

1 Short title (50 characters): **Aggregates of hybrid cyanobacteria-tobacco Rubisco**

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9 **Hybrid cyanobacterial-tobacco Rubisco supports autotrophic growth and pre-carboxysomal**  
10 **aggregation**

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17 **One Sentence Summary:**

18 Cyanobacterial Rubisco large subunits form functional hybrids with tobacco small subunits and pro-  
19 carboxysome micro-compartments via the linker protein CcmM35 in absence of cognate small subunits.

20

21 **Keywords:** carboxysome, CCM, cyanobacteria, Rubisco, photosynthesis

22

23 **FOOTNOTES:**

24 **List of author contributions:**

25 MAJP, MRH, MTL & ECS conceived research. All authors designed experiments. DJO, DW & MTL  
26 performed the experiments and analyzed data. All authors contributed to writing the manuscript.

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33

34 **Abstract**

35 Much of the research aimed at improving photosynthesis and crop productivity attempts to overcome  
36 shortcomings of the primary CO<sub>2</sub> fixing enzyme Rubisco. Cyanobacteria utilize a CO<sub>2</sub> concentrating  
37 mechanism (CCM), which encapsulates Rubisco with poor specificity but relatively fast catalytic rate  
38 within a carboxysome micro-compartment. Alongside active transport of bicarbonate into the cell, and  
39 localization of carbonic anhydrase within the carboxysome shell with Rubisco, cyanobacteria are able to  
40 overcome the limitations of Rubisco via localization within a high CO<sub>2</sub> environment. As part of ongoing  
41 efforts to engineer a β-cyanobacterial CCM into land plants, we investigated the potential for Rubisco  
42 large subunits (LSU) from the β-cyanobacteria *Synechococcus elongatus* (Se) to form aggregated Rubisco  
43 complexes with the carboxysome linker protein CcmM35 within tobacco chloroplasts. Transplastomic  
44 plants were produced that lacked cognate SeRubisco small subunits (SSU) and expressed SeLSU in place  
45 of tobacco LSU, with and without CcmM35. Plants were able to form a hybrid enzyme utilizing tobacco  
46 SSU and the SeLSU, allowing slow autotrophic growth in high CO<sub>2</sub>. CcmM35 was able to form large  
47 Rubisco aggregates with the SeLSU, and these incorporated small amounts of native tobacco SSU. Plants  
48 lacking the SeSSU showed delayed growth, poor photosynthetic capacity and significantly reduced  
49 Rubisco activity compared to both wild-type tobacco and lines expressing the SeSSU. These results  
50 demonstrate the ability of the SeLSU and CcmM35 to form large aggregates without the cognate SeSSU  
51 in planta, harboring active Rubisco that enables plant growth, albeit at much slower pace than plants  
52 expressing the cognate SeSSU.

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## 55 Introduction

56 The need to produce sufficient food for a growing population requires increasing the productivity and  
57 efficiency of agriculture in order to increase yields by the estimated 70% that will be needed by 2050  
58 (Lobell et al., 2009; Ray et al., 2012). Given its central role in crop growth and productivity, improving  
59 photosynthesis is one approach that has the potential to generate step-change improvements in crop  
60 yields and resource use efficiency (Long et al., 2006; Ort et al., 2015). One of the primary limitations to  
61 photosynthesis is the relative inefficiency of the central carbon fixing enzyme Rubisco (ribulose 1,5-  
62 bisphosphate carboxylase/oxygenase), in particular its lack of specificity for CO<sub>2</sub> versus O<sub>2</sub>, which leads to  
63 the energetically costly photorespiratory cycle (Whitney et al., 2011; Carmo-Silva et al., 2015; Sharwood  
64 et al., 2016; Flamholz et al., 2019). Exemplifying this, at current atmospheric levels of CO<sub>2</sub> and O<sub>2</sub>,  
65 Rubisco's tendency to oxygenate rather than carboxylate its substrate RuBP (ribulose 1,5-bisphosphate)  
66 is estimated to reduce yields by as much as 36% and 20% in US grown soybean and wheat, respectively  
67 (Walker et al., 2016). Recent work has shown that limiting the costs of photorespiration by increasing its  
68 efficiency can provide dramatic benefits to plant growth (South et al., 2019).

69 Synthetic biology approaches hold promise for improving a number of facets of photosynthetic  
70 efficiency in crop plants (Maurino and Weber, 2013; Erb and Zarzycki, 2016; Orr et al., 2017). One  
71 example is the introduction of CO<sub>2</sub>-concentrating mechanisms (CCM's) into C<sub>3</sub> crops to increase CO<sub>2</sub>  
72 concentrations at the site of Rubisco, a strategy which is likely to dramatically reduce the propensity of  
73 Rubisco to carry out oxygenation reactions by creating an environment which favors the beneficial  
74 carboxylation reaction (Price et al., 2011; McGrath and Long, 2014; Hanson et al., 2016; Long et al.,  
75 2016). Significant research efforts are being invested in this area, with varying sources for the CCMs  
76 being engineered, such as C<sub>4</sub> (Hibberd et al., 2008; Langdale, 2011) and CAM (Borland et al., 2014; Yang  
77 et al., 2015) systems from plants, and the pyrenoid and carboxysome-based systems of algae and  
78 cyanobacteria, respectively (Rae et al., 2017; Mackinder, 2018).

79 The CCM employed by cyanobacteria uses a combination of factors to create a high CO<sub>2</sub>  
80 environment localized around Rubisco (Price et al., 2008; Hanson et al., 2016). Aggregation and  
81 encapsulation of Rubisco within a highly ordered icosahedral protein micro-compartment, or  
82 carboxysome, allows co-localization of Rubisco and carbonic anhydrase (CA) to convert HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub>  
83 where it is needed, and permits the movement of key molecules while limiting CO<sub>2</sub> escape. Generating a  
84 high CO<sub>2</sub> environment is also facilitated by a complex system of inorganic carbon transporters on the  
85 cyanobacterial outer membrane that move either HCO<sub>3</sub><sup>-</sup> or CO<sub>2</sub> into the cytoplasm through active and  
86 passive mechanisms (Price, 2011). Modelling the incorporation of the various components of the CCM

87 into plants suggests that once a fully functioning system is established within a higher plant chloroplast,  
88 photosynthetic rates could be improved by as much as 60% (McGrath and Long, 2014). The resulting  
89 subsequent improvements in yield could facilitate a major change in crop productivity and resource use  
90 efficiency (Ort et al., 2015; Hanson et al., 2016).

91 Significant progress has been made during recent years to unravel the molecular mechanisms of  
92 CCMs involving either carboxysomes or pyrenoids. In *Synechococcus elongatus* PCC7942, which produces  
93  $\beta$ -carboxysomes, the *ccmM* gene gives rise to two proteins: CcmM58 and CcmM35, the latter arising  
94 from an internal ribosomal entry site (Long et al., 2007; Long et al., 2010). CcmM35 possesses three  
95 tandem repeats of Rubisco small subunit-like domains, and was initially thought to interact with Rubisco  
96 by replacing small subunits (Long et al., 2011). However, recent experiments suggest that CcmM35 binds  
97 Rubisco without releasing the small subunits (Ryan et al., 2019). A recent structural study revealed that  
98 the interaction between CcmM35 and Rubisco leads to dramatic phase separation (Wang et al., 2019).  
99 This nucleation of Rubisco holoenzymes by CcmM35 represents a critical first step in the assembly of  $\beta$ -  
100 carboxysomes (Cameron et al., 2013). In the pyrenoid of *Chlamydomonas*, similar phase separation was  
101 also observed when the Rubisco and a repeat protein called EYPC1 interact (Wunder et al., 2018).  
102 Likewise, in  $\alpha$ -carboxysomes, Rubisco holoenzymes interact with a highly disordered repeat protein  
103 called CsoS2 (Cai et al., 2015; Liu et al., 2018). In a recent breakthrough, Long and co-workers were able  
104 to assemble  $\alpha$ -carboxysomes in tobacco chloroplasts by co-expressing Rubisco large and small subunit  
105 genes along with CsoS2 and a shell protein called CsoS1A from *Cyanobium marinum* PCC7001 (Long et  
106 al., 2018). In another study, the shell proteins of  $\beta$ -carboxysome transiently expressed in the chloroplasts  
107 of *Nicotiana benthamiana* were able to assemble structures similar to micro-compartments (Lin et al.,  
108 2014a).

109 Our previous work demonstrated that replacing the Rubisco large subunit gene in tobacco with  
110 the Rubisco large and small subunit genes from *Synechococcus elongatus* PCC7942 (Se) resulted in plants  
111 that can support photosynthetic growth under elevated CO<sub>2</sub> conditions (Lin et al., 2014b; Occhialini et  
112 al., 2016). When CcmM35 was co-expressed in tobacco chloroplasts, the heterologous Rubisco was  
113 observed in a large aggregate with an appearance resembling a separate liquid phase (Lin et al., 2014a).  
114 In a previous study performed by another group, when the tobacco *rbcl* gene was replaced with that  
115 from *Synechococcus* PCC6301, no Rubisco large subunit (LSU) was detected in the transformed plant  
116 (Kanevski et al., 1999), and it was thought that the cyanobacterial LSU could not assemble with plant  
117 small subunit (SSU) to form a functional enzyme.

118 Here we investigated the assembly and functioning of cyanobacterial Rubisco within higher plant

119 chloroplasts when the Se LSU is expressed either with or without CcmM35 in the absence of cognate  
120 cyanobacterial SSU. Analysis of transplastomic tobacco lines incorporating some cyanobacterial  
121 components but lacking the cognate SSU revealed that the Se LSU and CcmM35 are able to form large  
122 aggregates of Rubisco within tobacco chloroplasts. Though only low amounts of tobacco SSUs were  
123 present, the transplastomic lines characterized differed significantly in physiology and biochemistry from  
124 comparable lines that also co-expressed the cognate cyanobacterial SSU. Remarkably, albeit at slow  
125 rates, in the absence of the cognate small subunits, the hybrid cyanobacterial LSU-tobacco SSU  
126 expressed in tobacco chloroplasts with and without CcmM35 was active and supported plant growth.

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## 129 **Results**

130

### 131 **Cyanobacterial Rubisco large subunits can support carbon fixation in tobacco chloroplasts in the** 132 **absence of cognate small subunits**

133 We generated two transplastomic tobacco lines named SeL and SeLM35 by replacing in-frame the entire  
134 tobacco Rubisco large subunit gene with that from *Synechococcus elongatus* PCC7942 (Se). In the  
135 SeLM35 line, the *ccmM35* gene was introduced downstream of the *Se-rbcL* gene to be co-expressed  
136 from the same chloroplast genome locus (Fig. 1A). We used the same regulatory elements at intergenic  
137 regions as described in our previous work namely, a terminator, an intercistronic expression element  
138 (IEE) and a Shine-Dalgarno (SD) or ribosome binding site (Lin et al., 2014b; Occhialini et al., 2016). In  
139 contrast to our previous work, the new transplastomic lines do not possess a corresponding  
140 cyanobacterial Rubisco small subunit gene. The *aadA* selectable marker gene was incorporated into the  
141 same operon as the *Se-rbcL* gene in the SeL construct instead of a separate operon as in the SeLM35  
142 construct. We obtained homoplasmic transformed shoots after two rounds of selection, and were able  
143 to transfer them to soil for growth under elevated CO<sub>2</sub> (9000 ppm). We collected seeds from two  
144 independent SeL lines and one SeLM35 line. Both DNA and RNA blots confirmed complete removal of  
145 the *Nt-rbcL* gene and its corresponding transcript in these plants (Fig. 1B, S1). We also analyzed the  
146 transcripts containing *Se-rbcL* and *ccmM35* genes in these lines together with SeLS and SeLSM35 lines  
147 generated in our previous study (Fig. S1). The RNA blots showed bands arising from incomplete  
148 processing of IEE as well as read-through transcription of the downstream *aadA* operon, consistent with  
149 our previous observations (Occhialini et al., 2016).

150

151 **Cyanobacterial Rubisco large subunits and CcmM35 aggregate in pro-carboxysome micro-**  
152 **compartments in tobacco chloroplasts**

153 Expression of Se CcmM35 together with the cyanobacterial LSU in the SeLM35 transformant resulted in  
154 the formation of aggregates, or pro-carboxysome micro-compartments, in tobacco chloroplasts (Fig. 2).  
155 These aggregates were similar in size and shape to those observed in plants containing both the large  
156 and small subunits of Rubisco, and CcmM35 (SeLSM35, Fig. S2), but were absent from tobacco plants  
157 expressing the Se LSU in the absence of CcmM35. Immuno-gold labelling confirmed the presence of the  
158 Se LSU and CcmM35 proteins within the SeLM35 pro-carboxysome compartments (Fig. 2, S3, S4). In  
159 comparison, in SeL plants, the Se LSU protein could be detected throughout the chloroplast and, as  
160 expected, the anti-CcmM antibody gave only background level signal.

161 Gel electrophoresis and immunoblotting of leaf extracts demonstrated the presence of  
162 cyanobacterial LSU and CcmM35 in SeLM35 transplastomic plants (Fig. 3). Visually, the two proteins  
163 appear to be more abundant on a total soluble protein basis in these plants compared to SeLSM35. As  
164 expected, both proteins were absent from WT leaf extracts, and in SeLS and SeL plants, Se LSU was  
165 present but CcmM35 was not observed. The tobacco SSU was detected in WT, SeL and SeLM35 leaf  
166 extracts, although its abundance in SeL was very low, and visualization of the ~13 kDa SSU required a  
167 higher TSP load to detect clearly using immunoblotting (Fig. 3C). Non-denaturing native-PAGE suggested  
168 that CcmM35 is present in functional complexes with Rubisco in the tobacco transplastomic lines  
169 SeLSM35 and SeLM35 (Fig. 3B).

170

171 **Cyanobacterial Rubisco activity is impaired by the lack of a cognate SSU within tobacco chloroplasts**

172 Consistent with previous efforts expressing Se Rubisco within tobacco chloroplasts, Rubisco content and  
173 activity on a leaf area basis were significantly lower in leaf extracts of all the transplastomic lines,  
174 representing less than 20% of the values in WT plants (Fig. 4). SeL plants in particular displayed minimal  
175 amounts of Rubisco. While Rubisco active sites in SeL were ca. 20% of SeLS plants expressing both Se  
176 Rubisco subunits (Fig. 4B), total activity in SeL was less than 5% of SeLS, and ca. 1% of WT tobacco,  
177 consistent with the extremely slow growth of these plants (see below). SeLM35 plants had significantly  
178 more Rubisco active sites than other transplastomic lines, including SeLSM35, which also expresses the  
179 CcmM35 linker protein (Fig. 4B,  $P < 0.001$ ), although Rubisco total activity was not significantly different  
180 between the two lines (Fig. 4A,  $P > 0.001$ ).

181 To ascertain the ability of tobacco chloroplasts to maintain active cyanobacterial Rubisco, we  
182 determined Rubisco activation states from WT and transplastomic plants under steady state conditions.

183 As anticipated, WT plants were observed to have a comparatively low activation state in high CO<sub>2</sub>  
 184 conditions (Fig. 4C). Lines expressing both Se Rubisco subunits, with or without CcmM35 showed  
 185 essentially fully active Rubisco. In contrast, in SeLM35 Rubisco, activation was ca. 70 %, and in SeL,  
 186 expressing just the cyanobacterial LSU, it was only ca. 20 %. These data indicate that these complexes,  
 187 although able to function, did not become fully active in these growth conditions.

188 All transplastomic lines displayed significantly lower total soluble protein compared to WT  
 189 tobacco (Fig. 4D,  $P < 0.001$ ) and this decrease was largely consistent with the decreased amount of  
 190 Rubisco on an area basis (Fig. S6). Alongside reduced total soluble protein and Rubisco content and in  
 191 agreement with visual observation of these transplastomic plants, levels of chlorophyll a, b, and thus  
 192 total chlorophyll were significantly reduced (Fig. S7). Chlorophyll a was more severely reduced, and with  
 193 the exception of SeLS, all lines had a significantly reduced chlorophyll a/b ratio compared to WT tobacco.

194 Cyanobacterial Rubisco has been characterized to have a very high catalytic rate, but also a poor  
 195 affinity for CO<sub>2</sub> (high  $K_C$  value). In SeLS and SeLSM35 plants, values obtained for carboxylation rate,  $V_C$ ,  
 196 and  $K_C$ , the Michaelis-Menten constant for CO<sub>2</sub>, were consistent with previous work (Table 1; Occhialini  
 197 et al., 2016). Rubiscos from SeLM35 and SeL, which contain the cyanobacterial LSU but lack a cognate  
 198 SSU, were able to carboxylate RuBP at significant rates. Immunoblotting suggested the presence of  
 199 tobacco SSU in the Rubisco complex, but this was likely at a stoichiometric ratio lower than 1:1 in  
 200 relation to the cyanobacterial LSU (Fig. 3). These two Rubisco enzymes had affinities for CO<sub>2</sub> comparable  
 201 to the enzyme from the transplastomic lines containing both the cyanobacterial LSU and SSU (Table 1).

202  
 203  
 204 **Table 1. Rubisco catalytic properties.** Maximum carboxylation rate ( $V_C$ ), and Michaelis-Menten  
 205 constant for CO<sub>2</sub> ( $K_C$ ) of Rubisco from wild-type (WT) tobacco and transplastomic lines expressing  $\beta$ -  
 206 cyanobacterial carboxysome components from *Synechococcus elongatus* (Se): Rubisco large subunit (L),  
 207 Rubisco small subunit (S), CcmM35 (M35). Values represent mean  $\pm$  SEM (n = 3-5 biological replicates).  
 208 \* Wild-type values from Occhialini et al. (2016). Letters denote significant differences ( $P < 0.05$ ) between  
 209 transplastomic lines as determined by Tukey's pairwise comparisons following ANOVA. For  $K_C$   
 210 differences were not significant at  $P = 0.05$  level.

Line	$V_C$			$K_C$		
	( $\mu\text{mol mg}^{-1} \text{min}^{-1}$ )			( $\mu\text{M}$ )		
Wild-type*	3.9	$\pm$ 0.2		9.0	$\pm$ 0.3	
SeLS	15.0	$\pm$ 0.9 a		168	$\pm$ 59 a	
SeL	0.6	$\pm$ 0.2 b		105	$\pm$ 9 a	
SeLSM35	10.9	$\pm$ 0.8 c		133	$\pm$ 12 a	
SeLM35	2.0	$\pm$ 0.3 b		110	$\pm$ 22 a	

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#### 214 **The lack of a cognate Rubisco small subunit also impairs photosynthetic gas exchange**

215 To evaluate the impact of the unusual Rubisco composition in the leaves of these transplastomic lines,  
216 gas exchange measurements were carried out. At the levels present in these transplastomic plants and in  
217 absence of a functional CO<sub>2</sub>-concentrating mechanism, the faster catalytic rate of Se Rubisco does not  
218 confer an advantage in photosynthetic rate per leaf area even at 2000 ppm CO<sub>2</sub> (Fig. 5A). Consistent with  
219 previous work, aggregating cyanobacterial Rubisco through the expression of CcmM35 in SeLSM35  
220 plants slightly reduced photosynthetic rates on an area basis (Fig 5A; Occhialini et al., 2016). SeLM35  
221 photosynthetic rates show that the lack of the cognate Se SSU decreases photosynthetic rates even  
222 further (Fig. 5A). Most transplastomic lines showed a noticeable increase of photosynthesis under low  
223 oxygen conditions (Fig. S8). However, even at the highest CO<sub>2</sub> concentration measured combined with  
224 2% oxygen, SeL plants displayed net photosynthetic rates that were barely above zero (Fig. S8C).

225 As a fully functional cyanobacterial CCM within tobacco will ideally require less Rubisco than  
226 wild-type plants, we also determined Rubisco content in the leaves used for gas exchange analyses.  
227 When CO<sub>2</sub> assimilation was normalized by Rubisco active site concentration, neither SeLM35 nor SeL  
228 outperformed WT plants even at 2000 ppm CO<sub>2</sub> (Fig 5B). Consistent with earlier work, at CO<sub>2</sub> levels well  
229 above ambient SeLS and SeLSM35 plants showed higher photosynthesis per Rubisco active site (Fig.  
230 5B;(Occhialini et al., 2016)). Even accounting for very low Rubisco content, SeL plants show null  
231 normalized rates even at C<sub>i</sub> of 2000 ppm CO<sub>2</sub> (Fig. 5B). This is consistent with the observation that even a  
232 short exposure of several hours in ambient CO<sub>2</sub> conditions leads to tissue damage, and that even in  
233 growth conditions of 4000 ppm CO<sub>2</sub> SeL plants are extremely slow to develop (see below).

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235

#### 236 **Replacement of tobacco Rubisco large subunits with cyanobacterial large subunits impairs growth** 237 **irrespective of other components**

238 Transplastomic plants where the native tobacco Rubisco large subunit (LSU) was replaced with the Se  
239 large subunit with or without the carboxysome linker protein CcmM35 (SeLSM35 and SeLM35) grew  
240 slowly even at 4000 ppm CO<sub>2</sub> when compared to both WT and lines expressing both Se Rubisco subunits  
241 (SeLS, Fig. 6A, S6, Table S2). Germination time was similar between all lines (~7 days). Plant height and  
242 total leaf area of SeLSM35 and SeLM35 plants started to visibly increase 60 days after sowing, and the  
243 growth rate for the subsequent 15 days was significantly slower in SeLM35 plants lacking the Se SSU

244 compared to SeLSM35 ( $P < 0.05$ , Fig. 6B, 6C, Table S2). SeL plants expressing only the Se LSU were  
245 dramatically slower in growth ( $P < 0.001$ ), which necessitated germination in tissue culture for  
246 establishment before transferring to soil. These plants took approximately three times as long as SeLS  
247 plants to reach a plant height of ~80 cm (Fig. 6B). SeL and SeLM35 plants produced numerous smaller  
248 leaves, consistent with the other line expressing CcmM35, SeLSM35 (Fig. S10). Both SeL and SeLM35  
249 were noticeably paler than WT controls and transplastomic lines expressing the Se SSU (Fig. S7, S9, S10).

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251

## 252 Discussion

253 The current study describes two new transplastomic tobacco lines, namely SeL and SeLM35, where the  
254 native *rbcl* gene has been replaced with its cyanobacterial counterpart without the *Se-rbcS* gene.  
255 Previous work had shown the ability of L<sub>8</sub>S<sub>8</sub> Rubisco from *Synechococcus elongatus* to assemble and  
256 function within higher plant chloroplasts and to form large aggregates of linked Rubisco complexes in the  
257 presence of CcmM35 (Lin et al., 2014a; Occhialini et al., 2016). Our current results show that  
258 cyanobacterial LSU interacts with the carboxysome linker protein CcmM35 in the absence of a cognate  
259 cyanobacterial SSU, and forms pro-carboxysome-like aggregates in tobacco chloroplasts. In contrast to a  
260 previous study where no cyanobacterial LSU was detected in a similar tobacco transformant (Kanevski et  
261 al., 1999), we were able to detect the cyanobacterial LSU as well as catalytic activity of Rubisco in both  
262 SeL and SeLM35 lines (Table 1). It should be noted that the cyanobacterial LSU expressed in the previous  
263 study had the first 8 residues at its N terminus replaced by the first 11 residues of the tobacco LSU,  
264 possibly leading to lower stability of the modified LSU or inhibition of its assembly with the tobacco SSU  
265 (Kanevski et al., 1999).

266 Relative to comparable lines expressing Se SSU, both SeLM35 and SeL plants showed delayed  
267 growth (Fig. 6) and developed more numerous, but smaller leaves (Fig. S9, S10). SeL was not able to  
268 grow autotrophically from seeds even in high CO<sub>2</sub> levels, and required establishment on tissue culture  
269 media. Similar effects have been seen when engineering Rubisco in tobacco where either the  
270 introduction of a foreign LSU (Whitney and Andrews, 2001; Sharwood et al., 2008) or mutation of the  
271 native tobacco LSU (Whitney et al., 1999) leads to very low Rubisco amount and/or very poor activity.

272 Rubisco from both SeLM35 and SeL had dramatically slower maximum catalytic rates compared  
273 to the native Se enzyme (SeLS, Table 1), consistent with the slower growth of these plants. Combined  
274 with the significantly lower Rubisco active sites, this led to much lower Rubisco activity on a leaf area  
275 basis (Fig. 4). In both lines containing CcmM35, Rubisco catalytic rate was worse than that of  $\beta$ -

276 cyanobacterial Rubisco extracted from SeLS where no aggregation occurs, which would suggest a  
277 putative negative impact of CcmM35 on Rubisco activity in the Se plants, and agrees with previous work  
278 with the SeLSM35 line (Occhialini et al., 2016). This is consistent with previous observations from plants  
279 expressing  $\alpha$ -cyanobacterial Rubisco within a minimal  $\alpha$ -carboxysome from *Cyanobium* (Long et al.,  
280 2018). The authors found that Rubisco catalytic rate was approximately halved when determined for  
281 Rubisco from tobacco chloroplasts; however, after high-speed centrifugation to remove insoluble  
282 carboxysomes, rates were consistent with those obtained from either the native cyanobacteria or  
283 expressed without linker proteins within tobacco. Movement of metabolites such as RuBP may be  
284 similarly inhibited by the formation of large  $\beta$ -pro-carboxysomes of LSU-CcmM35, as observed via  $K_{MRuBP}$   
285 measurements made on tobacco derived minimal  $\alpha$ -carboxysomes (Long et al., 2018). The large size of  
286 the observed pro-carboxysomes in SeLSM35 and SeLM35 plants, relative to native cyanobacterial  
287 carboxysomes, appears likely to have influenced metabolite movement. This highlights that an important  
288 part of balancing expression of the various components is not only to ensure correct formation of a  
289 functional carboxysome, but also to achieve a suitably sized microcompartment. However, Rubisco  
290 extracted from SeLM35 was significantly more active than the enzyme extracted from SeL plants,  
291 showing that in the absence of Se SSU, CcmM35 helps sequester more tobacco SSU, possibly by  
292 increasing stability of the hybrid  $L_8S_8$  enzyme or facilitating its assembly (Fig. 3).

293 The very low activity observed for SeL Rubisco that lacked the cognate SSU from cyanobacteria  
294 agrees with *in vitro* findings from a number of previous studies investigating the ability of LSU-only  
295 Rubisco to perform catalysis (Andrews and Ballment, 1984; Jordan and Chollet, 1985; Andrews, 1988). In  
296 studies including cyanobacterial Rubisco, *in vitro* preparations containing only  $L_8$  octameric cores  
297 typically had detectable activity corresponding to only ~1% of the cyanobacterial holoenzyme, and even  
298 addition of heterologous higher plant SSU from spinach led to dramatic increases in activity (Andrews,  
299 1988). The cyanobacterial  $L_8$  core binds spinach SSU with an affinity an order of magnitude lower than its  
300 native SSU, and the activity of the hybrid enzyme was only half that of the enzyme with homologous  
301 subunits (Andrews and Lorimer, 1985). This suggests that the minimal activity observed for SeL Rubisco,  
302 ~5% of SeLS (Fig. 4), may in part result from a substoichiometric amount of tobacco SSU's binding to  
303 cyanobacterial  $L_8$  cores.

304 A common theme in organization of Rubisco enzymes within both carboxysomes of  
305 photosynthetic bacteria and pyrenoids from green algae appears to be through interactions with a  
306 disordered repeat protein such as CcmM35 in  $\beta$ -carboxysomes, CsoS2 in  $\alpha$ -carboxysomes and EPYC1 in  
307 pyrenoids (Long et al., 2011; Cai et al., 2015; Mackinder et al., 2016). In the case of  $\beta$ -carboxysomes and

308 pyrenoids, the Rubisco enzymes were sequestered into a separate liquid phase by these linker proteins  
309 (Freeman Rosenzweig et al., 2017; Wunder et al., 2018; Wang et al., 2019). EPYC1 or CsoS2 were shown  
310 to interact only with the SSU (Liu et al., 2018; Atkinson et al., 2019), whereas both the large and small  
311 subunits are involved in binding CcmM35 based on a cryo-EM structural model, and the L<sub>8</sub> core alone  
312 was insufficient to form a separate liquid phase with CcmM35 (Wang et al., 2019). Thus, the tobacco  
313 SSUs are likely involved in the formation of CcmM35-Rubisco aggregates in SeLM35 plants although the  
314 stoichiometry between the Se LSU and tobacco SSUs was not determined. Indeed, the residues in Se SSU  
315 critical for interaction with CcmM35 are well conserved in tobacco SSU (Fig. S11; (Wang et al., 2019).

316 The poor photosynthetic performance of these transplastomic lines in the absence of a  
317 functional CCM with all the necessary components is unsurprising. However, the ability of some lines to  
318 outperform wild-type plants on a per Rubisco basis at higher CO<sub>2</sub> levels suggests that provided with high  
319 CO<sub>2</sub> concentrations such as those within a fully formed  $\beta$ -carboxysome shell in a complete CCM, the  
320 Rubisco levels within these plants may be sufficient to support improved rates of carbon assimilation.  
321 Consistent with this, Long and colleagues (2018) observed that leaf discs from plants expressing  $\alpha$ -  
322 cyanobacterial Rubisco produced similar photosynthetic rates to wild-type tobacco plants in 2% (v/v) CO<sub>2</sub>  
323 conditions within a membrane inlet mass spectrometry system (MIMS). Thus, and even considering the  
324 associated nitrogen costs of producing the shell components, reducing the typically very large  
325 investment into Rubisco by C<sub>3</sub> plants may represent an overall nitrogen saving (McGrath and Long, 2014).  
326 An issue that is highly likely to be encountered when dealing with the numerous other components of  
327 the carboxysome shell is to optimize expression levels, and this may also be necessary for Rubisco. An  
328 increasing understanding of the role of chaperones for Rubisco assembly (Feiz et al., 2014; Salesse-Smith  
329 et al., 2018; Wilson and Hayer-Hartl, 2018; Conlan et al., 2019) may provide avenues to increase Se  
330 Rubisco amounts, should this become necessary to support the desired number of carboxysomes per  
331 chloroplast, in order to drive higher photosynthetic rates within a fully formed CCM. It is also possible  
332 that adjusting the chloroplast regulatory sequences used to express Se Rubisco subunits may be  
333 sufficient to increase the Rubisco amount.

334 The ability of CcmM35 to link Se LSU *in planta* without a cognate SSU shows that tobacco SSU  
335 can not only substitute Se SSU to form functional hybrid Rubisco, but can also result in an enzyme to  
336 which CcmM35 can bind. While the Se SSU does not appear to be essential for formation of a pro-  
337 carboxysome, the differences shown here based on its presence in a pro-carboxysome highlight its  
338 importance for full Rubisco functionality and carboxysome structural organization. These results support  
339 the likely necessity of co-engineering cognate subunits from a distant foreign Rubisco, as part of efforts

340 to engineer both a foreign Rubisco into higher plants (Whitney and Andrews, 2001; Sharwood et al.,  
341 2008) and for more complex engineering of CO<sub>2</sub>-concentrating mechanisms such as carboxysomes and  
342 pyrenoids from cyanobacteria and algae, respectively (Atkinson et al., 2016; Rae et al., 2017).

343 The carboxysome alone will be insufficient to attain higher rates of photosynthesis without the  
344 removal of existing stromal carbonic anhydrase and the addition of transporters to pump high levels of  
345 HCO<sub>3</sub><sup>-</sup> into the chloroplast (Hanson et al., 2016; Long et al., 2018; Desmarais et al., 2019). There have  
346 been recent improvements in approaches to tackle the issue of localizing these inorganic carbon pumps  
347 (Rolland et al., 2016; Uehara et al., 2016), alongside advances in understanding the role of the various  
348 carbonic anhydrases (Hu et al., 2015; DiMario et al., 2016). Furthermore, there is now a better  
349 understanding of the actual ratios of components in β-carboxysomes (Sun et al., 2019), engineering of β-  
350 carboxysome shells to obtain cryoEM structural models (Cai et al., 2016; Sutter et al., 2019), an assembly  
351 of full β-carboxysomes in *E. coli* (Fang et al., 2018), and recent successes with α-carboxysomes (Long et  
352 al., 2018). These advances provide encouragement that ongoing research is steadily moving toward the  
353 ability to assemble these complex, powerful CCMs within plants to improve photosynthesis with the  
354 ultimate goal of improving global food security.

355

356

## 357 **Materials and Methods**

### 358 *Construction of chloroplast transformation vectors*

359 All primers used were obtained from Integrated DNA Technologies and listed in Table S1. Phusion™ high-  
360 fidelity DNA polymerase, FastDigest restriction enzymes and T4 DNA ligase from Thermo Scientific were  
361 used to generate amplicons, restriction digests and ligation products respectively. The ligation products  
362 were transformed into chemically competent DH5α *E. coli* and selected on LB agar medium with 100  
363 µg/mL ampicillin. A template vector to hold each DNA piece was first constructed as follows. The *aadA*  
364 operon from BJE-070 vector (Hanson et al., 2013) was removed by self-ligation of the NsiI digest. An  
365 amplicon was generated from the resulting vector using NsiI-BJF3 and BamHI-BJF5 primers and ligated  
366 into the BamHI and NsiI sites of the vector to introduce SbfI and NotI sites upstream of the NsiI locus.  
367 The resulting vector, BJFE-BB, was used as a vector to hold each DNA element between the SbfI and NotI  
368 sites using BB-XXX-f and BB-XXX-r primers where 'XXX' stands for the name of each DNA element. Once  
369 ligated into the BJFE-BB vector, each DNA element was flanked by SbfI-MluI upstream and MauBI-NotI  
370 downstream. Since MluI and MauBI restriction sites have compatible cohesive ends, these DNA parts can  
371 be assembled in any desired order using an approach similar to the BioBrick method (Shetty et al., 2008).

372 Specifically, we assembled an *aadA* module comprised of loxP-At\_TpsbA-IEE-SD-RBS-aadA-loxP. We then  
373 modified pGEM-F1-rbcL-F2 vector described previously (Lin et al., 2014b) by introducing a SbfI site  
374 immediately downstream of the *Se-rbcL* gene. It was accomplished by ligating the amplicon generated  
375 with HindIII-LSUE5 and T1L-IEE3 primers into the HindIII and XbaI sites to obtain the pCT-rbcL-BB2 vector.  
376 Next, XbaI+AscI digest of the amplicon from TrbcL5 and AscI-LSUFI2r primers was ligated into XbaI and  
377 MluI sites of pCT-rbcL-BB2 vector to obtain pCT-rbcL-BB vector. Finally, we introduced the *aadA* module  
378 between the SbfI and NotI sites of pCT-rbcL-BB vector to obtain pCT-rbcL-BB-aadA vector used to  
379 generate the SeL chloroplast transformant tobacco line. pCT-rbcL-ccmM35 described previously (Lin et  
380 al., 2014b) was used in the generation of SeLM35 tobacco chloroplast transformant.

381

### 382 *Generation of transplastomic tobacco plants*

383 We introduced transformation vectors into two-week-old tobacco (*Nicotiana tabacum* cv. Samsun)  
384 seedlings with the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad Laboratories) and tissue-  
385 culture based selection method as described previously (Occhialini et al., 2016). Briefly, about 10 µg of  
386 DNA was mixed with 100 µL of 50 mg/mL 0.6 µm gold nanoparticles, 100 µL of 2.5 M CaCl<sub>2</sub> and 40 µL of  
387 0.1 M spermidine free-base by vortexing for about one minute. The gold particles were then pelleted in  
388 a microcentrifuge at 1000 rpm for 8 seconds and resuspended in 180 µL of 70% ethanol. After the  
389 washing of the gold particles was repeated one more time, the pellet was resuspended in about 60 µL of  
390 100% ethanol and then spread on ten microcarrier discs used for bombardment. Two days later, the  
391 leaves from the bombarded seedlings were cut into halves and placed on RMOP agar medium with 500  
392 µg/mL spectinomycin for 4-6 weeks at 23°C under 14 h light per day. The shoots arising were cut into 5  
393 mm<sup>2</sup> pieces and subjected to a second round of selection on the same medium for another 4-6 weeks.  
394 The regenerated shoots were then transferred to MS agar medium for rooting and subsequently  
395 transferred to soil for growth in a chamber with elevated CO<sub>2</sub> (~9000 ppm) until the seeds were  
396 collected. Total DNA was extracted from leaf tissues using CTAB buffer, digested with EcoRV+KpnI  
397 restriction enzymes, separated on a 1% agarose gel, transferred to a Nylon membrane and detected with  
398 a DIG-label DNA probe as described previously (Lin et al., 2014b).

399

### 400 *Analyses of transgenes' transcripts on RNA blots*

401 The transcripts were analyzed on RNA blots using the procedure described previously with the same  
402 DIG-labeled RNA probes (Occhialini et al., 2016). Briefly, RNA samples were prepared from leaf tissues  
403 with a PureLink<sup>®</sup> RNA mini kit (Life Technologies) and their concentrations were estimated with a Qubit<sup>®</sup>

404 RNA BR assay kit. About 1 µg each RNA sample was mixed with NorthernMax<sup>®</sup> formaldehyde load dye  
405 (Life Technologies) with 50 µg/mL ethidium bromide and incubated at 65 °C for 15 min before they were  
406 loaded to 1.3% agarose gel with 2% formaldehyde. After separation at 7 V cm<sup>-1</sup> for about 2 h, the gel was  
407 washed three times in diethylpyrocarbonate-treated water for 10 min each and incubated in 20x SSC for  
408 45 min before the RNAs were transferred to a positively charged nylon membrane under capillary action.  
409 The membrane was then exposed to UV radiation with a Stratalinker<sup>®</sup> UV Crosslinker, hybridized with 200  
410 ng of each DIG-labeled RNA probe in ~ 4 mL of DIG EasyHyb buffer (Roche) at 68 °C overnight, and  
411 detected with anti-digoxigenin-AP antibody and CDP-Star chemiluminescent substrate (Roche).

412

#### 413 *Plant material*

414 Seeds of wild type (WT) and transplastomic tobacco (*Nicotiana tabacum* cv. Samsun) were sown into  
415 trays of a commercial potting mix (Petersfield Products, UK) with a slow-release fertiliser (Osmocote,  
416 Scotts UK Professional, UK). Seedlings were thinned out after *ca.* two weeks, with individual plants  
417 transferred to 1 L pots after three weeks. Seeds of SeL were sown into tissue culture pots containing agar  
418 solidified MS medium containing 1% sucrose before transferring to soil after three weeks. Plants were  
419 grown in a controlled environment chamber (Microclima 1750, Snijders Scientific B.V., Netherlands). The  
420 chamber was set at day/night temperatures of 24/22 °C with a 16 h photoperiod, 60 % humidity. The  
421 ambient CO<sub>2</sub> concentration within the chamber was maintained at 4000 ± 400 ppm using the integrated  
422 CO<sub>2</sub> controller. CO<sub>2</sub> levels were also monitored in the chamber with a Vaisala hand held GM70 meter  
423 (Vaisala, UK). Plants were kept well-watered. Space limitations within growth chambers necessitated  
424 growing plants in batches for growth analysis.

425

#### 426 *Fixation and embedding of plant tissue, immunogold labelling and TEM*

427 Small pieces (1x1.5mm) of tissue from fully expanded leaves of plants equivalent in size to 33 DAS WT  
428 plants were incubated in fixative (4% paraformaldehyde, 2.5 % glutaraldehyde in 0.05 M sodium  
429 phosphate buffer pH 7.2) for 2 hours at room temperature with rotation. A vacuum was used to aid  
430 infiltration. After washing 3x 10 minutes in 0.05 M sodium phosphate buffer pH 7.2, the tissue was  
431 dehydrated in an ethanol series (50%, 70%, 80%, 90%) at room temperature for 30 minutes each step  
432 and finally 100% ethanol for 1 hour. Tissue was infiltrated with LR white resin (Agar Scientific, UK), first  
433 by incubating for 1 hour in 100% ethanol:LR white 1:1 (v/v), then for 2 hours in 100% LR white and finally  
434 overnight in 100% LR white. Specimens were transferred to Eppendorf tubes charged with fresh 100%  
435 LR white resin. The tubes were sealed with plastic film and the resin polymerised at 50 °C for 16 hours.

436 Ultrathin sections (~90 nm) of embedded leaf material were captured on gold gilded grids (Agar  
437 Scientific, Stansted, UK) and used for immunogold labelling. Samples were blocked for 30 minutes in 1%  
438 BSA in phosphate buffered saline (PBS) and then incubated in primary antibody solution (antibody  
439 diluted 1/100 in 1% BSA in PBS) for 1.5 hours. Grids were washed 3x 10 minutes with 1% BSA in PBS  
440 before incubation for 1 hour with secondary goat anti-rabbit antibody conjugated to 10 nm gold particles  
441 (Agar Scientific, UK, 1/100 antibody dilution prepared in 1% BSA in PBS). Grids were washed 3x 10  
442 minutes in 1% BSA in PBS and 3x 5 minutes in distilled water before air-drying. Images were obtained at  
443 80kv using a JEOL 1010 (JEOL, Japan) microscope equipped with a digital AMT NanoSprint500 camera  
444 (Deben, UK).

445

#### 446 *Gel electrophoresis and immunoblotting*

447 Soluble protein extracts were analysed for the presence of proteins via both denaturing (SDS-PAGE) and  
448 non-denaturing (Native-PAGE) gel electrophoresis. SDS-PAGE and immunoblotting was carried out as  
449 described in Perdomo *et al.* (2018) using Bio-Rad Mini-Protean TGX gels (Bio-Rad, UK). Non-denaturing  
450 gels were run using a Tris-glycine buffering system at 4°C as per the manufacturer's instructions. For both  
451 types of electrophoresis, immunoblotting was as described by Perdomo *et al.* (2018) using SeLSU and  
452 CcmM antibodies described previously (Lin *et al.*, 2014b) and a plant SSU antibody (Agri-Sera AS07 259,  
453 Agri-Sera, Sweden).

454

#### 455 *Rubisco biochemistry*

456 Rubisco activities and activation state in leaf extracts were determined as described by Carmo-Silva *et al.*  
457 (2017), except that homogenate centrifugation was at a reduced 300 *g* for 1 min. Chlorophyll content in  
458 the homogenates was determined by the method of Wintermans and de Mots (1965) using ethanol and  
459 measuring absorbance in a microplate reader (SPECTROstar Nano, BMG LabTech, UK). Total soluble  
460 protein (TSP) in the same supernatant as used for Rubisco activity assays was determined via Bradford  
461 assay (1976). The amount of Rubisco was also quantified in the same supernatant by a [<sup>14</sup>C]CABP  
462 [carboxyarabinitol-1,5-bisphosphate] binding assay (Whitney *et al.*, 1999).

463 Rubisco catalytic properties were determined essentially as described previously (Prins *et al.*,  
464 2016; Orr and Carmo-Silva, 2018) with the following changes: leaf discs were ground in extraction buffer,  
465 followed by centrifugation at 300 *g* and 4°C for 1 min. Supernatants were immediately used for assays,  
466 which was previously found to be suitable with similar cyanobacterial Rubisco complexes (Lin *et al.*,  
467 2014b). Additional higher CO<sub>2</sub> concentrations (180, 280, and 410 μM) were also used for catalysis assays

468 to enable determination of the Michaelis-Menten constant for CO<sub>2</sub> ( $K_M^{CO_2}$  or  $K_c$ ).

469

#### 470 *Photosynthesis measurements*

471 Photosynthetic gas exchange was measured in healthy leaves that had recently reached full expansion,  
472 typically leaf 4 or 5 on plants of approximately 45 cm in height. A LI-6800F portable gas exchange system  
473 (LI-COR, Lincoln, NE, USA) was used to enclose a 6 cm<sup>2</sup> portion of leaf, with constant irradiance of 600  
474  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  supplied by the cuvette head LEDs, a vapour pressure deficit of  $1.20 \pm 0.03$  kPa and  
475 a flow rate of  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Leaf temperature was maintained at  $24 \pm 1^\circ\text{C}$ . For all measurements, the  
476 entire gas exchange system was positioned inside the plant growth chamber, and controlled remotely via  
477 Ethernet connection. After the cuvette was clamped onto a leaf, the chamber door was kept closed to  
478 minimise fluctuations in CO<sub>2</sub> levels and the plant allowed to stabilise for at least 15 min at 3000 ppm CO<sub>2</sub>  
479 prior to commencing measurements. For transplastomic tobacco lines, the ambient CO<sub>2</sub> concentration  
480 (Ca) was subsequently decreased to 100 ppm, followed by increases to 200, 400, 800, 1200, 1600, 2000  
481 and 2500 ppm CO<sub>2</sub>. For wild-type tobacco, additional concentrations were used such that increases in  
482 CO<sub>2</sub> went from 50 to 100, 150, 200, 250, 300, 400, 600, 800, 1200, 1600, 2000 and 2500 ppm. For all  
483 leaves measured, a separate CO<sub>2</sub> response curve was determined under 2% (v/v) O<sub>2</sub> conditions using a  
484 balanced air gas cylinder for input, using otherwise identical settings.

485

#### 486 *Plant biomass*

487 Leaf numbers and leaf measurements were taken every 3-7 days from four or five individuals for each  
488 line (2 in the case of the SeL line). Plant height was measured from soil level to growing point.  
489 Measurements were initiated at 28 DAS for WT and SeLS, 46 DAS for SeLSM35 and SeLm35 and 127 DAS  
490 for SeL, due to the differing growth rates between lines and continued until the initiation of flowering. At  
491 the end of the growth period, final leaf measurements were taken and area measured using a LI-COR  
492 3100 leaf area machine (LI-COR, Europe). Leaf areas were then derived for all time points.

493

#### 494 *Statistical analysis*

495 Statistical differences between trait means were tested via one-way analysis of variance (ANOVA). In  
496 cases where an effect of genotype was observed ( $P < 0.05$ ), a post-hoc Tukey test was used to conduct  
497 multiple pairwise comparisons. Statistical analyses were performed using RStudio (version 1.1.453, (R  
498 Studio Team, 2019)) and R (version 3.5.0, (R Core Development Team, 2013)). Plots were prepared with  
499 ggplot2 (Wickham, 2016). Plant height and leaf area data analyses involved fitting curves to the

500 exponential phase of growth and comparing means of the curve coefficients using ANOVA. Where a  
501 significant difference was observed between lines, a post-hoc Holm-Sidak test was used for multiple  
502 pairwise comparisons. Analyses were performed in SigmaPlot (version 13, Systat Software, UK).

503

504

#### 505 **Accession Numbers**

506 Sequence data for cyanobacterial RbcL and CcmM35 can be found in the GenBank data library under  
507 accession numbers AIM40198.1 and AIM40200.1 respectively.

508

#### 509 **Supplemental Data**

510 **Supplemental Figure S1.** RNA blots of WT and transplastomic tobacco lines.

511 **Supplemental Figure S2.** Presence of pro-carboxysome compartments in tobacco transplastomic plants  
512 containing cyanobacterial Rubisco large subunits and CcmM35, with and without Rubisco small subunits.

513 **Supplemental Figure S3.** Electron micrographs of tobacco plants expressing cyanobacterial Rubisco large  
514 subunits and CcmM35 contain a pro-carboxysome compartment in the chloroplast.

515 **Supplemental Figure S4.** Additional examples of electron micrographs of tobacco plants expressing  
516 cyanobacterial Rubisco large subunits and CcmM35 with a pro-carboxysome compartment in the  
517 chloroplast.

518 **Supplemental Figure S5.** Western blots of SDS-PAGE and Native-PAGE gels used to examine protein  
519 composition of wild-type (WT) tobacco and transplastomic lines expressing  $\beta$ -cyanobacterial  
520 carboxysome components.

521 **Supplemental Figure S6.** Rubisco content expressed as grams per square metre.

522 **Supplemental Figure S7.** Chlorophyll content of transplastomic lines.

523 **Supplemental Figure S8.** Response of leaf CO<sub>2</sub> assimilation to intercellular CO<sub>2</sub> concentrations (C<sub>i</sub>) under  
524 atmospheric levels and 2 % oxygen.

525 **Supplemental Figure S9.** Plant photographs at a comparable growth stage.

526 **Supplemental Figure S10.** Comparison of leaf size in transplastomic plants.

527 **Supplemental Figure S11.** Multiple sequence alignment of cyanobacterial and tobacco Rubisco small  
528 subunits.

529

530 **Supplemental Table S1.** Oligonucleotide sequences used in the construction of transformation vectors.

531 **Supplemental Table S2.** Plant growth data analyses.

532

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536 comments on the manuscript.

537

538 **Tables**

539

540 **Table 1. Rubisco catalytic properties.** Rubisco maximum carboxylation rate ( $V_c$ ), and Michaelis-Menten  
541 constant for CO<sub>2</sub> ( $K_c$ ) of wild-type (WT) tobacco and transplastomic lines expressing  $\beta$ -cyanobacterial  
542 carboxysome components from *Synechococcus elongatus* (Se): Rubisco large subunit (L), Rubisco small  
543 subunit (S), CcmM35 (M35). Values represent mean  $\pm$  SEM (n = 3-5 biological replicates). \* Wild-type  
544 values from Occhialini *et al.* (2016). Letters denote significant differences ( $P < 0.05$ ) between  
545 transplastomic lines as determined by Tukey's pairwise comparisons following ANOVA.

546

547

548

549 **Figure legends**

550

551 **Figure 1. Replacement of the *rbcl* gene in tobacco chloroplasts with the *Se-rbcl* with or without the**  
552 ***ccmM35* gene.** (A) The gene arrangements of WT, SeL and SeLM35 tobacco lines along with the locations  
553 of the EcoRV and KpnI restriction sites used in the DNA blot. The binding site for the DIG-labeled DNA  
554 probe is shown in green bars. Seeds were obtained from two independent SeL lines and one SeLM35  
555 line. (B) DNA blot analysis of the WT, SeL and SeLM35 samples digested with EcoRV and KpnI. All samples  
556 produced the expected band on the DNA blot.

557

558 **Figure 2. Tobacco plants expressing cyanobacterial Rubisco large subunits and CcmM35 contain a pro-**  
559 **carboxysome compartment in the chloroplast.** Immunolocalization of *Synechococcus elongatus* (Se)  
560 proteins in the chloroplasts of transplastomic tobacco lines expressing the Rubisco large subunit and  
561 CcmM35 (SeLM35) or the large subunit alone (SeL). Electron micrographs of ultrathin sections of  
562 mesophyll cells probed with the indicated primary antibody and a secondary antibody conjugated to 10  
563 nm gold particles. Scale bars indicate size. Additional images are presented in Supplemental Figures S3  
564 and S4.

565

566 **Figure 3. Protein composition of wild-type (WT) tobacco and transplastomic lines expressing  $\beta$ -**  
567 **cyanobacterial carboxysome components.** Polypeptides in leaf extracts prepared from plants of each  
568 line were separated by denaturing SDS-PAGE (A) and non-denaturing Native-PAGE (B) and either stained  
569 with Coomassie Blue (upper panels) or used for immunoblotting with antibodies against cyanobacterial  
570 Rubisco large subunit (SeLSU) and CcmM35, and against tobacco Rubisco small subunit (NtSSU) (lower  
571 panels). Panels showing blotting of PAGE gels are slices from blots (see Fig. S5) and show the indicated  
572 size regions where the respective antibodies detect proteins of interest. For SDS-PAGE and Native-PAGE,  
573 10 and 20  $\mu$ g total soluble protein was loaded per lane, respectively. (C), SDS-PAGE and Native-PAGE gels  
574 immunoblotted with antibody against NtSSU, loaded with 20 and 40  $\mu$ g total soluble protein,  
575 respectively.

576

577 **Figure 4. Rubisco and total soluble protein.** Rubisco total activity (A), activation state (B), and content  
578 (C), and total soluble protein (D), of wild-type (WT) tobacco and transplastomic lines expressing  $\beta$ -  
579 cyanobacterial carboxysome components from *Synechococcus elongatus* (Se): Rubisco large subunit (L),  
580 Rubisco small subunit (S), CcmM35 (M35). Values represent mean  $\pm$  SEM (n = 3-4 biological replicates).

581 Letters denote significant differences ( $P < 0.05$ ) as determined by Tukey's honestly significant difference  
582 [HSD] mean-separation test following ANOVA ( $P$ -values indicated on each panel).

583

584 **Figure 5. Response of net CO<sub>2</sub> assimilation (A) to intercellular CO<sub>2</sub> concentrations (C<sub>i</sub>).** Rates are  
585 expressed on an area basis (A) and on a Rubisco active site basis (B) for leaves of wild-type (WT) tobacco  
586 and transplastomic lines expressing  $\beta$ -cyanobacterial carboxysome components from *Synechococcus*  
587 *elongatus* (Se): Rubisco large subunit (L), Rubisco small subunit (S), CcmM35 (M35). Values represent  
588 mean  $\pm$  SEM (n = 3-4 biological replicates).

589

590 **Figure 6. Plant development and growth traits.** Photographs of 33 day old plants grown in parallel in  
591 4000 ppm CO<sub>2</sub> (A), plant height (B) and leaf area (C) development during the growth cycle wild-type (WT)  
592 tobacco and transplastomic lines expressing  $\beta$ -cyanobacterial carboxysome components from  
593 *Synechococcus elongatus* (Se): Rubisco large subunit (L), Rubisco small subunit (S), CcmM35 (M35).  
594 Values represent mean  $\pm$  SEM (n = 3-5 biological replicates). DAS, days after sowing.

595

596

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598

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