A procedure to introduce point mutations into the Rubisco large subunit gene in wild-type plants

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SUMMARY
Photosynthetic inefficiencies limit the productivity and sustainability of crop production and the resilience of agriculture to future societal and environmental challenges. Rubisco is a key target for improvement as it plays a central role in carbon fixation during photosynthesis and is remarkably inefficient. Introduction of mutations to the chloroplast-encoded Rubisco large subunit rbcL is of particular interest for improving the catalytic activity and efficiency of the enzyme. However, manipulation of rbcL is hampered by its location in the plastome, with many species recalcitrant to plastome transformation, and by the plastid’s efficient repair system, which can prevent effective maintenance of mutations introduced with homologous recombination. Here we present a system where the introduction of a number of silent mutations into rbcL within the model plant Nicotiana tabacum facilitates simplified screening via additional restriction enzyme sites. This system was used to successfully generate a range of transplastomic lines from wild-type N. tabacum with stable point mutations within rbcL in 40% of the transformants, allowing assessment of the effect of these mutations on Rubisco assembly and activity. With further optimization the approach offers a viable way forward for mutagenic testing of Rubisco function in planta within tobacco and modification of rbcL in other crops where chloroplast transformation is feasible. The transformation strategy could also be applied to introduce point mutations in other chloroplast-encoded genes.

Keywords: chloroplast transformation, food security, homologous recombination, Rubisco, photosynthesis, site-directed mutagenesis, Nicotiana tabacum, technical advance.

INTRODUCTION
The transformation of chloroplast or plastid genomes in higher plants represents a promising technology in multiple biotechnological applications such as the introduction of agronomically important traits, metabolic engineering, recombinant protein expression and the production of high-value therapeutic compounds (Maliga and Bock, 2011). Chloroplast transformation offers several benefits: precise manipulation of its genome via homologous recombination, no transgene silencing and better control over the escape of transgenes into the environment. In addition to the introduction and expression of foreign genes, chloroplast transformation can also be used to delete or mutate plastid-encoded protein subunits for functional studies. Since many of these proteins are involved in photosynthesis, the technology has great potential to improve photosynthesis and productivity in crops (Hanson et al., 2013; Bock, 2015; Martin-Avila et al., 2020).

One of the most commonly studied plastid genes is rbcL, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). In plants, Rubisco catalyzes two competing reactions in the stroma of chloroplasts: carboxylation or oxygenation of ribulose-1,5-bisphosphate (RuBP) (Ogren and Bowes, 1971; Tcherkez et al., 2006; Andersson and Backlund, 2008). The carboxylation of RuBP is an essential step in photosynthesis in plants, while its oxygenation generates 2-phosphoglycolate, which is recycled through the photosynthetic pathway, spanning multiple organelles (Ogren and Bowes, 1971; Ogren, 1984; Keys, 1986; Busch, 2020). Rubisco in C3 plants is a relatively slow enzyme with a catalytic turnover number, kcat, of about 3–4
CO₂ sec⁻¹ (Orr et al., 2016; Flamholz et al., 2019). As a result, C₃ plants generally express a large amount of Rubisco within the chloroplast stroma in order to achieve sufficient carbon fixation. Since the reactions of Rubisco control major metabolic fluxes, manipulating its kinetics has been an important target for improving photosynthesis in plants (Sharwood, 2017; Zhu et al., 2020). Two general goals of engineering Rubisco are to improve its carboxylation efficiency under ambient O₂, which is defined as $k_{cat}^{c}/K_{M}^{c}$, where $K_{M}$ is the Michaelis-Menten constant, and to increase its CO₂/O₂ specificity factor, which is the ratio of its carboxylation efficiency to oxygenation efficiency or $k_{cat}^{c}K_{M}^{c}O_{2}^{c}K_{M}^{c}$ (Whitney et al., 2011a). Increasing the Rubisco content in maize and rice led to higher plant biomass, supporting the hypothesis that Rubisco represents a bottleneck in photosynthesis and crop yields can be improved with a more efficient Rubisco (Salesse-Smith et al., 2018; Yoon et al., 2020). However, thus far, attempts to engineer vascular plants with such a Rubisco enzyme have been unsuccessful.

Rubisco enzymes with different kinetic properties exist in nature despite the well-characterized catalytic constraints (Galmès et al., 2015, 2016; Sharwood et al., 2016a; Flamholz et al., 2019). As organisms adapt to different environments they have evolved Rubisco enzymes that are optimized to their immediate surroundings (Tcherkez et al., 2006; Savir et al., 2010). For example, Rubiscos in C₄ plants are generally associated with a lower affinity for CO₂ or higher $K_{M}$ and a higher $k_{cat}^{c}$ compared with those in C₃ plants (Whitney et al., 2011a). Modeling studies indicated that a typical C₃ Rubisco is optimized for 220 p.p.m. of atmospheric CO₂ and Rubiscos from several C₃ plants would improve carbon fixation in C₃ plants (Zhu et al., 2004; Sharwood et al., 2016b). Rubiscos from red algae such as *Griffithsia monilis* were shown to have the highest known specificity factors and are generally assumed to be promising candidates for improving photosynthesis in C₃ plants (Whitney et al., 2011a).

Plants possess form I Rubisco, which is a hexadecameric complex made up of eight chloroplast-encoded large subunits (LSus) and eight small subunits (SSus) encoded by a family of *RbcS* nuclear genes and imported to the chloroplast stroma (Whitney et al., 2011a). Each holoenzyme complex consists of four LSu dimers with two active sites located inside each dimer at the interface between the two LSus (Andersson and Backlund, 2008), and capped with four SSu monomers at each end. Plants produce specific chaperonins to prevent irreversible aggregation of LSus as well as multiple chaperones for step-by-step assembly of functional LₚS₂ complexes (Bracher et al., 2017; Wilson and Hayer-Hartl, 2018). Thus, engineering plants with a more efficient Rubisco that can accumulate the enzyme at sufficiently high levels (Carmo-Silva et al., 2015) has been a major challenge.

Engineering Rubisco in higher plants has historically been carried out exclusively in tobacco (*Nicotiana tabacum*), where well-established procedures for chloroplast transformation allowed precise modification of its *rbcL* gene through homologous recombination and, recently, co-engineering of both Rubisco subunits (Sharwood, 2017; Martin-Avila et al., 2020). Although LSus from sunflower, Arabidopsis and C₃ and C₄ *Flaveria* species were able to assemble with native tobacco SSus to form functional enzymes in tobacco transformant plants, the accumulation of such hybrid enzymes in the leaves was significantly lower than the normal amount, probably due to suboptimal interactions between the foreign LSus and native chaperones or SSus (Kanevski et al., 1999; Whitney et al., 2011b, 2015). Attempts to replace the *rbcL* gene in tobacco with red algal Rubisco genes produced transformants without functional Rubisco due to incompatibility with the chaperonin machinery and chaperones in tobacco chloroplast stroma (Whitney et al., 2001; Lin and Hanson, 2018). A recent study demonstrated that a red-type Rubisco from *Rhodobacter sphaeroides* was able to assemble and function in tobacco chloroplasts, but its poor compatibility with native Rubisco activases led to low activation levels in the absence of its cognate Rubisco activase from *R. sphaeroides* (Gunn et al., 2020). Thus, successfully expressing Rubisco that is sufficiently phylogenetically distant from the host plant is likely to require manipulation of ancillary proteins such as Rubisco activase and/or assembly related factors.

Unless chaperones and SSus can also be optimized to work with a foreign LSu, an alternative approach could be to introduce carefully selected site-directed mutations in the native LSu via chloroplast transformation. However, generating such transformants from wild-type tobacco has been inefficient because the desired mutations introduced by recombination between the mutated *rbcL* and the native version of the gene can be removed by the plastid’s repair system before transformants reached homoplasmy (Kanevski et al., 1999; Whitney et al., 1999). Thus, a tobacco master line has been created in which the native *rbcL* gene is replaced with the codon-modified *rbcL* gene from *Rhodospirillum rubrum* with low sequence homology followed by removal of the selectable marker gene to facilitate the introduction of mutated and foreign *rbcL* genes and polycistrons into tobacco (Whitney and Sharwood, 2008). This tobacco master line has been successfully used to study the residues critical for the catalytic properties of C₄ Rubisco enzymes in *Flaveria* species as well as introduction of Arabidopsis LSus into tobacco (Whitney et al., 2011b, 2015). In a recent study, inhibiting the expression of native SSus in the tobacco master line with RNA interference allowed the simultaneous transformation of both Rubisco subunits and investigation of the effects of novel Rubisco complexes in tobacco (Martin-Avila et al., 2020). This tobacco master line has been a great resource for carrying out Rubisco engineering in a model species.
although the generation of such a master line in other plants is a lengthy process and an efficient procedure to modify chloroplast genes including \(rbcl\) using a wild-type line would therefore be helpful.

In this study, we developed an approach to effectively introduce specific mutations into chloroplast-encoded genes. As a proof of concept, we synthesized a tobacco \(rbcl\) gene with synonymous or silent mutations resulting in unique restriction sites. Using this modified \(rbcl\) gene as a template, we introduced single- or double-residue substitutions that were predicted in previous phylogenetic and biochemical studies to be potentially important for the enzyme’s kinetic properties (Kapralov et al., 2012; Galmés et al., 2014; Studer et al., 2014; Orr et al., 2016). This approach allowed us to successfully replace the \(rbcl\) gene with the modified \(rbcl\) genes directly within wild-type tobacco plants with high retention of the mutations. A procedure to screen for transformants that possess the mutant \(rbcl\) genes is described, as well as preliminary analyses of Rubisco activities in 10 different transplastomic lines.

RESULTS

Modified restriction sites in the \(rbcl\) gene allowed efficient screening of the transformants

We synthesized part of a modified \(rbcl\) gene (Nt-rbcl\(^m\)) by introducing 26 silent mutations such that four restriction sites were removed while 10 were added (Figure 1a and Figure S1 in the online Supporting Information). The Nt-rbcl\(^m\) gene was then seamlessly joined with the native \(rbcl\) promoter from tobacco and inserted into a chloroplast transformation plasmid, pCT-rbcl, described previously (Lin et al., 2014). In the resultant chloroplast transformation vector pCT-Nt-rbcl\(^m\), the Nt-rbcl\(^m\) gene followed by the native \(rbcl\) terminator, Nt-Trbcl, and a selectable marker operon expressing the aadA gene driven by the tobacco psbA promoter are flanked between a 980 bp upstream homologous region or Flank 1, which contains the native \(rbcl\) promoter, and a 1 kbp downstream homologous region, or Flank 2 (Figure 1b). The homologous recombination between the plastid genome and the pCT-Nt-rbcl\(^m\) plasmid through the two flanking regions should introduce the aadA marker gene into the plastid genome and facilitate the selection of transformants with spectinomycin on regeneration medium. However, it is expected that the intended mutations in Nt-rbcl\(^m\) will be incorporated into only a portion of the transformants with the aadA gene. If the cross-over site upstream of the aadA gene is located within Nt-rbcl\(^m\), the resulting transformants will not possess those mutations upstream of the cross-over site (Figure 1c). Our strategy is to use the unique restriction sites in Nt-rbcl\(^m\) to screen for the transformants with the intended mutations.

Figure 1. Modification of the tobacco \(rbcl\) gene for chloroplast transformation.
(a) Schematic diagram of the synthesized Nt-rbcl\(^m\) gene with modified restriction sites.
(b) The chloroplast transformation vector pCT-Nt-rbcl\(^m\) for replacing the tobacco \(rbcl\) gene with Nt-rbcl\(^m\).
(c) Comparison of two hypothetical scenarios of cross-over events between the \(rbcl\) locus of the plastid genome and pCT-Nt-rbcl\(^m\) plasmid upstream of the selectable marker aadA gene. The location of a hypothetical point mutation in Nt-rbcl\(^m\) gene on pCT-Nt-rbcl\(^m\) plasmid is indicated with an ‘X’. The two cross-over events are indicated with dashed lines between the plastid genome and pCT-Nt-rbcl\(^m\) plasmid. If the cross-over takes place in Flank 1 or inside Nt-rbcl upstream of ‘X’, as in event 1, the point mutation will be introduced into the transformant. On the other hand, the cross-over taking place downstream of ‘X’ will fail to introduce the point mutation into the transformant. Note that although the segment upstream of Nt-rbcl has been marked as ‘Flank 1’, Nt-rbcl should be considered as part of Flank 1 due to high homology between Nt-rbcl and Nt-rbcl\(^m\).
Introducing point mutations into chloroplast genes

Table 1 Summary on the generation of tobacco chloroplast transformants with point mutations in the Rubisco large subunit and the number of independent shoots obtained at each step of selection

| Cultivar      | Mutations            | Predicted roles and references                                                                 | Number of independent shoots obtained at each stage of selection |
|---------------|----------------------|------------------------------------------------------------------------------------------------|---------------------------------------------------------------|
|               |                      |                                                                                               | First round | First round with expected restriction fragments | Homoplasmic shoots after the second selection round | Final independent transformants |
| Samsun        | None (NtLwt)         | Unmodified Rubisco                                                                           | 8           | 2                                      | 1                                           | 1*                                 |
| Samsun        | V101I                | C$_3$-C$_4$ transition (Christin et al. 2008; Studer et al. 2014)                             | 2           | 1                                      | 1                                           | 1                                   |
| Samsun        | V255A                | C$_3$-C$_4$ transition (Kapralov and Filatov, 2007; Christin et al. 2008; Studer et al. 2014) | 4           | 1                                      | 1                                           | 1                                   |
| Samsun        | A281S                | C$_3$-C$_4$ transition (Christin et al. 2008; Studer et al. 2014)                             | 8           | 3                                      | 3                                           | 3                                   |
| Samsun        | H282N                | C$_3$-C$_4$ transition (Studer et al. 2014)                                                   | 5           | 3                                      | 3                                           | 3                                   |
| Samsun        | A281S,H282N          | See A281S and H282N above                                                                     | 4           | 2                                      | 2                                           | 2                                   |
| Petit Havana  | L270I                | C$_3$-C$_4$ transition (Christin et al. 2008; Studer et al. 2014)                             | 7           | 4                                      | 3                                           | 3                                   |
| Petit Havana  | L225I,K429Q          | C$_3$ branch for L225I (Kapralov and Filatov, 2007; Studer et al. 2014)                       | 5           | 2                                      | 2                                           | 2                                   |
| Petit Havana  | K429Q                | Found in paternal parent (Nicotiana tomentosiformis)                                           | 4           | 3                                      | 3                                           | 2                                   |
| Petit Havana  | C449G                | Improved catalytic efficiency (Orr et al. 2016)                                               | 3           | 1                                      | 1                                           | 1                                   |

*The NtLwt transformant possesses all the silent mutations in the Nt-rbcLm gene except for a single mutation necessary for the new HindIII site. All the other transformants have the entire set of silent mutations in the Nt-rbcLm gene as well as the intended non-synonymous mutations.

Phylogenetic studies of Rubisco had previously suggested that several amino acid substitutions in LSus were positively selected during the C$_3$-C$_4$ transition (Kapralov et al., 2011, 2012; Studer et al., 2014). As detailed in Table 1, we selected five of those mutations (V101I, V255A, L270I, A281S and H282N) and introduced each into the Nt-rbcLm gene as well as a double mutant with both A281S and H282N. We also included the Nt-rbcLm gene with a C449G mutation that was suggested by a previous wide survey of Rubisco kinetic properties from 75 plant species to be associated with improved catalytic efficiency (Orr et al., 2016). In addition, we introduced the K429Q mutation to obtain the enzyme from tobacco’s paternal parent, Nicotiana tomentosiformis (accession YP_398871.1). We also created the Nt-rbcLm gene that encodes both K429Q and L225I mutations as the inverse I225L change was found to be selected in evolution of C$_3$ branches, and ancestors of tobacco Rubisco potentially possessed an L225I mutation (Studer et al., 2014).

We introduced each of the pCT-Nt-rbcLm vectors with these mutations into tobacco seedlings of either the Samsun or Petit Havana tobacco cultivar with biolistics and performed restriction digestion of the PCR-amplified rbcL gene from the transformed shoots arising from the first selection round (Figure 2a). We found that typically 40% of the shoots at this stage possessed the restriction sites corresponding to the Nt-rbcLm gene. Our result was comparable to that in a previous study, which introduced a different set of silent mutations to remove commonly used restriction sites in the tobacco rbcL gene and found that 6 out of 12 (50%) of the transformants had the silent mutations (Sinagawa-Garcia et al., 2009). After the shoots we produced with the modified restriction sites at the rbcL locus had been subjected to a second round of selection, we analyzed restriction fragment length polymorphisms (RFLPs) in the transformants with DNA blotting (Figure 2b,c). Those transformants that had achieved homoplasy with the Nt-rbcLm gene were transferred to rooting medium and subsequently to soil until they set seeds. We obtained multiple independent transformants for six out of nine LSu mutations and one each for the remaining three mutations (Table 1). Sequencing of the rbcL locus in the final transformants confirmed that all but one of the transformants possessed the entire set of silent mutations in the Nt-rbcLm gene along with the intended non-synonymous mutations. The NtLwt transformant inherited all the silent mutations in the Nt-rbcLm gene minus the single nucleotide change for the new HindIII site (Table 1). Our results are consistent with the previous study which found that the majority of transformants (five out of six) with silent mutations in the rbcL gene, possessed the entire set of mutations and only one originated from a cross-over event within the rbcL gene (Sinagawa-Garcia et al., 2009).
Mutations in the Rubisco large subunit did not prevent assembly of Rubisco holoenzyme

We further investigated one tobacco transformant for each Rubisco LSu mutant to determine the effects of the introduced mutations. DNA blotting with a probe hybridized to a region upstream of the \(rbc\) gene locus confirmed that all transformants were homoplasmic and possessed a restriction site from the \(Nt-rbc\) gene (Figure 3). Likewise, an RNA blot of the same samples with a probe to detect the \(Nt-rbc\) gene showed that all transformants had an extra dicistronic transcript with both the \(Nt-rbc\) and downstream \(aad\) genes in addition to the monocistronic \(Nt-rbc\) transcript (Figure 4a). Both the \(rbc\) transcripts present in the transformants were much less abundant than the single monocistronic transcript from the wild-type sample; however, the total \(rbc\) transcript levels in the transformants were comparable to those in the single transcript in the wild type (Figure 4b). The proportion of dicistronic mRNA amongst the transgenic lines did not significantly vary (\(\text{ANOVA}, P = 0.848\)). Soluble protein samples from the leaf tissues of the wild-type plant and all transformants displayed a similar band for the L8S8 holoenzyme on blue native PAGE (Figure S2), indicating these mutations did not prevent Rubisco assembly, although measurements indicate an effect on enzyme abundance in the leaf (Table 2).

Introduced mutations often affected Rubisco carboxylation

To compare the potential impact of these introduced mutations on Rubisco activity an analysis was conducted using glasshouse-grown plants of each transplastomic line and wild-type controls. Leaf disks were taken from 26-day-old plants and analyzed for their maximum Rubisco carboxylation rate (\(k_{\text{cat}}\)) and other related parameters including total soluble protein and chlorophyll content (Tables 2 and S1). There was significant variation amongst genotypes in the cv. Samsun background (Table 2). Two mutations, V101I and V255A, showed a significant negative impact on the maximum carboxylation rate of Rubisco. Amongst cv. Petit Havana genotypes there was some evidence for the negative impacts of the C449G mutation, with variation among these genotypes approaching statistical significance (\(P = 0.054, \text{ANOVA}\)). In cv. Petit Havana genotypes there was
also some support for the effect of mutations on Rubisco content, reflected in total soluble protein content (Table 2).

Plants of each transplastomic line grew to a comparable size and displayed no obvious phenotype under the conditions used (Figure 5). There were no significant differences in chlorophyll content (Table S1). Growth under additional environmental conditions will be needed to further explore the effect of the mutations on plant phenotype and enzymatic activity.

DISCUSSION

Engineering the chloroplast genome, or plastome, to utilize the unique characteristics of this organelle is of increasing interest for goals such as improving photosynthesis and the use of plants as bio-factories. Due its inefficiencies, Rubisco, the central CO$_2$-fixing enzyme, has long been considered a promising target to improve photosynthesis and increase biomass and yield in crops. Manipulation of Rubisco subunit genes presents contrasting complications. Chloroplast transformation allows precise site-directed mutagenesis of the large subunit (rbcL) gene in the chloroplast genome, and although this has long been successfully used to investigate Rubisco biogenesis and biochemistry, the technology is currently limited to a relatively small set of species. Understanding of the pervasive role, in some cases, of the small subunit (encoded by the nuclear RbcS family) on catalysis is rapidly increasing, and despite the complexity of manipulating highly similar gene families, often involving a large number of homologs, advances are being made in this area (e.g. Khumsupan et al., 2020; Martin-Avila et al., 2020). In this study, we introduced 10 restriction sites with 26 silent mutations in a modified tobacco rbcL gene (Nt-rbcL$^{m}$), which allowed PCR-RFLP screening of transformants after the first round of selection. The elimination of null transformants without the intended mutations after the first selection round also improved efficiency of the workflow to obtain the final stable transformants. Compared with a previous report where only one in eight transformants had the desired mutation in the rbcL gene (Whitney et al., 1999), about 40% of our transformants obtained after the first selection had Nt-rbcL$^{m}$, with the majority of these giving rise to stable transplastomic plants with the entire set of mutations in the Nt-rbcL$^{m}$ gene. Our results are generally consistent with a previous study that removed common restriction sites in the rbcL gene with silent mutations (Sinagawa-García et al., 2009).
It proved useful to generate multiple shoots or subclones from each transformant after the first selection round since more than half of the shoots tested after the second selection round had lost Nt-rbcLm. This is not surprising since Nt-rbcLm is over 98% identical to the wild-type sequence and can be removed before the transformants reach homoplasy (Kanevski et al., 1999; Whitney et al., 1999). There may be potential to further improve the transformation efficiency by increasing the number of silent mutations in Nt-rbcLm such that there is no sequence homology to the wild-type gene. However, having too many silent mutations could possibly interfere with translation efficiency and other underlying sequence-specific regulatory processes, which are not yet fully understood.

One such regulatory element that is widely conserved among plants is a major translation pause site within rbcL transcripts caused by an internal ribosome-binding site and mRNA structure (Gawroński et al., 2018).

### Table 2

| Genotype     | Rubisco kcat (sec⁻¹) | Rubisco (µmol sites m⁻²) | TSP (g m⁻²) | TSP (% Rubisco) | Activation state (%) |
|--------------|----------------------|--------------------------|-------------|----------------|---------------------|
| WT-SS        | 2.6 ± 0.2            | 24.6 ± 2.8               | 4.8 ± 0.5   | 36.2 ± 2.7     | 82.4 ± 2.6          |
| NtLwt        | 2.4 ± 0.2            | 18.6 ± 2.5               | 4.0 ± 0.7   | 36.0 ± 4.6     | 78.0 ± 2.9          |
| V101I        | 1.6 ± 0.1            | 18.5 ± 1.1               | 4.7 ± 0.1   | 29.1 ± 1.3     | 71.2 ± 6.2          |
| V255A        | 1.6 ± 0.0            | 18.2 ± 2.9               | 3.7 ± 0.2   | 34.1 ± 3.1     | 69.4 ± 5.9          |
| A281S        | 1.8 ± 0.2            | 17.8 ± 2.7               | 3.5 ± 0.3   | 35.4 ± 4.3     | 74.4 ± 5.1          |
| H282N        | 2.2 ± 0.3            | 15.3 ± 2.8               | 3.2 ± 0.2   | 33.6 ± 4.4     | 71.1 ± 4.5          |
| A281S/H282N  | 2.1 ± 0.2            | 18.9 ± 3.3               | 3.5 ± 0.3   | 37.2 ± 3.8     | 72.4 ± 2.1          |
| WT-PH        | 0.007                | 0.337                    | 0.249       | 0.842          | 0.216               |
| C449G        | 1.5 ± 0.2            | 26.6 ± 1.3               | 5.6 ± 0.4   | 33.9 ± 2.5     | 71.1 ± 2.5          |
| K429Q        | 1.9 ± 0.1            | 22.1 ± 4.2               | 5.2 ± 0.5   | 29.3 ± 3.2     | 68.3 ± 1.9          |
| L225I/K429Q  | 2.4 ± 0.3            | 19.7 ± 2.2               | 5.0 ± 0.6   | 28.0 ± 2.6     | 68.4 ± 2.8          |
| L270I        | 1.9 ± 0.2            | 19.3 ± 2.4               | 4.4 ± 0.3   | 30.9 ± 3.3     | 74.7 ± 3.9          |
| P            | 0.054                | 0.018                    | 0.025       | 0.612          | 0.165               |

P values < 0.05 are highlighted in bold.

It proved useful to generate multiple shoots or subclones from each transformant after the first selection round since more than half of the shoots tested after the second selection round had lost Nt-rbcLm. This is not surprising since Nt-rbcLm is over 98% identical to the wild-type sequence and can be removed before the transformants reach homoplasy (Kanevski et al., 1999; Whitney et al., 1999). There may be potential to further improve the transformation efficiency by increasing the number of silent mutations in Nt-rbcLm such that there is no sequence homology to the wild-type gene. However, having too many silent mutations could possibly interfere with translation efficiency and other underlying sequence-specific regulatory processes, which are not yet fully understood. One such regulatory element that is widely conserved among plants is a major translation pause site within rbcL transcripts caused by an internal ribosome-binding site and mRNA structure (Gawroński et al., 2018).

**Figure 5.** Visual comparison of plant development. (a) Wild-type tobacco cv. Samsun (WT-SS) and rbcL transplastomic lines. (b) Wild-type tobacco cv. Petite Havana (WT-PH) and rbcL transplastomic lines. Photos were taken of plants at the same age (35 days after sowing) and growth stage.
Our analyses indicate that many of the transformants with the *Nt-rbcL*<sup>m</sup> gene had a lower Rubisco content than the wild-type plants, although in some cases the difference was not statistically significant. One reason for reduced Rubisco levels could be the 23 modified codons in the *Nt-rbcL*<sup>m</sup> gene, with the majority of these changes resulting in the incorporation of less frequently used codons that could have a negative impact on the protein’s translation efficiency. Although translation efficiencies in chloroplasts cannot always be predicted from codon usage, and chloroplasts do not possess rare codons similar to those found in *Escherichia coli*, different codons have varying translation efficiencies so that it may still be desirable to avoid unnecessary codon changes (Nakamura and Sugiuira, 2011). For example, strategically targeting a single restriction site closest to each non-synonymous mutation instead of introducing the entire set of silent mutations in the *Nt-rbcL*<sup>m</sup> gene should minimize unintentional influence on translation efficiencies.

The processing of the *rbcL* mRNA 3′ end in our transformants was not efficient, giving rise to a dicistronic transcript with *rbcL* and *aadA* genes. This is probably due to insufficient length of the *rbcL* 3′-untranslated region (UTR), which was 205 nucleotides in the transformants. In addition to the dicistronic transcript, we also observed two sizes for monocistronic mRNAs, indicating a second transcript processing site that is probably in the *psbA* promoter downstream of the *rbcL* 3′-UTR. In a previous study in which an *rbcL* 3′-UTR that was 269 nucleotides long was incorporated downstream of the *rbcL* gene, the dicistronic transcripts were much less abundant, probably due to more efficient processing of the transcripts (Whitney and Sharwood, 2008). It was previously shown that 410 nucleotides following *rbcL* were necessary for proper maturation of the *rbcL* transcript (Sinagawa-García *et al*., 2009). Thus, future work should consider incorporation of a complete *rbcL* 3′-UTR that is at least 410 nucleotides long so that the transformants can produce *rbcL* transcripts that are similar in size and abundance to those in the wild type. In addition, stem–loop structures at the *loxP* sites flanking the *aadA* operon were suggested to interfere with the processing of both *rbcL* and *aadA* transcripts (Sinagawa-García *et al*., 2009). Thus, it may be preferable to replace *loxP* sites with long direct repeats that can spontaneously trigger removal of the marker gene through homologous recombination (Iamtham and Day, 2000). Alternatively, the marker operon can be flanked with *attB* and *attP* sequences and subsequently removed with the expression of PhiC31 phage integrase (Kittiwongwattana *et al*., 2007).

Previously, the development of a tobacco master line, where the *rbcL* gene had been replaced with a homolog from *Rhodospirillum rubrum* encoding a form II Rubisco, allowed modification of the *rbcL* gene and rapid characterization of the subsequent mutant Rubisco enzymes within 6–9 weeks of transformation, although this master line required a high-CO<sub>2</sub> environment to grow in soil (Whitney and Sharwood, 2008). Recently, functional Rubisco enzymes from Arabidopsis and tobacco were successfully assembled in *E. coli* with the co-expression of at least five chaperones (Aigner *et al*., 2017; Wilson *et al*., 2019; Lin *et al*., 2020). Thus, modified Rubisco enzymes from plants can now be readily produced in *E. coli*, and once modifications that lead to superior carboxylation kinetics are identified they can be introduced into host plants for further characterization of their effects on photosynthesis and plant growth. These new tools are complemented by directed evolution approaches with cyanobacterial Rubisco, which have shown promising improvements to catalytic efficiency and specificity (Wilson *et al*., 2018).

As a proof of concept for this approach to mutating chloroplast genes, we generated seven tobacco transformants each with one residue substitution and two transformants each with two residue substitutions in the Rubisco LSU. Most of these residue substitutions were predicted to be selected during the C<sub>3</sub>–C<sub>4</sub> transitions or associated with a higher catalytic efficiency, while L225I and K429Q were potentially present in ancestors of tobacco Rubisco (Studer *et al*., 2014). Mutations that are bona fide kinetic switches between C<sub>3</sub> and C<sub>4</sub> Rubisco should result in enzymes with a higher *k<sub>cat</sub>* (Whitney *et al*., 2011b). However, our analyses indicated a lack of significant improvement in the carboxylation rate of Rubisco from these transformants; indeed, a number of the mutant Rubiscos displayed lower maximum carboxylation activities than the control plants. Importantly, the transformants exhibited Rubisco with similar mobility on native PAGE to the wild-type plants, suggesting no impairment of Rubisco biogenesis as a result of the mutations introduced in *rbcL*.

A previous study identified a residue substitution in the Rubisco LSU of *Flaveria* species as a C<sub>3</sub>–C<sub>4</sub> kinetic switch, but the same residue substitution which is native to the tobacco enzyme did not convey the same kinetic effect (Whitney *et al*., 2011b). Thus, kinetic switches are likely to require accessorial components, which may be one or more additional complementary mutations in the large or even the small subunit of Rubisco. Indeed, evidence that the residues at the interface between the two subunits play important roles in determining the kinetic properties was previously reported by studies of the Rubisco from *Chlamydomonas* (Spreitzer *et al*., 2005; Genkov and Spreitzer, 2009) and cyanobacteria (Wilson *et al*., 2018). Understanding the role of the small subunit in plant Rubisco catalysis is an expanding area, and will be an important complement to engineering the LSU via chloroplast transformation (e.g. Ishikawa *et al*., 2011; Morita *et al*., 2014; Atkinson *et al*., 2017; Khumsupan *et al*., 2020), alongside newly developed tools for rbcL–rbcS co-engineering in the plastome (Martin-Avila *et al*., 2020).
Reliable plastid transformation procedures for many agriculturally important crops are not yet available. A recent study showed that spectinomycin-resistant Arabidopsis plastid transformants could be selected with reasonably high efficiency once nuclear-encoded ACC2, an enzyme subunit involved in the fatty acid biosynthesis pathway inside plastids, was knocked out, rendering the plants hypersensitive to spectinomycin (Yu et al., 2017). Fertile Arabidopsis plastid transformants were then successfully generated when root-derived microcalli of acc2 knockout lines were used for transformation and regeneration (Ruf et al., 2019). These latest developments have the potential to inform future attempts to extend plastid transformation technology to other species. Once the plastid genome in crops can be readily transformed, an important goal would be to employ changes within the Rubisco LSU to improve its properties in key crops such as rice and wheat. Since our approach does not require initial generation of master lines in target plant species, which can be time-intensive, it can greatly expedite future work in introducing targeted changes not only in the Rubisco LSU, but also in other chloroplast-encoded protein subunits.

**EXPERIMENTAL PROCEDURES**

**Construction of the chloroplast transformation vectors with modified *Nt-rbcL* genes**

All primers used were obtained from Integrated DNA Technologies (https://eu.idtdna.com/) and are listed in Table S2. Phusion™ high-fidelity DNA polymerase, FastDigest restriction enzymes and T4 DNA ligase from Thermo Scientific (http://www.thermofisher.com/) were used to generate amplicons, restriction digests and ligation products, respectively. The partial *Nt-rbcLm* gene (base pairs 172–1362) with silent mutations was synthesized by GenScript (http://www.genscript.com/) and amplified with NtLm-154f and NtLm-1343r primers (Figures 1a and S1). The upstream and downstream fragments were amplified from tobacco DNA with LSU-FL1f+NtLm-171r and NtLm-1363f+NtLrev primers, respectively. The three amplicons were joined with overlapping PCR with LSU-FL1f and NtLrev primers, digested with Clal and MauBI and ligated into the similarly digested pCT-rbcL vector (described in Lin et al., 2014) to obtain the pCT-Nt-rbcLm vector (Figure 1b). To introduce V101I, L225I, A281S, H282N or A281S/H282N amino acid substitutions, amplicons obtained with the respective forward primers and NtLrev were digested with *MluI*, *AgeI* or *HindIII* and ligated into the similarly digested pCT-Nt-rbcLm vector. The V255A, L270I and C449G mutations were introduced by ligating the amplicons from NtL-97SF and NtLrev primers. The amplicons were purified, digested with *MluI*, *AgeI* or *HindIII* and analyzed on an agarose gel. The shoots possessing the restriction sites in the *rbcL* gene were cut into 25 mm² pieces and placed on RMOP agar medium with 500 µg ml⁻¹ spectinomycin. After shoots appeared 4–6 weeks later, DNA was extracted from each shoot with cetyltrimethylammonium bromide solution (Allen et al., 2006), 1 µg each was digested with *NheI* and *XhoI* and the RFLP was analyzed on a DNA blot with a digoxigenin (DIG)-labeled probe as described previously (Orr et al., 2020). The shoots homoplasmic with *Nt-rbcLm* genes were then placed on MS agar medium with 500 µg ml⁻¹ spectinomycin for rooting and subsequently transferred to soil. The *Nt-rbcLm* genes in those plants were confirmed by Sanger sequencing.

**Analyses of *Nt-rbcLm* transcripts in the tobacco transformants**

The RNA was extracted and purified from young leaves of 5-week-old plants using a PureLink RNA mini kit from Life Technologies (http://www.thermofisher.com/), and 200 ng of each sample was separated in a denaturing gel with 1.3% agarose gel and 2% formaldehyde, transferred to a nylon membrane and detected with a DIG-labeled RNA probe as described previously (Occhialini et al., 2016). Chemiluminescence detection was performed with a ChemiDoc MP imaging system (Bio-Rad) and the band intensities were quantified with Gel Analyzer options in ImageJ software (https://imagej.nih.gov/ij/). The experiment was performed for two sets of plants. Five different RNA concentrations (25, 50, 100, 200 and 400 ng) from a wild-type cv. Samsun plant were applied along with each set of samples, and a quadratic function obtained from their band intensities was used to estimate the relative *rbcL* transcript quantity in each band. Each *rbcL* transcript quantity was then normalized with the corresponding 25S rRNA band intensity obtained from the UV exposure of the agarose gel before transfer.

**Analyses of Rubisco holoenzyme on blue native PAGE**

Leaf tissues were homogenized in an extraction buffer consisting of 100 mM bicine-NaH₂O pH 7.9, 5 mM MgCl₂, 1 mM EDTA, 5 mM ε-aminoacaproic acid, 50 mM 2-mercaptoethanol, 5% (w/v) polyethylene glycol 4000, 10 mM NaHCO₃, 10 mM TDT and Pierce™ protease inhibitor mini tablets (Thermo Scientific part no. A32995) and insoluble materials were removed with centrifugation at 16 000g at 4°C for 5 min. The protein concentrations were estimated with the Bradford method using a protein assay dye reagent concentrate (Bio-Rad part no. 5000006), and 3 µg of each total soluble extract mixed with a sample buffer consisting of 50 mM 2-bis(2-hydroxyethyl)amino-2-(2-hydroxyethyl)methyl-1,3-propanediol (BIS-TRIS), 50 mM NaCl, 10% w/v glycerol and 0.001% w/v Ponceau S pH 7.2 was loaded to a NativePAGE™ 3–12% BIS-TRIS protein gel (Thermo Scientific part no. BN10003BOX). The electrophoresis was...
carried out in an XCell™ SureLock™ Mini-Cell with an anode buffer consisting of 50 mM BIS-TRIS and 50 mM Tricine pH 6.8 and a cathode buffer consisting of additional 0.002% (w/v) Coomassie G-250 dye at 4°C 150 V for 30 min and 250 V for about 60 min. The gel was fixed with 100 ml of 40% methanol and 10% acetic acid solution for 15-30 min, stained with 100 ml 0.02% Coomassie R-250 in 30% methanol and 10% acetic acid solution for 15-30 min and destained with 100 ml 8% acetic acid solution for 2-5 h.

**Plant material**

To obtain plant material for protein analyses, seeds of wild-type and transgenic *N. tabacum* cv. Petit Havana and cv. Samsun were sown into planter trays of a commercial potting mix (Petersfield Products, http://www.petersfieldgrowing.com/) with a slow-release fertilizer (Osmocote, Scotts UK Professional, https://www.lovethegarden.com/uk-en/scotts). All lines germinated at similar time post-sowing (6 days), and seedlings were thinned out after about 2 weeks, with individuals transferred to 1 L pots after about 3 weeks. Plants were grown inside a heated glasshouse at Lancaster University, UK, during June and July with minimum day/night temperatures of 25/18 °C and a 16 h photoperiod. Supplemental lighting was supplied by sodium lamps when light levels fell below 200 µmol m⁻² sec⁻¹. Plants were kept well-watered.

**Protein extraction and analysis**

Leaf samples for Rubisco analyses were collected from the youngest fully expanded leaf 32 days after sowing. Samples were collected 3 h after the beginning of the photoperiod, and plants were positioned to avoid shading for at least 1 h prior to sampling. Three 0.5 cm² leaf disks were rapidly collected using a cork borer and immediately snap frozen, then stored at −80°C prior to analysis.

Frozen leaf samples were homogenized in an ice-cold mortar and pestle in 0.6 ml of extraction buffer, the soluble proteins collected after centrifugation for 1 min at 4°C and 14 700g, followed immediately by assays of initial and total Rubisco activity as described by Carmo-Silva et al. (2017). The Rubisco activation state was calculated as the ratio of initial/total activity. An aliquot (100 µl) of the same soluble protein extract was incubated at room temperature (22°C) for 30 min with 100 µl of carboxyarabinitol-1,5-bisphosphate (CABP) binding buffer (Carmo-Silva et al., 2017) including [¹⁴C]CABP for determination of Rubisco content via [¹⁴C] CABP binding (Sharwood et al., 2016c).

The same soluble protein extract was used to determine total soluble protein (TSP) via Bradford assay (Bradford, 1976). The method of Wintermans and de Mots (1965) was used to determine chlorophyll content, using 20 µl of the homogenate taken in duplicate prior to centrifugation. This was added to 480 µl of ethanol, inverted to mix and kept in the dark for 2-3 h. Absorbance was measured using a SPECTROstar Nano (BMG LabTech, http://www.bmglabtech.com/) to determine concentrations of chlorophyll a and b.

**Statistical analysis**

Statistical differences between biochemical trait means were assessed via ANOVA. Where a genotype effect was observed (P < 0.05), a post-hoc Tukey’s honestly significant difference test was used to conduct multiple pairwise comparisons. Analyses were performed using RStudio version 1.2.5033 (R Studio Team, 2019) and R version 3.6.2 (R Core Development Team, 2013). Plots were prepared with ggplot2 (Wickham, 2016). Outliers were detected using the Tukey fences method, where outliers are defined as extreme values that are 1.5 times the interquartile range (1.5 IQR) below the first quartile or 1.5 IQR above the third quartile.

**ACCESSION NUMBERS**

Tobacco *RbcL* sequence data can be found in the GenBank data library under accession number NP_054507.1.

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**CONFLICTS OF INTEREST**

The authors declare that they have no conflicts of interest.

**AUTHOR CONTRIBUTIONS**

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

**DATA AVAILABILITY STATEMENT**

The data generated in this study can be obtained from the corresponding author upon request.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Sequence alignment of native *rbcL* and modified *rbcL* (*Nt*-rbcL™).

**Figure S2.** Blue native PAGE analyses of soluble proteins in the transformants.

**Table S1.** Chlorophyll data from analysis of tobacco transplastomic lines.

**Table S2.** Oligonucleotide sequences used in the construction of transformation vectors.

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