub>2</sub>, 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 CO<sub>2</sub> concentrations, exhibited a reduced affinity to inorganic carbon (C<sub>i</sub>) compared with the wild-type cells, and showed reduced C<sub>i</sub> uptake. We propose that these bestrophin-like proteins are essential components of the CCM that deliver HCO<sub>3</sub><sup>-</sup> accumulated in the chloroplast stroma to CAH3 inside the thylakoid lumen.

*Chlamydomonas* | CO<sub>2</sub> 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 (C<sub>i</sub>) forms. Thus, most aquatic autotrophs have developed an adaptation called the CO<sub>2</sub> concentrating mechanism (CCM) that increases the concentration of CO<sub>2</sub> around Ribulose biphosphate carboxylase/oxygenase (Rubisco) to increase its carboxylase activity. Aside from Rubisco's slow rate of catalysis, O<sub>2</sub> can compete with CO<sub>2</sub> for the active site of the enzyme, resulting in the wasteful process of photorespiration (2). Since CO<sub>2</sub> and O<sub>2</sub> 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 (HCO<sub>3</sub><sup>-</sup>) transporters that help increase the HCO<sub>3</sub><sup>-</sup> concentration in the chloroplast stroma relative to the external HCO<sub>3</sub><sup>-</sup> concentration. These transporters are located on the plasma membrane (LC11 and HLA3) as well as the chloroplast envelope (NAR1.2/LCIA). Loss of any one of these transporters reduces the ability of the cell to accumulate HCO<sub>3</sub><sup>-</sup> 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 HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub> near the site of Rubisco (8, 9), increasing photosynthetic and growth rates at otherwise growth-limiting CO<sub>2</sub> 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 CO<sub>2</sub>, even though these mutants tend to overaccumulate HCO<sub>3</sub><sup>-</sup> (11). *Chlamydomonas* CCM models propose that mutants missing CAH3 accumulate the HCO<sub>3</sub><sup>-</sup> brought into the chloroplast by the transport proteins but cannot convert that HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub>, 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 HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub>. The apparent acid dissociation constant (pK<sub>a</sub>) of the interconversion of HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> 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 CO<sub>2</sub> concentrations (14). Therefore, as HCO<sub>3</sub><sup>-</sup> is brought from the stroma to the thylakoid lumen, it goes from an environment favoring HCO<sub>3</sub><sup>-</sup> to one favoring CO<sub>2</sub>. Therefore, the

## Significance

Models of the CO<sub>2</sub> concentrating mechanism (CCM) of green algae and diatoms postulate that chloroplast CO<sub>2</sub> is generated from HCO<sub>3</sub><sup>-</sup> brought into the acidic thylakoid lumen and converted to CO<sub>2</sub> by specific thylakoid carbonic anhydrases. However, the identity of the transporter required for thylakoid HCO<sub>3</sub><sup>-</sup> 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 HCO<sub>3</sub><sup>-</sup> transporters, a critical currently missing step of the CCM required for future engineering efforts of the *Chlamydomonas* CCM into plants to improve photosynthesis.

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The authors declare no conflict of interest.

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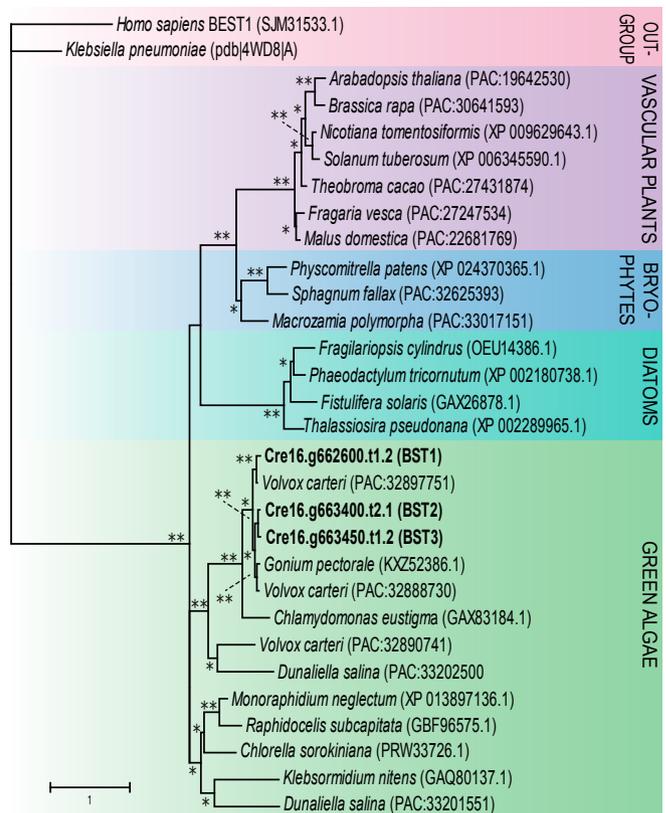
acidification of the thylakoid lumen is important to the functioning of the CCM.

The CCM models also proposed the presence of a thylakoid  $\text{HCO}_3^-$  transporter that brings in  $\text{HCO}_3^-$  from the stroma to the lumen for dehydration by CAH3 (12, 13). In a recent interactome study, the CCM complex LCIB/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  $\text{CO}_2$  conditions in a transcriptomic study showing they belonged to a cluster of genes that had increased expression in low  $\text{CO}_2$  and were controlled by CIA5 (16). Bestrophins are typically chloride channels, including the *Arabidopsis* bestrophin-like protein AtVCCN1 (17). However, they have also been shown to transport a range of anions, with some showing high  $\text{HCO}_3^-$  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  $\text{HCO}_3^-$  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 with low expression of all 3 genes grow poorly in limiting  $\text{CO}_2$  conditions, exhibit a poor affinity for external  $\text{C}_i$ , and have a severely reduced ability to accumulate  $\text{HCO}_3^-$ . This study sheds light on the intracellular location and function of these bestrophin-like proteins in the CCM of *Chlamydomonas*.

## Results

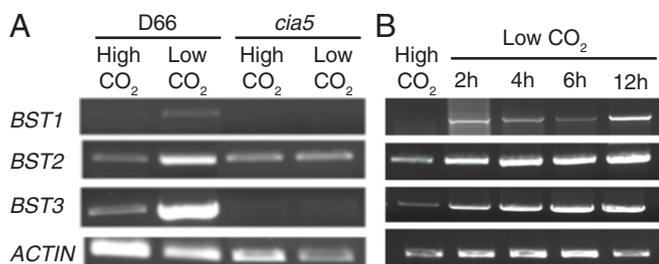
***Chlamydomonas* Has 3 Very Similar Bestrophin-Like Proteins on the Thylakoid Membrane.** *BST1* (Cre16.g662600), *BST2* (Cre16.g663400), and *BST3* (Cre16.g663450) (collectively *BST1–3*) are paralogous bestrophin-like genes located within a 130-kilobase pair (kbp) region on the 16th chromosome of *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 TMHMM, 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 approximately 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 entry pocket of *BST1* has a predominantly neutral/negative electrostatic potential, and the selective pore is positively charged, supporting the hypothesis that *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 (5 categories) and 500 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. \*Bootstrap value  $\geq$  50, \*\*Bootstrap value  $\geq$  90.

***BST1–3* Are Up-Regulated under Low  $\text{CO}_2$  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  $\text{CO}_2$  or ambient  $\text{CO}_2$  conditions. For this work, we have used 5%  $\text{CO}_2$  (vol/vol) in air as high  $\text{CO}_2$ , 0.04% as ambient  $\text{CO}_2$ , and <0.02% as low  $\text{CO}_2$ . 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  $\text{CO}_2$  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  $\text{CO}_2$  conditions, a transcriptional pattern observed with other CCM genes. *BST2* transcript levels in *cia5* cells showed reduced induction in ambient  $\text{CO}_2$  when compared with D66 cells, where *BST2* transcript levels increase in ambient  $\text{CO}_2$  conditions. A time course study of the expression of these 3 genes during induction of the CCM was done by transferring high  $\text{CO}_2$ -grown cells to ambient  $\text{CO}_2$  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  $\text{CO}_2$ , 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



**Fig. 2.** Transcript analysis of *BST1-3*. (A) Semi-quantitative RT-PCR showing *BST1-3* accumulation in ambient  $\text{CO}_2$  (0.04%  $\text{CO}_2$ ) vs. high  $\text{CO}_2$  (5% [vol/vol]  $\text{CO}_2$  in air) in D66 and *cia5* cells. (B) Semi-quantitative RT-PCR time course showing the expression of *BST1-3* in complementary DNA obtained from high  $\text{CO}_2$  (5%  $\text{CO}_2$  [vol/vol] in air) and in cells switched to ambient  $\text{CO}_2$  (0.04%  $\text{CO}_2$ ) for the indicated times. Actin has been used as a loading control.

studies visually showed that *BST1*, *BST2*, and *BST3* were preferentially concentrated near the pyrenoid (Fig. 3B). To confirm that expression using the constitutive *PSAD* promoter was not affecting localization or pyrenoid periphery enrichment, we constructed a *BST3-Venus* line with the *BST3* gene under its native promoter. This line showed the same localization pattern as *BST3* under the constitutive *PSAD* promoter (Fig. 3C), and quantification of enrichment showed a 1.46-fold enrichment ( $P < 0.01$ , Student's paired *t* test) around the pyrenoid relative to the rest of the chloroplast. Thus, *BST1*, *BST2*, and *BST3* are thylakoid localized anion transporters enriched at the pyrenoid periphery that are expressed coordinately with the expression of other *Chlamydomonas* CCM proteins.

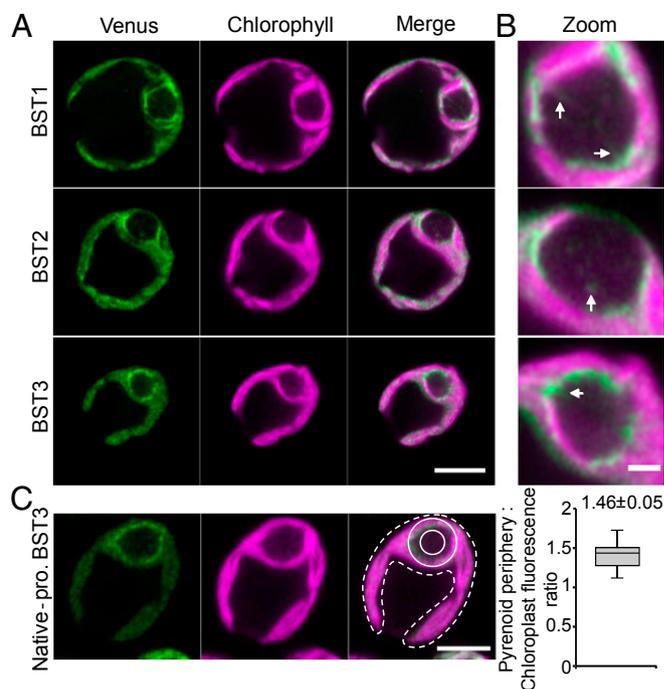
**Reduction of *BST1-3* Expression Results in Cells that Grow Slowly under Low  $\text{CO}_2$  Conditions.** A *BST3* knockout (*bst3*) was obtained from the *Chlamydomonas* Library Project (CLiP) mutant collection (23) with a paromomycin insert in the last exon of the *bst3* gene (SI Appendix, Fig. 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  $\text{CO}_2$  (SI Appendix, Fig. S5 A and B), but no clear phenotype on plates at pH 7 or pH 8.4 at 100  $\mu\text{mol}$  of photons per  $\text{m}^{-2}\cdot\text{s}^{-1}$  at low  $\text{CO}_2$  (SI Appendix, Fig. S5C). However, there was no significant difference in  $\text{C}_i$  affinity between wild type and *bst3* grown at ambient  $\text{CO}_2$  (SI Appendix, Fig. S5D), and  $\text{C}_i$  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  $\text{CO}_2$ . Colonies were then screened for growth on high  $\text{CO}_2$  versus low  $\text{CO}_2$ , and *BST1-3* expression was quantified using RT-qPCR. Three independent colonies from 2 different transformations were chosen for further study and designated as *bst1-1*, *bst1-2*, and *bst1-3* (*BST* RNAi triple-knockdown lines 1, 2, and 3).

The growth of *bst1-1*, *bst1-2*, and *bst1-3* on high and low  $\text{CO}_2$  was compared with D66 and the *CAH3* knockout mutant, *cia3* (Fig. 4A). In low  $\text{CO}_2$ , *bst1-1* showed severely reduced growth that was further exacerbated at high pH, resembling the growth of *cia3* (Fig. 4A). The *bst1-2* and *bst1-3* also grew more slowly than wild-type cells, but better than *bst1-1*. However, at high  $\text{CO}_2$ , the growth of all 3 strains was comparable to wild type. RT-qPCR showed that *bst1-1* had significantly reduced expression of *BST1*, *BST2*, and *BST3* compared with D66 (Fig. 4B), and *bst1-2* and *bst1-3* had a more moderate knockdown of expression of the 3 genes. To see if reduced transcript levels resulted in decreased protein abundance,

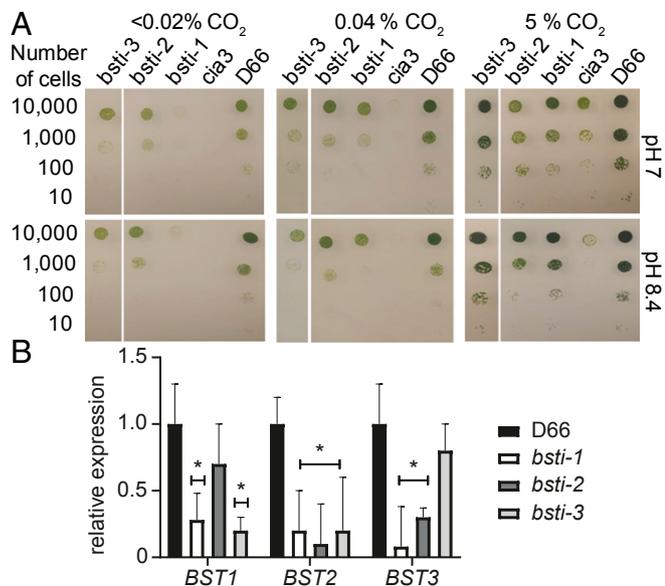
we checked *BST3* protein levels in the knockdown lines. All showed reduced levels relative to D66, although this was only significant for *bst1-2* and *bst1-3* ( $P < 0.05$ , Student's *t* test; SI Appendix, Fig. S6A). Thus, the *BSTs* are required for wild-type-like growth of *Chlamydomonas* under low  $\text{CO}_2$  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  $\text{C}_i$  and the ability to accumulate  $\text{C}_i$  to levels higher than can be obtained by diffusion. The *bst1-1*, *bst1-2*, and *bst1-3* acclimated to ambient  $\text{CO}_2$  exhibited a lower affinity for  $\text{C}_i$  as judged by their measured  $\text{C}_i$  concentration needed for half-maximum oxygen evolution [ $K_{1/2}(\text{C}_i)$ ] (Fig. 5). When grown at high  $\text{CO}_2$ , *bst1-3* and D66 exhibited similar  $\text{C}_i$  affinities (SI Appendix, Fig. S6B). These results indicate that the expression of *BST1-3* is required for optimal  $\text{C}_i$  affinity when cells are grown on ambient levels of  $\text{CO}_2$ . At pH 8.4, the  $K_{1/2}(\text{C}_i)$  values for *bst1-3* are elevated in sharp contrast to a low  $K_{1/2}(\text{C}_i)$  for D66 (Fig. 5 A and B). At the higher pH of 8.4, the predominant  $\text{C}_i$  species in the medium would be  $\text{HCO}_3^-$ . Thus, the increased affinity of the cells for  $\text{C}_i$  reflects their ability to actively take up and utilize  $\text{HCO}_3^-$ . For *bst1-1*, where the expression of all 3 *BST* genes is between 60 and 90% reduced, there is a reduced  $\text{C}_i$  affinity at both pH 8.4 (Fig. 5 A and B) and pH 7.8 (Fig. 5 C and D). In contrast, *bst3*, the mutant missing only *BST3*, the difference in  $\text{C}_i$  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*.

$\text{C}_i$  uptake activity was measured in D66, *bst1-1*, *bst1-2*, and *bst1-3* to evaluate the importance of *BST1-3* in accumulation and



**Fig. 3.** Localization of *BST1-3*. (A) Confocal microscopy of *BST1-3* proteins fused with Venus (green) and driven by the constitutive *PSAD* promoter. Chlorophyll autofluorescence is shown in magenta. (Scale bar, 5  $\mu\text{m}$ .) (B) Zoomed-in images of *BST1-3* pyrenoids shown in A. Arrows highlight where Venus fluorescence is seen overlapping with chlorophyll fluorescence in the pyrenoid matrix. (Scale bar, 1  $\mu\text{m}$ .) (C) Localization and quantification of *BST3* distribution under its native promoter. The ratio of fluorescence intensity at the pyrenoid periphery (solid line region) and chloroplast (dotted line region) was quantified. The value above the plot denotes the mean  $\pm$  SE ( $n = 23$ ). (Scale bar, 4  $\mu\text{m}$ .)



**Fig. 4.** Growth of *bsti-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-3*. Cells were diluted to  $6.6 \times 10^5$  cells per milliliter, followed by 1:10 serial dilution 3 times to compare growth in low  $\text{CO}_2$  ( $<0.02\% \text{ CO}_2$ ), ambient  $\text{CO}_2$  ( $0.04\% \text{ CO}_2$ ), and high  $\text{CO}_2$  ( $5\% \text{ CO}_2$  [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  $\text{CO}_2$  for 12 h before harvesting the RNA. \* $P < 0.05$  by Student *t* test.

fixation of  $\text{C}_i$ . Ambient  $\text{CO}_2$ -acclimated *bsti-1* had a notably lower accumulation and fixation of  $^{14}\text{C}_i$  compared with D66 at pH 8.4 (Fig. 6), and *bsti-2* and *bsti-3* also had inhibited  $^{14}\text{C}$  uptake and fixation, although not as reduced as *bsti-1* (Fig. 6). The most severely affected mutant, *bsti-1*, accumulated  $^{14}\text{C}_i$  to only 30 to 40% of the levels observed in D66 cells. These results indicate that *BST1-3* play an important role in  $\text{C}_i$  uptake and fixation in low  $\text{CO}_2$  conditions in *Chlamydomonas*.

A bestrophin-like protein recently discovered in *Arabidopsis*, AtVCCN1, is a  $\text{Cl}^-$  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  $\Delta\text{pH}$  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 ( $\Delta\psi$ ) and  $\Delta\text{pH}$  components of the pmf, leading to photodamage or to an adenosine 5'-triphosphate (ATP)/NADPH imbalance. To investigate if *BST1-3*, in addition to being critical for  $\text{C}_i$  affinity and accumulation, have a role in regulating pmf similar to AtVCCN1, we measured electrochromic shift to estimate the pmf in the knockdown lines under  $\text{HCO}_3^-$ -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  $F_v/F_m$  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.

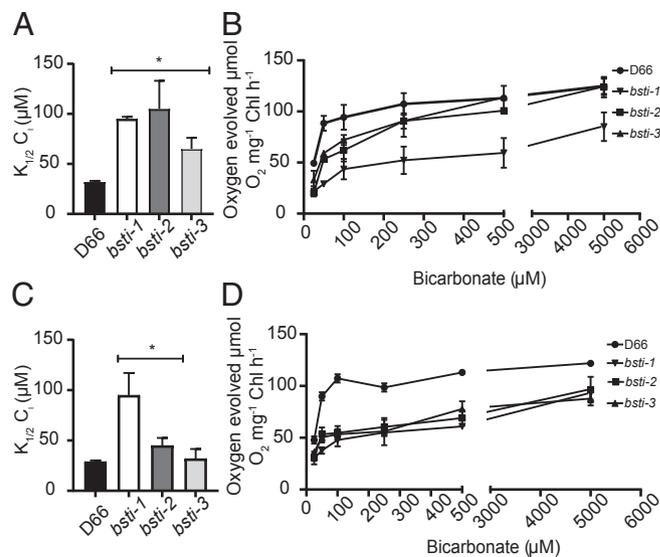
In conclusion, the localization, the  $\text{C}_i$  affinity, and the  $\text{C}_i$  accumulation phenotypes of the *bsti* triple-knockdown mutants support an essential role for *BST1-3* in the CCM.

## Discussion

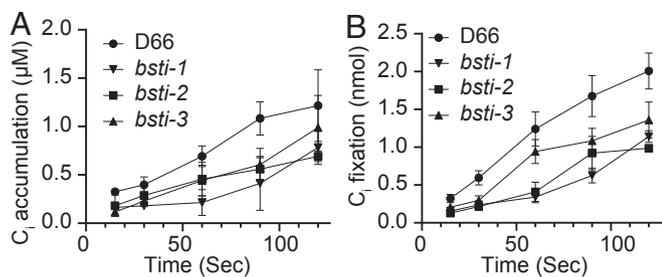
We present evidence here that *BST1-3* are chloroplast thylakoid localized anion transporters that are important components of the *Chlamydomonas* CCM. Cells that have reduced *BST1-3* transcript levels fail to grow on low  $\text{CO}_2$  (Fig. 4), have a lower affinity for  $\text{C}_i$  (Fig. 5), and have a reduced ability to accumulate added  $^{14}\text{C}_i$  (Fig. 6). A key aspect of current *Chlamydomonas* CCM models is that accumulated  $\text{HCO}_3^-$  is converted to  $\text{CO}_2$  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  $\theta$ -type carbonic anhydrase within the thylakoid of *Phaeodactylum tricornutum* that was required for CCM function. These CCM models predict that a thylakoid  $\text{HCO}_3^-$  transporter is required to deliver  $\text{HCO}_3^-$  from the chloroplast stroma to the thylakoid lumen. We propose that *BST1-3* are the transporters that bring  $\text{HCO}_3^-$  to *CAH3* inside the thylakoid.

Members of the human bestrophin family transport both  $\text{HCO}_3^-$  and  $\text{Cl}^-$  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  $\text{CO}_2$  conditions, and their expression is controlled by the transcription factor *CIA5/CCM1* (22, 25). We have observed that all 3 *BST* genes are induced when *Chlamydomonas* is grown under ambient  $\text{CO}_2$  conditions (Fig. 2) and that this induction is absent in the *cia5* mutant (Fig. 2A). In addition, *LCIB* and *LCIC*, possible  $\theta$ -carbonic anhydrases (24, 26) essential to the CCM (4) that interact with *BST1-3* (15), have the same expression pattern



**Fig. 5.** Photosynthetic oxygen evolution activity of *bsti-3* RNAi lines and D66.  $\text{C}_i$  affinity was estimated for *bsti-3* and D66 acclimated to ambient  $\text{CO}_2$  for 12 h at pH 8.4 (A and B) and for *bsti-3* and D66 at pH 7.8 (C and D). Oxygen evolving activity was measured at the indicated pH, and the  $K_{1/2}(\text{C}_i)$  values were calculated from the  $\text{O}_2$  evolution versus  $\text{C}_i$  curves. Triplicate runs were made at each  $\text{C}_i$  concentration. The differences in  $K_{1/2}(\text{C}_i)$  were significant (\* $P < 0.05$  by Student's *t*-test). At pH 7.8, the maximum velocity ( $V_{\text{max}}$ ) of D66 is  $121 \mu\text{mol}$  of  $\text{O}_2$  per milligram of chlorophyll (Chl) per hour ( $\text{O}_2 \text{ mg}^{-1} \text{ Chl h}^{-1}$ ), the  $V_{\text{max}}$  of *bsti-1* is  $105 \mu\text{mol}$  of  $\text{O}_2 \text{ mg}^{-1} \text{ Chl h}^{-1}$ , the  $V_{\text{max}}$  of *bsti-2* is  $87 \mu\text{mol}$  of  $\text{O}_2 \text{ mg}^{-1} \text{ Chl h}^{-1}$ , and the  $V_{\text{max}}$  of *bsti-3* is  $90 \mu\text{mol}$  of  $\text{O}_2 \text{ mg}^{-1} \text{ Chl h}^{-1}$ . At pH 8.4, the  $V_{\text{max}}$  of D66 is  $124 \mu\text{mol}$  of  $\text{O}_2 \text{ mg}^{-1} \text{ Chl h}^{-1}$ , the  $V_{\text{max}}$  of *bsti-1* is  $85.5 \mu\text{mol}$  of  $\text{O}_2 \text{ mg}^{-1} \text{ Chl h}^{-1}$ , the  $V_{\text{max}}$  of *bsti-2* is  $124 \mu\text{mol}$  of  $\text{O}_2 \text{ mg}^{-1} \text{ Chl h}^{-1}$ , and the  $V_{\text{max}}$  of *bsti-3* is  $123 \mu\text{mol}$  of  $\text{O}_2 \text{ mg}^{-1} \text{ Chl h}^{-1}$ . Error bars indicate SD.



**Fig. 6.**  $C_i$  uptake of D66 and *bsti1-3*.  $C_i$  uptake and  $C_i$  accumulation were measured in D66 and *bsti1-3* using the silicone oil uptake method (*Materials and Methods*). Cells were grown in high  $CO_2$  and then acclimated to ambient  $CO_2$  for 12 h prior to the assays. Cells were harvested and depleted of endogenous  $C_i$  before running the assays. A time course of intracellular  $C_i$  accumulation (A) and  $CO_2$  fixation (B) is shown for pH 8.4. Triplicate samples were run for each time point. The added  $H^{14}CO_3^-$  concentration was  $50 \mu M$ .

(16). Thus, the expression of the *BST1-3* genes is consistent with these proteins playing a role in the uptake and accumulation of  $C_i$  when *Chlamydomonas* is exposed to low  $CO_2$  conditions.

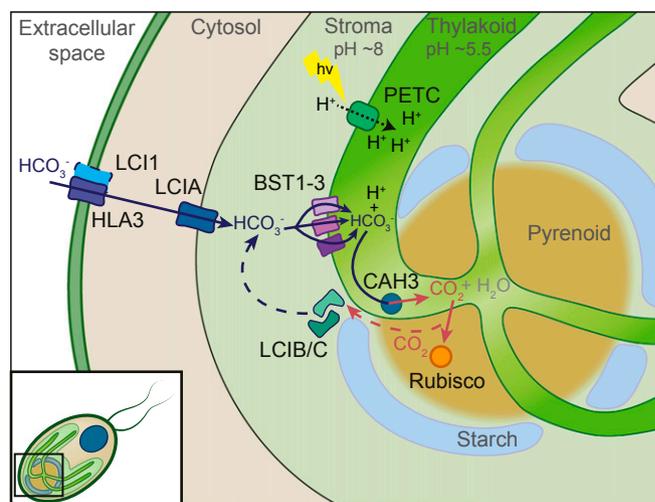
An alternative hypothesis is that the 3 BST proteins have a function similar to AtVCCN1 (20) and are involved in  $Cl^-$  transport to regulate the pmf across the thylakoid. The presence of AtVCCN1 decreases pmf in *Arabidopsis*, but the presence of the 3 BST proteins increases pmf in *Chlamydomonas*. 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  $C_i$  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  $CO_2$  (3). However, when more than 1 transporter is knocked down, a significant change in  $C_i$  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  $C_i$  affinity at ambient levels of  $CO_2$  (*SI Appendix, Figs. S4 and S5*). However, when the expression of all 3 genes is decreased in *bsti1-3*, cells could not grow at low  $CO_2$  and  $C_i$  uptake was severely compromised. In addition, *BST1-3* transcript levels in the RNAi strains correlated to  $C_i$  affinity and  $C_i$  uptake, supporting their  $C_i$  transport role and functional redundancy. This redundancy likely explains why *BST1-3* were not identified in earlier mutant screens, as these screens typically knock out only 1 gene at a time. The 3 BST proteins do, however, have sequence differences, particularly at their C termini. Therefore, they might have specific (or slightly different) physiological roles that cannot be differentiated under the conditions employed in this present study.

Fig. 7 shows a refined model for the *Chlamydomonas* CCM, which now includes our proposed function for *BST1-3*. In this model, HLA3 and LCI1 transport  $HCO_3^-$  across the plasma membrane, bringing  $HCO_3^-$  into the cell (12, 13). At the chloroplast envelope, NAR1.2 (LCIA) transports  $HCO_3^-$  into the chloroplast stroma. Then, *BST1-3* on the thylakoid bring  $HCO_3^-$  into the thylakoid lumen, where CAH3 in the pyrenoid thylakoid tubules converts  $HCO_3^-$  to  $CO_2$  to be fixed by Rubisco. Since *BST1-3* are found throughout the thylakoid, a potential futile cycle is possible. However, for the futile cycle to take place, CAH3 needs to be present and active in the thylakoid membranes away from the pyrenoid. There is published work that CAH3 is preferentially located and activated in the pyrenoid tubules under low ( $<0.02\%$ )  $CO_2$  conditions (8, 9). The location of *BST1-3* is also likely to be important in the recapture of  $CO_2$  that is generated by the CCM (Fig. 7). Any  $CO_2$  in the pyrenoid

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  $CO_2$  (27) by directionally driving  $CO_2$  to  $HCO_3^-$  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). Having *BST1-3* throughout the thylakoid (Fig. 3) would increase the surface area for the reuptake of  $HCO_3^-$  in the stroma. As such, we propose that the recapture of  $C_i$  is a 2-step process, with leaked  $CO_2$  from the pyrenoid converted to  $HCO_3^-$  by LCIB/C and *BST1-3* transporting the  $HCO_3^-$  back into the thylakoid, creating an overall cyclic recapture mechanism. Loss of either *BST1-3* or LCIB/C results in cells that cannot accumulate  $C_i$  to normal levels, which agrees with experimental observations.

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  $pK_a$  of the interconversion of  $HCO_3^-$  to  $CO_2$  is about 6.4. The pH of the chloroplast stroma, thought to be near 8.0, is well above the  $pK_a$ , while the pH of the thylakoid lumen is thought to be close to 5.7 (14), below the  $pK_a$ . When  $HCO_3^-$  moves from the chloroplast stroma to the thylakoid lumen, it moves from an environment that favors  $HCO_3^-$  to one that favors  $CO_2$ . This effectively allows the algal cells to increase the  $CO_2$  concentration to levels higher than could be obtained by the action of carbonic anhydrase alone. Thus, a transthylakoid pH gradient is necessary for this proposed “ $CO_2$  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  $CO_2$  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  $H^+$  would be consumed per  $CO_2$  generated, which is the



**Fig. 7.** Tentative model showing the proposed physiological role of *BST1-3* in the CCM of *Chlamydomonas*. Known transporters (LCI1, HLA3, and LCIA) are indicated on the plasma membrane and chloroplast, respectively. Solid line arrows indicate the movement of  $HCO_3^-$  into the thylakoid by *BST1-3*. Dashed lines indicate the proposed leakage-reducing pathway that involves recycling  $CO_2$  by LCIB/C back to  $HCO_3^-$ . The dotted black line represents the light-driven establishment of a proton gradient across the thylakoid membrane by PSII and the cytochrome *b6f* complex of the photosynthetic electron transport chain (PETC).

equivalent of less than one-third of an ATP per CO<sub>2</sub> generated. This cost is far less than the 2 additional ATPs required for C<sub>4</sub> photosynthesis, and C<sub>4</sub> photosynthesis has been shown to be energetically competitive with C<sub>3</sub> photosynthesis once the costs of photorespiration are considered (33). 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, they are excellent candidates to be the HCO<sub>3</sub><sup>−</sup> transporters that not only bring HCO<sub>3</sub><sup>−</sup> into the thylakoid lumen for CO<sub>2</sub> generation but may also play a role in C<sub>i</sub> 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 (*nit2*<sup>−</sup>, *cw15*, *mt*<sup>+</sup>) was obtained from Rogene Schnell (University of Arkansas, Fayetteville, AR), and CMJ030 (CC-4533; *cw15*, *mt*<sup>−</sup>) and *bst3* (*BST3* knockout LMJ.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<sub>2</sub> (5% [vol/vol] CO<sub>2</sub> in air) to reach an optical density at 730 nm between 0.2 and 0.3 (~2 to 3 × 10<sup>6</sup> cells per milliliter). This was followed by CCM induction when the cells were transferred to ambient CO<sub>2</sub> (0.04% CO<sub>2</sub>) bubbling. For photosynthetic assays, cells acclimated to 5% or 0.04% CO<sub>2</sub> were resuspended in C<sub>i</sub>-depleted buffer at pH 7.8 or pH 8.4, and O<sub>2</sub> evolution was measured at different C<sub>i</sub> concentrations. K<sub>1/2</sub>(C<sub>i</sub>) was

calculated as the C<sub>i</sub> 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*.

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