Construction of a tobacco master line to improve Rubisco engineering in chloroplasts

Spencer M. Whitney* and Robert E. Sharwood
Molecular Plant Physiology Group, Research School of Biological Sciences, Australian National University, Canberra, Australian Capital Territory 0200, Australia

Received 21 September 2007; Revised 26 October 2007; Accepted 12 November 2007

Abstract
The inability to assemble Rubisco from any photosynthetic eukaryote within *Escherichia coli* has hampered structure–function studies of higher plant Rubisco. Precise genetic manipulation of the tobacco chloroplast genome (plastome) by homologous recombination has facilitated the successful production of transplastomic lines that have either mutated the Rubisco large subunit (L) gene, *rbcL*, or replaced it with foreign variants. Here the capacity of a new tobacco transplastomic line, cmtrL, to augment future Rubisco engineering studies is demonstrated. Initially the *rbcL* was replaced with the selectable marker gene, *aadA*, and an artificial codon-modified cm*rbcM* gene that codes for the structurally novel Rubisco dimer (L2, ~100 kDa) from *Rhodosporillum rubrum*. To obtain cm*trL*, the *aadA* was excised by transiently introducing a T-DNA encoding CRE recombinase biolistically. Selection using *aadA* enabled transplantation of mutated and wild-type tobacco Rubisco genes into the cm*trL* plastome with an efficiency that was 3- to 10-fold higher than comparable transformations into wild-type tobacco. Transformants producing the re-introduced form I tobacco Rubisco variants (hexadecamers comprising eight L and eight small subunits, ~520 kDa) were identified by non-denaturing PAGE with fully segregated homoplasmic lines (where no L2 Rubisco was produced) obtained within 6–9 weeks after transformation which enabled their Rubisco kinetics to be quickly examined. Here the usefulness of cm*trL* in more readily examining the production, folding, and assembly capabilities of both mutated tobacco and foreign form I Rubisco subunits in tobacco plastids is discussed, and the feasibility of quickly assessing the kinetic properties of those that functionally assemble is demonstrated.

Key words: CO₂ fixation, mutagenesis, photosynthesis, plastome transformation, Rubisco.

Introduction
Significant advances have been made over 50 years into various aspects of Rubisco biology (Portis and Parry, 2007), with considerable efforts still pursuing a more comprehensive appreciation of the enzymes complex catalytic chemistry and identifying what features afford the natural kinetic variability found amongst the different Rubiscos, in the hope of engineering improved variants into crop plants (Andrews and Whitney, 2003; Raines, 2006; Tcherkez et al., 2006; Parry et al., 2007). The inability to assemble Rubisco readily from any eukaryote in *Escherichia coli* (Gatenby et al., 1987; Cloney et al., 1993) or cyanobacteria (Whitney et al., 2001) had thwarted mutagenic studies of the higher plant Rubisco holoenzyme until the development of techniques to transform the chloroplast genome (plastome) of some plants efficiently. While the range of species whose plastome can be transformed continues to expand (Koop et al., 2008), the transplantation of foreign or modified Rubisco variants into higher plant plastids has so far been exclusively performed in tobacco where the transformation efficiency is highest. Efforts are therefore focused on using tobacco as a model higher plant species to appreciate more fully the requirements and limitations to modifying the endogenous Rubisco into higher plant plastids as well as...

For the Rubisco large (L) subunit gene (*rbc*L), located in the plastome, genetic manipulation by plastome transformation in tobacco is a routine but protracted process. Generally at least 6 months is required to obtain transplastomic *T*₀ tobacco lines with a completely segregated population of transformed plastomes (homoplasmic) and be of suitable maturity to measure the Rubisco kinetics and undertake physiological analyses, such as measuring its photosynthetic properties by whole leaf gas exchange and chlorophyll fluorescence. Previous efforts have been able to mutate the hexadecameric form I Rubisco in tobacco (Whitney et al., 1999) and replace it either totally, with the form II dimer (*L₂*) from the bacterium *Rhodospirillum rubrum* (Whitney and Andrews, 2001a, 2003), or partially, by replacing *rbc*L with the sunflower gene (*rbc*L₅) to produce a hybrid form I Rubisco comprising eight sunflower L subunits and eight cytosolically synthesized tobacco small (S) subunits (Kanevski et al., 1999). However, success was not forthcoming in producing a comparable hybrid Rubisco comprising cyanobacterial L subunits, or with expressing Rubisco L and S subunits from two non-green algae whose folding and assembly requirements were not met by the tobacco chloroplast chaperone complexes (Whitney et al., 2001). An appropriate means for engineering the native (or foreign) S subunit genes (*Rbc*S) in higher plants also remains an elusive challenge due to the multiple *Rbc*S copies that are located in the nucleus in higher plants which essentially precludes *Rbc*S from targeted mutagenic or replacement strategies. Multiple attempts to assemble appreciable levels of plastid-synthesized tobacco S subunits showed an apparent necessity for very high levels of plastomic *rbc*S mRNA expression and reduced levels of the native *Rbc*S message (Whitney and Andrews, 2001b; Zhang et al., 2002; Dhingra et al., 2004).

The present focus is on addressing five primary issues related to Rubisco engineering in higher plant chloroplasts. The first is identifying ways to control the level of Rubisco expression in chloroplasts with some degree of predictability by examining the influence of different 5′ and 3′ regulatory sequences and codon usage. Two other issues involve examining what classes of foreign Rubiscos can assemble in higher plant chloroplasts without chaperone engineering and whether the L subunits of other plant species can functionally assemble with tobacco S subunits without catalytic detriment, as has been found for the sunflower L–tobacco S hybrid Rubisco (Sharwood et al., 2008). The roles of the multiple L subunit post-translational modifications on their expression, folding, and assembly also remain to be established (Houtz and Portis, 2003), as does ascertaining to what extent foreign S subunits co-transplanted with their cognate foreign L subunit can outcompete the tobacco S subunits for functional assembly.

In this study, the development of a master tobacco line called *cm*₅ *L*₅, that may be effective at addressing many of these issues, is demonstrated. The transplastomic line expresses *R. rubrum* Rubisco whose gene lacks sequence homology with re-introduced Rubisco genes, which beneficially excluded undesired, alternative recombination events and accelerated obtaining homoplasmic lines. The distinct structure (*L₂*) and antigenicity of *R. rubrum* Rubisco in this master line from that of form I (*L₈S₈*)Rubiscos enabled the subunit expression, functional assembly, and catalytic prowess of re-introduced mutated and wild-type tobacco Rubisco to be assessed within 2 months following transformation.

**Materials and methods**

**Plasmid construction, sequencing, and transformation**

All plasmids and PCR products were sequenced using BigDye terminator sequencing (Applied Biosystems) on an ABI 3730 sequencer (Biomolecular Resource Facility, JCSMR, ANU). Plasmid p*cm*₅ *L*₅ contains 1126 bp and 1176 bp of flanking tobacco plastome sequence that directs the homologous replacement of *rbc*L and most of its 3′-untranslated sequence with a codon-modified *R. rubrum* *rbc*M gene (*cm*₅ *rbc*M) and an inversely oriented *aadA* selective marker gene (GenBank accession no. AY827488). The *cm*₅ *rbc*M gene was assembled by primer extension overlap PCR using 80-mer primers that overlapped with adjacent primers by 15 nucleotides as described (Whitney and Sharwood, 2007). The codon bias of *cm*₅ *rbc*M was modified to mimic tobacco *rbc*L (Supplementary Fig. S1 available at JXB online). Plastome transforming plasmids directing the re-insertion of the tobacco *rbc*L were derived from pLEV1 (L-subunit Encoding Vector; Whitney et al., 1999). Nucleotide 163 in the *rbc*L coding region was deleted following erroneous digestion and re-ligation with SacII to give pLEV-L. pL335V codes a leucine to valine mutation in *rbc*L at codon 335 and contains an adjacent silent mutation that introduces a unique EcoRV site (Whitney et al., 1999). The *H₆Ub-Rbc*M gene codes for a fusion protein comprising the 6×histidine-tagged ubiquitin sequence from *pflu* (Baker et al., 2005) and the tobacco *Rbc*S sequence from *pRVS*Su (Whitney and Andrews, 2001b). Sequence comprising 29 bp of the intergenic sequence (*IS₃⁹*) immediately 5′ to the *Rbc*S gene in the *Phaeodactylum tricornutum* *rbc*L operon (that is highly expressed in tobacco plastids; Whitney et al., 2001) was cloned immediately 5′ to the *H₆Ub-Rbc*M gene and the *IS₃⁹*, *H₆Ub-Rbc*S sequence inserted 3′ to the *rbc*L stop codon in pLEV1 to give plasmid pLEVUbS. The plasmids were transformed into *E. coli* XL1-Blue cells, grown at 25 °C in LB media containing ampicillin (0.2 mg ml⁻¹), purified using the Wizard mini-prep kit (Promega), and eluted in water then concentrated to 1 mg aliquot of freshly prepared plasmid was coated onto tungsten for biolistic transformation as described (Svab and Maliga, 1993). p*cm*₅ *L*₅ was transformed into the plastome of *Nicotiana tabacum* L. cv. Petit Havana (N,N), producing *L*₅ lines. The *aadA* gene was excised following biolistic transformation of homoplasmic *T₂₀* tissue with the T-DNA plasmid pKO27 that codes for CRE recombinase containing an N-terminal fusion for the pea Rubisco S subunit plastid-targeting sequence (Corneille et al., 2001). Homoplasmic *aadA*-free *cm*₅ *L*₅ lines were obtained (see below), their flowers successively backcrossed with wild-type pollen, and the *T₃* progeny of *cm*₅ *L*₅ line 1 transformed with pLEV1, pLEVAL, pL335V, and pLEVUbS.
**Propagation of material in tissue culture**

Eight \(^{14}C\) CARLA transplastomic lines from 10 spectinomycin-resistant (spec\(^{+}\)) plants that grew on selective medium [agar-solidified Murashige–Skoog salts containing 3% (w/v) sucrose, 0.5 mg ml\(^{-1}\) spectinomycin, and hormones (Swab and Maliga, 1993)] were identified by PCR (see below). Leaf tissue from two \(^{14}C\) CARLA lines (1 and 3) was carried through an additional round of regeneration on selective media and homoplasmicity was confirmed by DNA blot analysis (see below). Replicate plants for both \(^{14}C\) CARLA lines were regenerated aseptically in Magenta pots (Sigma) on MS medium (selective medium without hormones or spectinomycin) and were either grown to maturity in soil under high CO\(_2\) pressures [1% (v/v) CO\(_2\) in air] as described (Whitney and Andrews, 2001a), or the sterile leaves of \(^{14}C\) CARLA line 1 were transformed with pKO27 (Corneille et al., 2001) using the biolistic method (Swab and Maliga, 1993). After 2 d on MS medium the leaves were dissected (~0.5 cm\(^2\)) and propagated in kanamycin-selective medium (selective medium containing 15 µg ml\(^{-1}\) kanamycin in place of spectinomycin). The first six plantlets to develop were transferred to MS\(^{spec}\) medium (MS medium containing 0.5 mg ml\(^{-1}\) spectinomycin) and after 3 weeks two \(^{14}C\) CARLA lines were identified by PCR and both grown to maturity in soil under high CO\(_2\) pressures.

Four to six weeks after propagation on selective medium, the first six Spec\(^{+}\) plants from \(^{14}C\) CARLA leaves transformed with pLEV1, pl335V, pLEbUbS, and pLEVAL were transferred to MS\(^{spec}\) medium and transplastomic lines identified using non-denaturing PAGE and DNA blots (see below). Genomic DNA was isolated from all Spec\(^{+}\) plants that developed up to 8 weeks post-bombardment, and the transplastomic lines were identified by PCR using primers LSA (Whitney et al., 1999) and LSE (Whitney and Andrews, 2001a).

**Leaf sampling**

Samples from a leaf from 6–8 cm high plants growing in MS\(^{spec}\) medium at ~7 weeks post-bombardment were taken for DNA (0.5 cm\(^2\)) and RNA (1 cm\(^2\)) blot analyses, and the remainder (2–10 cm\(^2\)) were used for PAGE, Rubisco purification, and kinetic analyses. The harvested leaf sections were rapidly frozen in liquid nitrogen and stored at ~80 °C.

**Protein extraction**

Leaf discs were extracted in 0.5–1.0 ml of ice-cold extraction buffer [50 mM HEPES-NaOH, pH 8.0, 1 mM EDTA, 2 mM dithiothreitol (DTT), 1% (v/v) plant protease inhibitor cocktail (Sigma-Aldrich), and 1% (w/v) polyvinylpyrrolidone (PVPP)] in a Wheaton glass homogenizer and the lysate centrifuged (35 000 g, 10 mins, 4 °C). Aliquots of the supernatant were either assayed relative to bovine serum albumin (BSA) for protein content by dye binding (Coomassie Plus kit, Pierce), treated with an equal volume of SDS buffer [125 mM TRIS-Cl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 150 mM 2-mercaptoethanol, 0.01% (w/v) bromophenol blue], diluted 2-fold with non-denaturing PAGE buffer [30% (v/v) glycerol, 0.001% (w/v) bromophenol blue], or incubated with 10–20 mM NaHCO\(_3\) and MgCl\(_2\) at 25 °C for 10 min before incubating for 5 min with or without 100 µM [\(^{14}C\)]carboxypentitol-P\(_2\) before chromatography through a Superdex 200HR 10/30 column (see below).

Protein expression in the XL1-Blue *E. coli* cells used to prepare the transforming plasmid was examined by harvesting 5 ml of the cell cultures by centrifugation (5000 g, 5 min, 20 °C), suspending the cell pellet in extraction buffer (no PVPP), then lysing by passage through a French pressure cell (140 MPa). An aliquot of lysate sample was mixed with an equal volume of SDS buffer (total cellular protein), and the remaining sample was centrifuged (38 000 g, 15 min, 4 °C) and aliquots of the supernatant assayed for soluble protein content and treated with a equal volume of SDS buffer (soluble cellular protein).

**PAGE and immunoblot analysis**

Protein samples were separated by SDS–PAGE (BIS-TRIS-buffered 4–12% NuPAGE gels, Invitrogen), blotted onto nitrocellulose, probed with appropriate polyclonal antiserum raised in rabbits to pure spinach Rubisco, tobacco Rubisco S subunits, or *R. rubrum* Rubisco, and the immunoreactive bands visualized using AttoPhos (Promega) as described previously (Whitney et al., 2001). Proteins were separated by non-denaturing PAGE at 4 °C using 4–12% TRIS-glycine gels (Invitrogen) in 60 mM TRIS, 191 mM glycine buffer at 100 V for 4 h. Protein bands were visualized using Gelcode Blue reagent (Pierce).

**Rubisco content, chromatography, and kinetics**

Radiolabelled [\(^{14}C\)]carboxypentitol-P\(_2\) (an isomeric mixture of carboxyribitol-P\(_2\) and carboxyribitol-P\(_2\)) was synthesized as described (Pierce et al., 1980) and used to measure Rubisco content following pre-activation of Rubisco with 10–200 mM each of NaHCO\(_3\) and MgCl\(_2\) as described (Ruuska et al., 1998; Whitney and Andrews, 2001a). Soluble leaf protein was chromatographed through a Superdex 200HR 10/30 column equilibrated with specificity buffer (30 mM triethanolamine pH 8.3, 30 mM Mg acetate; Kane et al., 1994) using an AKTA explorer system (APBioTech). Fractions (0.3 ml) containing Rubisco were identified following SDS–PAGE by immunoblot analysis using antibodies to spinach or *R. rubrum* Rubisco and by scintillation counting of the samples incubated with \[^{14}C\]carboxypentitol-P\(_2\). The first fractions only containing form I Rubisco from non-carboxypentitol-P\(_2\)-treated samples were pooled (~100–150 pmol L subunit sites) and used to measure CO\(_2\)/O\(_2\) specificity at 25 °C as described (Kane et al., 1994) after equilibrating with an atmosphere containing 500 ppm CO\(_2\) in O\(_2\) controlled using three Wösthoff precision gas-mixing pumps.

Rubisco from LEbUbS leaves was purified by IMAC (immobilized metal affinity chromatography). Pieces of leaf material of 2–8 cm\(^2\) were homogenized in 1–2 ml of ice-cold affinity extraction buffer [50 mM HEPES-NaOH, pH 8.0, 1 mM EDTA, 2 mM dithiothreitol (DTT), 1% (v/v) plant protease inhibitor cocktail (Sigma-Aldrich), and 1% (w/v) polyvinylpyrrolidone (PVPP)] in a Wheaton glass homogenizer and the lysate centrifuged (35 000 g, 10 mins, 4 °C). Aliquots of the supernatant were either assayed relative to bovine serum albumin (BSA) for protein content by dye binding (Coomassie Plus kit, Pierce), treated with an equal volume of SDS buffer [125 mM TRIS-Cl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 150 mM 2-mercaptoethanol, 0.01% (w/v) bromophenol blue], diluted 2-fold with non-denaturing PAGE buffer [30% (v/v) glycerol, 0.001% (w/v) bromophenol blue], or incubated with 10–20 mM NaHCO\(_3\) and MgCl\(_2\) at 25 °C for 10 min before incubating for 5 min with or without 100 µM [\(^{14}C\)]carboxypentitol-P\(_2\) before chromatography through a Superdex 200HR 10/30 column (see below).

Protein expression in the XL1-Blue *E. coli* cells used to prepare the transforming plasmid was examined by harvesting 5 ml of the cell cultures by centrifugation (5000 g, 5 min, 20 °C), suspending the cell pellet in extraction buffer (no PVPP), then lysing by passage through a French pressure cell (140 MPa). An aliquot of lysate sample was mixed with an equal volume of SDS buffer (total cellular protein), and the remaining sample was centrifuged (38 000 g, 15 min, 4 °C) and aliquots of the supernatant assayed for soluble protein content and treated with a equal volume of SDS buffer (soluble cellular protein).

**Genetic manipulation of Rubisco in tobacco plastids**
containing 15 mM NaH\textsubscript{14}CO\textsubscript{3} (specific activity \sim 2000 cpm nmol\textsuperscript{-1} CO\textsubscript{2}) by the concentration of Rubisco active sites measured by \textsuperscript{14}C\textsubscript{2}carboxyarabinitol-P\textsubscript{2} binding. Ribulose-P\textsubscript{2} was synthesized according to Kane et al. (1998).

**Leaf DNA and RNA blot analyses**

Total leaf DNA and RNA were extracted, separated by agarose gel electrophoresis, and blotted onto membranes as described previously (Whitney et al., 1999; Whitney and Andrews, 2001b). Hybridization was performed at 55 °C using buffer supplied in the AlkPhos Direct Labelling Kit (GE Healthcare). The DNA blots were probed with the 1176 bp RFR or 1856 bp \textit{LS} DNA fragment amplified from pLEV1 using primers \textit{LSA} and \textit{LSB} (Whitney et al., 1999) or the 336 bp \textit{RbcS} DNA fragment excised from pLEV1 using primers \textit{RbcL1} probe (Whitney and Andrews, 2001a) and the RNA blots probed with the 991 bp \textit{RbcL2} DNA fragment amplified from pLEV1 using primers \textit{LSA} and \textit{LSB} (Whitney et al., 1999) or the 336 bp \textit{RbcS} DNA fragment excised from pLEV1 using primers \textit{RbcL1} probe. The probes were labelled with [\textalpha\textsuperscript{32}P]dATP using the MegaPrime Labelling Kit (GE Healthcare), and the autoradiograph signals were detected on storage phosphor screens and imaged using a BioRad Pharo Imaging System.

**Results and discussion**

**Alternative recombination events can frustrate Rubisco engineering**

Genetic manipulation of higher plant plastomes, which typically target the insertion or modification of a gene of interest, is accompanied by the introduction of a selectable marker gene (\textit{smg}) coding for antibiotic resistance (Maliga, 2004). Transformation occurs by flanking the heterologous sequence with an appropriate flanking plastome sequence to direct the insertion of the genetic modifications with surgical precision via homologous recombination (Svab and Maliga, 1993). Previous attempts to mutate codon 335 in the tobacco \textit{rbcL} gene (substituting Leu with Val, L335V; Whitney, 1999) or to replace \textit{rbcL} with the gene from sunflower (\textit{rbcL}5; Kanevski et al., 1999) were hampered by the preservation of sequence homology in the transforming plasmids between the mutated sequences and the commonly used \textit{aadA} \textit{smg} (which confers resistance to the antibiotics streptomycin and spectinomycin; spec\textsuperscript{B}). Generating the L335V mutant proved particularly difficult as the intermediary region of homologous sequence was longer, with most transplastomic lines incorporating the \textit{aadA} gene and not the mutation. Only one L335V line (from eight independent transformants) was shown by PCR and DNA blot analyses to incorporate the \textit{rbcL} mutation. However, during subsequent growth on selective medium towards a fully segregated transformed plastome population (homoplasmicity), most of the regenerating vegetative tissue underwent secondary recombination events with non-transformed plastome copies. This second population of transformed plastomes that incorporated the \textit{aadA} gene but not the \textit{rbcL} mutation were exclusively selected for. This selective pressure against retaining the plastome copies containing the \textit{rbcL} mutations might have resulted from the kinetic impairment to Rubisco imparted by the L335V mutation (Whitney et al., 1999).

Another unwanted recombination event also occurred when replacing \textit{rbcL} with \textit{rbcL}5. As both genes retain large regions of sequence homology, a transformant producing a recombinant \textit{rbcL}5-L\textsuperscript{5} variant containing the first 233 nucleotide of \textit{rbcL} was produced (Kanevski et al., 1999). In contrast, total replacement of \textit{rbcL} with the \textit{R. rubrum} \textit{rbcM} occurred without artefact (Whitney and Andrews, 2001a) as both genes, and the intervening sequence between \textit{rbcM} and \textit{aadA}, shared little sequence homology. The growth rate, phenotype, and leaf development of the resultant tobacco–\textit{rubrum} plants in tissue culture were comparable with those of wild-type tobacco, which it was rationalized made them highly suited for efficiently re-transforming with mutated tobacco, and even foreign, \textit{rbcL} variants.

**Transformation efficiency is highest using the \textit{aadA} gene**

The initial study objective was to re-introduce variant \textit{rbcL} genes into the existing tobacco–\textit{rubrum} lines using an alternative \textit{smg} to the \textit{aadA} already stably in place (Andrews and Whitney, 2003). Towards this goal, the transformation efficiency of two alternative \textit{smgs} was compared using the versatile transforming plasmid pRV112a (Zoubenko et al., 1994). The \textit{aadA} coding sequence in pRV112a was replaced with the spinach \textit{badh} gene (encoding betaine aldehyde dehydrogenase) or the entire \textit{aadA} cassette replaced by the \textit{Prrn-atpB} 5'UTR-neo-TrbcL sequence from plasmid pNK30 (Kuroda and Maliga, 2001). Bombardment of pRV112a-\textit{badh} into 20 tobacco leaf sections yielded >100 plantlets that were resistant to 10 mM betaine aldehyde, none of which were plastome transformants (data not shown). In contrast, two plastome transformants were isolated from 20 tobacco leaf sections bombarded with pRV112a-neo after selecting on medium containing 50–100 μg ml\textsuperscript{-1} kanamycin, while eight \textit{specR} plastome transformants were obtained following transformation of 10 leaves with pRV112A (data not shown). Clearly transformation efficiency was highest using spectinomycin selection, consistent with its preferential use in plastome transformation research (Maliga, 2004; Koop et al., 2008).

**Construction of marker-less \textit{cm} \textit{trl} tobacco–\textit{rubrum} lines**

As proposed in Andrews and Whitney (2003), a two-step approach was undertaken to engineer a photosynthetic tobacco master line that lacked sequence homology to form I Rubisco genes and enabled selection of transformants using the \textit{spec}\textsuperscript{B}–coding \textit{aadA} gene. To achieve this, new tobacco–\textit{rubrum} plants were first generated using \textit{aadA} selection, and then \textit{aadA} was excised to
allow its re-use. To complete the first step, four new cmtrLA tobacco–rubrum lines were selected (from five bombardments) where the tobacco rbcL coding sequence (excluding the first 42 nucleotides; Whitney and Andrews, 2001a) and 269 bp of its 3'-untranslated region (UTR) were replaced by 2960 bp of sequence containing a codon-modified rbcM gene (cmrbcM, Supplementary Fig. S1 at JXB online), 222 bp of the tobacco psbA 3'-UTR sequence, and an inversely oriented Prrn–aadA–Trps16 cassette that was flanked by directly oriented 34 bp loxP sites (Fig. 1A). The codon bias of the synthetic cmrbcM matched that of rbcL but maintained the same low level of sequence homology shown between rbcM and rbcL (Supplementary Fig. S1 at JXB online). The second step of excising aadA from a cmtrLA line was achieved by the P1 bacteriophage site-specific Cre recombinase using a novel single-step protocol. Two aadA-free (marker-less) cmtrLA lines were isolated following transient expression of CRE in cmtrLA leaves after biolistically introducing the T-DNA plasmid, pKO27, that encodes the cre gene fused in-frame with the 5' sequence coding for a pea Rubisco MT subunit plastid-targeting sequence with expression regulated by the P2' Agrobacterium promoter (Corneille et al., 2001). Stable or transient introduction of pKO27, and other CRE-expressing T-DNA variants, by agroinfiltration has been highly successful in yielding marker-free transplastomic lines (Corneille et al., 2001; Hajdukiewicz et al., 2001; Lutz et al., 2006). Notably, agroinfiltration can result in both stable transformation with the T-DNA and transient expression of cre. Plants in which CRE was only transiently produced have to be identified and if cre has integrated into the nucleus the gene has to be segregated away in the seed progeny. This complication was avoided by using a simpler approach that biolistically introduced pKO27 into three sterile leaves of a homoplasmic T0 cmtrLA plant (line 1) that had been grown in MS medium following two rounds of regeneration in selective medium. As plasmid DNA can persist for >2 weeks in bombarded leaf tissue (Ye et al., 2003), the shot leaves were regenerated on selective medium containing 15 μg ml⁻¹ kanamycin to provide some selection pressure for growth of cells containing pKO27. Plantlets emerged after 4 weeks from the segmented shot leaves that had become bleached. The first six sufficiently developed plantlets were transferred to growth in 0.75 l pots on MS medium. After 3 weeks, the plants were 4–7 cm in height, had developed roots, and were phenotypically indistinguishable from cmtrLA lines being grown under identical conditions for transfer to growth in soil. DNA from the youngest leaf was extracted, and two marker-less cmtrLA lines were identified using PCR (Fig. 1B), with DNA blots indicating that both lines were already homoplasmic with no cmtrLA (or wild-type tobacco) plastome copies detected (Fig. 1C). The homoplasmic cmtrL1 and cmtrL2 lines were transferred to soil, grown to maturity under high CO2 pressures, and their flowers pollinated with wild-type pollen. All the cmtrL1 and cmtrL2 T1 progeny (>10³ T0 seed from each line screened) were sensitive to growth on sucrose-free MS medium containing spectino- mycin (0.5 mg ml⁻¹) or kanamycin (0.1 mg ml⁻¹), indicating that neither line carried a plastid aadA gene and were unlikely to contain stably incorporated nuclear neo copies and the T-DNA-associated cre gene. To

![Fig. 1](https://academic.oup.com/jxb/article-abstract/59/7/1909/639629/639629)

**Fig. 1.** Generation and screening of homoplasmic tobacco–rubrum lines with and without the aadA selectable marker gene. (A) Tobacco plastome organization following transformation with plasmid pcmtrLA (GenBank accession no. AY027488) that directed the replacement of rbcL (excluding the first 42 bp of coding sequence) and 285 bp of its 3' sequence (T) in the wild-type tobacco plastome with a codon-modified R. rubrum Rubisco gene (cmrbcM), 222 bp of the psbA 3'-untranslated sequence (T), and a p-aadA-T gene cassette [p, 16S rDNA rrn promoter and 5'-untranslated region; t, rps16 3'-untranslated region (Svab and Maliga, 1993)] that was flanked by 34 bp loxP sites (white triangles). The homologous sequence in pcmtrLA used to direct recombination with the tobacco plastome is indicated by the dotted lines and numbering ([Shinozaki et al., 1986] GenBank accession no. Z00044). The aadA gene in a pcmtrLA line was excised by transient Cre recombinase expression producing independent cmtrLA lines (see Materials and methods for details). Shown are the annealing positions of the rbcL1, KFR (right flanking region), and rbcL2 probes, the size of XbaI (Xb) fragments that hybridize with the RFR probe, the primers 5'TpsbA (5' TGGATCTCCTGGCCTAGTCTATAGGAGGT-3') and LSe (Whitney and Andrews, 2001b), and the size of their amplified products. P, rbcL promoter and 5'-UTR. (B) PCR identification of the independent cmtrL1 and cmtrL2 lines and (C) confirmation of homoplasmicity on blots of XbaI-digested total leaf DNA probed with the RFR probe.
confirm this, PCR analyses using leaf DNA from 10 T1 progeny from each cmtrL line and primers specific for cre were unable to amplify a product, indicating that both were cre free (data not shown).

**Testing the ability to transform rbcl variants into cmtrL leaves**

Transforming plasmids based on pLEV1 (Whitney et al., 1999; Whitney and Andrews, 2003) were used to reintroduce either the native tobacco rbcl or the L335V-coding mutated rbcl gene (pL335V), or to co-introduce rbcl with a tobacco RbcS gene that contained a sequence coding for an N-terminal 6×histidine-tagged ubiquitin fusion (pLEVUbS, Fig. 2A). As shown previously, proteins whose genes are regulated by the tobacco rbcl promoter and 5′-UTR are constitutively expressed in E. coli, which can lead to the insertion of transposons in the 5′-UTR or coding sequence if the recombinant protein is detrimental to bacterial growth (Whitney and Andrews, 2001). Therefore, in addition to sequencing, the integrity of the transforming plasmids shot into tobacco was examined by SDS–PAGE and immunoblot analyses to confirm expression of the Rubisco subunits (Fig. 2B). Consistent with that shown previously (Whitney and Sharwood, 2007), large amounts of the tobacco L subunits were produced in the E. coli transformed with pLEV1, pL335V, and pLEVUbS, and these were almost entirely insoluble as the folding and assembly requirements of higher plant Rubiscos are not met by the E. coli chaperone complexes (Gatenby et al., 1987). The small amount of soluble L detected appeared to be complexed with chaperonin complexes when separated by non-denaturing PAGE (data not shown). Approximately 10% of the ubiquitin–S subunit fusion produced was soluble, as seen previously for the native tobacco S subunit expressed in E. coli (Whitney and Andrews, 2001; Whitney and Sharwood, 2007).

As transcription of the nuclear RbcS genes is heightened in tobacco–rubrum but the unassembled S subunit is degraded (Whitney and Andrews, 2003), it was reasoned that successful re-introduction of their cognate L subunits would readily facilitate production of form I hexadecamers (L8S8). As L8S8 Rubiscos are both structurally and antigenically distinct from the R. rubrum L2 enzyme, the feasibility of detecting, selectively purifying, and measuring the CO2/O2 specificity of the form I enzyme from heteroplasmic leaf tissue as soon as possible following biolistic transformation was examined. As summarized in Fig. 3A, specR plantlets began to develop 4–6 weeks after transformation, of which the first six were transferred directly into MSspec medium to promote normal vegetative development (Fig. 3B). After 2–3 weeks, the plants were 4–8 cm in height and had developed roots. Soluble protein from a leaf was analysed by non-denaturing PAGE for production of the R. rubrum L2 enzyme alone (non-transformed), both L2 and L8S8 Rubisco

![Fig. 2. Transplanting variant tobacco Rubisco genes into cmtrL plastids. (A) Modified pLEV1 transforming plasmids (Whitney et al., 1999) used to replace the 5′rbclM gene and psbA 3′-UTR sequence (T) with tobacco rbcl genes that contain either no mutation (pLEV1), a frameshift mutation that encoded a 6 kDa ΔL-subunit (pLEVΔL), or a nucleotide substitution mutating codon Leu35 to Val (pL335V; Whitney et al., 1999), or incorporated an additional 3′ sequence coding for a 6×histidine-tagged ubiquitin (Ub)–tobacco S subunit (RbcS) fusion (pLEVUbS, see Materials and methods for more detail). The annealing positions of primers LSA and LSE used to identify transformants by PCR and the RbcS DNA probe are shown. The plasmids incorporate a promoter-less adaA-t gene cassette (t, 148 bp of the rps16 3′-UTR, nucleotides 4942–5090 in the tobacco plastome, GenBank accession no. Z00044) inserted within the rbcl 3′-UTR (T) 269 bp downstream of the rbcl stop codon with 18 bp of the rbcl 5′ sequence repeated 5′ to the adaA initiator codon (Whitney et al., 1999). The flanking homologous sequence used to direct recombination into the cmtrL plastome is indicated by the dotted lines. Numbering correlates with the tobacco plastome sequence (GenBank accession no. Z00044). N, Neol; X, Xhol. (B) SDS–PAGE and immunoblot detection of constitutively expressed tobacco Rubisco L2 and Ub-tagged S (UbS) subunits in the total (soluble+insoluble) and soluble cellular protein from XL1-Blue E. coli transformed with the variant plasmids. M, molecular mass marker with sizes shown.](https://academic.oup.com/jxb/article-abstract/59/7/1909/639629 by guest on 28 March 2019)
(heteroplasmic transformant), or just the L8S8 enzyme (homoplasmic transformant) (Fig. 3C). Transformants and the extent of their segregation were subsequently confirmed by DNA blot analysis of total genomic DNA isolated from the same leaf (Fig. 3D). Curiously the L8S8 enzyme in the LEVUbS lines appeared to resolve as normal-sized and larger oligomeric complexes, suggesting that the plastid-synthesized H6UbS subunits had successfully assembled into some of the hexadecamers (see below).

Blots of total RNA extracted from the same leaf were probed with rbcL DNA and showed that the LEV1 and L335V lines produced an abundant monocistronic (rbcL) transcript and an ~80% less abundant bicistronic (rbcL–aadA) transcript as shown previously for other LEV1 transformants (Whitney and Andrews, 2003) (Fig. 3E). As the aadA gene is promoter-less and does not produce a monocistronic mRNA, production of sufficient rbcL–aadA transcript is requisite for the specR phenotype. In the LEVUbS transformants, two rbcL transcripts were made, an abundant bicistronic rbcL–UbRbcS mRNA that exceeded the amount of nuclear RbcS mRNA by >6-fold, and a 70–80% less abundant tricistronic rbcL–aadA–UbRbcS mRNA whose translation is necessary for spectinomycin selection (Fig. 3E).

From the non-denaturing PAGE (Fig. 3C) and DNA blots (Fig. 3D), it was evident that five LEV1, three L335V, and five LEVUbS of the six specR plants analysed were transplastomic lines, with all except the LEV1#2 and L335V#3 lines already being homoplasmic. As the plants already had well-developed roots, they were immediately transferred to growth in soil without the need for repeated propagation on selective medium, thus reducing the time required to obtain mature transplastomic plants. Twelve weeks after beginning the transformation process, all three L335V and three of the LEVUbS transformants had been grown to maturity in soil and were beginning to set seed. This contrasts with the 8 months that was required previously to propagate the single L335V transformant to the same level of development (Whitney et al., 1999).
The transformation efficiency of cmtrL leaves is high

The production of transplastomic lines was more efficient using the leaves of cmtrL plants than wild-type tobacco leaves. To examine the transformation efficiency of cmtrL, the genomic DNA from all the spec R plants that developed up to 8 weeks post-plasmid bombardment was analysed by PCR using one primer specific to rbcL (LSA) and one that anneals downstream of the aadA insertion site (LSE, Fig. 2A). In bona fide LEV1 and L335V transformants, this produced a 2.5 kb amplification product and a 3.2 kb product in transplastomic LEVUbS lines (data not shown). Comparable high transformation efficiencies (~2–3 transformants per bombardment) were found in generating LEV1, L335V, and pLEVUbS transformants using cmtrL leaves (Table 1). Notably, the transformation efficiency incmtrL leaves was not at a high transformation frequency and a 3.2 kb product in transplastomic LEVUbS lines transformants, this produced a 2.5 kb amplification product (LSE, Fig. 2A). In bona fide LEV1 and L335V the genomic DNA from all the spec R plants that developed up to 8 weeks post-plasmid bombardment was analysed by PCR using one primer specific to rbcL, cmtrL1, and one that anneals downstream of the aadA insertion site (LSA, Fig. 2A). The mutation introduced a stop codon after 57 codons, shortening the rbcL open reading frame to a 6 kDa A subunit whose production in transformed pLEVAL-XL1-Blue-E. coli cells was not detected (Fig. 2B). Comparable with what was seen previously in

<table>
<thead>
<tr>
<th>Table 1. Transformation frequencies for wild-type tobacco and cmtrL leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transforming</strong></td>
</tr>
<tr>
<td><strong>plasmid</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>LEV1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>L335V</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>LEVUbS</td>
</tr>
<tr>
<td>LEVAL</td>
</tr>
</tbody>
</table>

a Data from Whitney and Andrews (2003) and Whitney et al. (1999) using wild-type tobacco. 
b Calculated by dividing the number of transformants by the number of leaves transformed.

Rubisco purification

The variation in the size of R. rubrum L2 Rubisco (~100 kDa) and L8S8 Rubisco (~520 kDa) enabled purification of the latter by size exclusion chromatography. The capacity of a Superdex 200HR 10/30 size exclusion column to resolve L8S8 and L2 Rubisco was examined by incubating CO2-Mg2+-activated soluble leaf protein from heteroplasmic lines producing both Rubiscos with the tight binding inhibitor [14C]carboxyarabinitol-P2. Both scintillation counting and immunoblot analyses showed the L8S8 and L2 Rubisco peaks were not totally resolved to baseline (Fig. 4A). This was in contrast to previous findings (S Whitney and H Ashida, unpublished data), and subsequent separation of protein standards indicated that the resolving capacity of the ageing Superdex 200HR 10/30 column was suboptimal. Nevertheless, fractions containing only the L8S8 Rubisco were obtainable (shaded region in Fig. 4A), which were pooled and used to measure Sc (see below).

The capacity of plastid-synthesized H6UbS subunits to assemble in the variant L8S8 enzymes produced in LEVUbS (Fig. 3B, see above) was examined using IMAC. A H6Ub ubiquitin fusion was chosen because (i) it can be specifically removed by Ub protease and not leave any residual N-terminal sequence on the fused target protein (Baker, 1996; Baker et al., 2005); (ii) chloroplasts do not appear to accumulate Ub proteases (Vierstra, 1996); and (iii) the appended Ub sequence can increase the stability of protein fusions due to the chaperonin-like effect of Ub on protein folding (Baker, 1996; Staub et al., 2000). SDS–PAGE and immunoblot analysis of the LEVUbS soluble leaf protein confirmed that small amounts of H6UbS subunits were produced and showed that tobacco L and S subunits were selectively purified with H6UbS by IMAC (Fig. 4B). On non-denaturing PAGE the ‘IMAC-pure’ protein (not digested with Ub protease, UbP) resolved as an enzyme complex larger than L8S8, suggesting that it incorporated one or more H6UbS subunits. Once the H6Ub sequence was removed by UbP treatment, the mobility of the ‘pure’ enzyme through the gel resembled that of the wild-type L8S8 enzyme (Fig. 4B).
While the H6Ub fusion strategy was successful in specifically isolating L8S8 tobacco Rubisco, its versatility was compromised by the low yield of hexadecamers that incorporated a plastid-synthesized H6UbS subunit. From [14C]carboxyarabinitol-P2 binding analyses it was determined that 1.5% of the total Rubisco (5 pmol active sites) extracted from 2 cm² of a LEVUbS leaf was recovered by rapid IMAC purification (total processing time <15 min). The poor incorporation of plastid-synthesized H6UbS subunits into hexadecamers (despite the abundant UbRbcS mRNA pool size; Fig. 3E) correlates with that observed previously where similar levels (1.2% of the assembled S subunit pool) of plastid-synthesized, C-terminal histidine-tagged S subunits (SH7) were assembled into L8S8 enzyme even though the plastid pool of RbcS mRNA exceeded the pool of endogenous nuclear RbcS message >10-fold (Zhang et al., 2002). Even in anti-RbcS tobacco, when the pool of cytosolic S subunits was reduced >80%, the production of plastid-synthesized S subunits had no influence on Rubisco levels (Zhang et al., 2002) until the RbcS gene was incorporated within the trnI–trnA intergenic region of the 16S rDNA operon within the tobacco plastosome inverted repeat region which is ascribed as being a transcriptional hot spot (Dhingra et al., 2004). Whether improvements in incorporation of H6UbS subunits into Rubisco hexadecamers can be attained by transforming its gene into the trnI–trnA intergenic region, or by reducing RbcS levels in the cmtrL line by antisense or RNAi techniques, remains to be examined.

The yield of Rubisco isolated from LEVUbS leaves by IMAC appeared compromised by cleavage of the H6Ub fusion during purification. On immunoblots of the IMAC-purified Rubisco, the proportion of mature S subunits exceeded the H6UbS signal >20-fold (Fig. 4B). This contrasts with previous IMAC-purified L8S8 complexes from the transplastomic lines producing plastid-synthesized S:H6UbS subunits where the cytosolic-synthesized S:H7 ratio was 7:1 (Whitney and Andrews, 2001b). As wild-type tobacco Rubisco (or any other soluble leaf protein) does not bind to IMAC (Whitney and Andrews, 2001b), the higher S:H6UbS stoichiometry in the IMAC-purified

---

**Fig. 4.** Purification and kinetic analysis of form I Rubisco. (A) CO2-Mg2+-activated soluble protein (0.4–0.5 mg in 0.2 ml) from heteroplasmic and homoplasmic L335V leaves (lines 3 and 4, respectively, Fig. 3C and D) was incubated with and without 5 μM [14C]carboxypentitol-P2 and chromatographed through a Superdex 200HR 10/30 column at 4 °C (see Materials and methods for further detail). Fractions (0.3 ml) containing mutated Val335 tobacco Rubisco (L8S8) and *R. rubrum* Rubisco (L2) were detected, and the content of Rubisco L subunit sites measured, by scintillation counting, and separation of L2 from L8S8 confirmed by immunoblot analysis using antibodies raised against form I (spinach) and form II (*R. rubrum*) Rubisco. The fractions only containing L8S8 enzyme (grey region) from non-[14C]carboxypentitol-P2-treated samples were used to measure CO2/O2 specificity (S0/S1) according to Kane et al. (1994). (B) SDS–PAGE, non-denaturing PAGE, and immunoblot detection of the Rubisco L, 6×His-tagged ubiquitin–Rubisco S (H6UbS), and S subunits in leaf protein from LEVUbS line 1 during IMAC purification. The samples analysed were the total leaf soluble protein (total sol.), protein that did not bind to IMAC (non-bind), IMAC-purified protein before (IMAC pure) and after digestion with USP (pure), and the soluble protein from a fully expanded leaf from a cmtrL plant growing in soil in air containing 1% (v/v) CO2. M, marker proteins (sizes shown).
Rubisco from LEVUbS leaves indicates cleavage of the $^{18}$O sequence during the purification process. Notably, similar processing of the $^{18}$O sequence has been observed during the purification of recombinant $^{15}$N fusion proteins from *E. coli* (Baker *et al.*, 2005) and tobacco chloroplasts (Staub *et al.*, 2000). Clearly this premature processing of $^{15}$N coupled with the apparent preferential assembly of cytosolically synthesized S subunits (or poor translation of $^{15}$N) reduces the effectiveness of the $^{15}$N fusion strategy for purifying tobacco Rubisco from small amounts of leaf tissue and questions the merit of its use in future Rubisco transplantation studies.

**Kinetic screening**

Leaf material from LEV1 and L335V plants growing in MSppm medium (Fig. 3C) was used to measure quickly the kinetic parameters that are used to model the ribulose-P$_2$-saturated CO$_2$ assimilation rate of C$_3$ photosynthesis (Farquhar *et al.*, 1980; Andrews and Whitney, 2003; Parry *et al.*, 2007). CO$_2$O$_2$ specificity ($S_{c/o}$) was measured using Superdex 200HR 10/30-purified L$_8$S$_8$ Rubisco from heteroplasmic L335V (line 3) and LEV1 (line 2) leaf tissue (Fig. 4A). The measured $S_{c/o}$ values matched that previously measured using highly purified enzyme from homoplasmic lines (Table 2). Likewise, the maximal carboxylation rates ($V_{c}^{\text{max}}$) and Michaelis constants for CO$_2$ in the presence of air levels of 20.6% (v/v) O$_2$ ($K_c^{21\%O_2}$) measured using freshly isolated soluble leaf protein from homoplasmic lines (L335V line 4, LEV1 line 5) also matched those measured previously (Table 2; Whitney *et al.*, 1999).

**Features of cmtriL that make it a good recipient for form I Rubisco transformations**

While comprehensively assessing the versatility of cmtriL lines for transplanting non-tobacco Rubiscos into tobacco chloroplasts remains to be examined there is optimism that the high transformation efficiency of cmtriL and the unique structure and antigenic specificity of the *R. rubrum* enzyme make it a more suitable recipient than wild-type tobacco for screening the production, folding, and assembly capabilities of both foreign and mutated tobacco form I Rubiscos. The ease with which numerous L335V transformants were readily obtained using cmtriL leaves contrasted with previous attempts where significant experimental effort was required to obtain one transformant (Whitney *et al.*, 1999). Evidently the ease of obtaining the L335V, LEV1, LEVUbS, and even LEVAL transformants correlates with the poor sequence similarity between the cmtriM gene in cmtriL and the introduced rbcL (and $^{15}$N-RbcS) sequences (Supplementary Fig. S1 at JXB online).

The quick segregation of transformed plastome populations enabled prompt analysis of the L$_8$S$_8$ Rubisco kinetics. In most cases the transformants were already homoplasmic 6–9 weeks after bombarding the cmtriL leaves with transforming plasmids, which contrasts with previous efforts to generate homoplasmic LEV1 lines from transformed wild-type tobacco leaves that necessitated multiple rounds of regeneration on selective media (Whitney *et al.*, 1999). Once homoplasmic, reliable, and reproducible measurements of $V_{c}^{\text{max}}$ and $K_c^{21\%O_2}$ can be made using leaf protein extract (Kubien *et al.*, 2003; Ghannoum *et al.*, 2005; Sharwood *et al.*, 2008), while $S_{c/o}$ determination using the method of Kane *et al.* (1994) requires at least partial purification (Whitney and Andrews, 1998) which, as shown here, can be sufficiently achieved by Superdex 200HR 10/30 chromatography (Table 2).

While the re-introduced tobacco L$_8$S$_8$ Rubiscos examined in this study were abundantly expressed, producing foreign L$_8$S$_8$ Rubisco in tobacco plastids may be more problematic. As evidenced in previous Rubisco transplantation studies, reductions in transcript abundance (Whitney and Andrews, 2001a), perturbations to translational processing (Whitney and Andrews, 2003), and problems with the folding requirements of introduced L subunits and their assembly with tobacco S subunits (Kanevski *et al.*, 1999; Sharwood *et al.*, 2008) significantly constrain the production of foreign Rubiscos in tobacco plastids, sometimes even precluding assembly of functional enzyme (Whitney *et al.*, 2001). The high transformation efficiency and prompt isolation of Rubisco null LEVAL lines suggest producing transformants where the introduced foreign Rubisco subunits do not, or poorly, assemble into functional Rubisco should not be problematic. Indeed, an indication of assembly problems should be evident by the production of pale green leaves during growth in MSppm medium (Fig. 3B). The detection of foreign Rubisco subunits is also simplified by the antigenic distinctiveness of the *R. rubrum* Rubisco relative to form I Rubiscos which, as shown previously (Whitney *et al.*, 2001), can enable the level of assembled and unassembled introduced Rubisco subunits to be examined.

### Table 2. Measurement of CO$_2$/O$_2$ specificity ($S_{c/o}$), maximal ribulose-P$_2$-dependent carboxylase activity ($V_{c}^{\text{max}}$), and Michaelis constant for CO$_2$ under ambient O$_2$ levels ($K_c^{21\%O_2}$)

<table>
<thead>
<tr>
<th>Rubisco (source)</th>
<th>$S_{c/o}$</th>
<th>$K_c^{21%O_2}$ ($\mu$M)</th>
<th>$V_c^{\text{max}}$ ($\text{s}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (LEV1 line #1)</td>
<td>84±2° (82)</td>
<td>21.5±1.6° (19.8)</td>
<td>3.3±0.2° (3.4)</td>
</tr>
<tr>
<td>Val335 (L335V line #3)</td>
<td>21±1° (20)</td>
<td>29.6±2.5° (31.3)</td>
<td>0.6±0.1° (0.8)</td>
</tr>
</tbody>
</table>

*Measured using Superdex 200HR 10/30-purified protein (Fig. 4A) (n=2).*

*Measured in leaf soluble protein extracted from homoplasmic transformants growing in MSppm medium 7 weeks post-bombardment (n=2).*

*Kinetic values measured previously (Whitney *et al.*, 1999).*
rapidly using immunoblot analyses. As shown in Fig. 3C, the assembly of \( \text{L}_8\text{S}_8 \) complexes can also be quickly ascertained using non-denaturing PAGE which additionally provides a means to assess whether the plants are homoplasmic according to whether or not the \( \text{R}. \text{rubrum} \) L\(_2\) enzyme is present.

**Alternative screening strategies to consider**

Like \( \text{cm}^{\text{tr}}\text{L} \), transplanting foreign or modified Rubiscos into higher plant plastids may be equally successful using a marker-less Rubisco null (\( \Delta rbc \)) line as a transformation recipient. Similar to the LEVAL lines produced here, previous Rubisco null tobacco produce bleached leaves that required grafting onto wild-type stock to produce seed (Kanevski and Maliga, 1994). While the production of a marker-less \( \Delta rbc \) line has not been reported, it would, in theory, make a suitable candidate for transforming in mutated or foreign Rubisco genes. Generating an \( rbc \) knockout using an alternative selectable marker gene, such as \( \text{neo} \), would enable subsequent transformations using \( \text{aad}A \) selection without needing to remove \( \text{neo} \). It might also be possible to omit antibiotic selection altogether with a \( \Delta rbc \) line and use the restoration of green pigmentation as a phenotypic indicator of functional Rubisco production. Unlike the \( \text{cm}^{\text{tr}}\text{L} \) leaves that are photosynthetic and have a wild-type-like phenotype that makes them amenable to biotic transformation, the sensitivity of \( \Delta rbc \) leaves to permanent tissue damage following even minor handling (as evidenced with LEVAL leaves) may prevent their plastomes from being transformed by this procedure. This potential limitation might be circumvented by generating \( \Delta rbc \) protoplast suspensions and transforming their plastomes by PEG treatment (Koop et al., 2008). An alternative strategy is to transform wild-type tobacco leaves and introduce sufficient silent nucleotide mutations into the candidate Rubisco \( rbc \) to prevent unwanted recombination events occurring with tobacco \( rbc \). Notably the cost involved in making multiple changes or re-synthesizing an entire gene may curb the general applicability of this approach.

**Future Rubisco transplantation applications**

Defining the \( \text{cm}^{\text{tr}}\text{L} \) plants as ‘master lines’ stems from the simplicity with which transplastomic tobacco expressing variant \( rbc \) genes can be generated using \( \text{cm}^{\text{tr}}\text{L} \) which provides unique opportunities to examine more readily the structure–function relationships of tobacco Rubisco. The capacity now exists to scrutinize the importance and function of the \( \text{L} \) subunit post-translational modifications (Houtz and Portis, 2003) and manipulate those residues that are thought to regulate strongly, and specify, the interactivity of Rubisco with its helper protein, Rubisco activase (Larson et al., 1997; Ott et al., 2000). The \( \text{cm}^{\text{tr}}\text{L} \) lines should simplify efforts to examine whether the ability of catalytically normal cyanobacterial Rubisco complexes comprised of S subunit–L subunit (SL) fusions (Whitney and Sharwood, 2007) can be emulated in tobacco chloroplasts using tobacco SL fusion peptides. Mimicking many of the practical mutations already examined in form I Rubiscos from cyanobacteria and *Chlamydomonas* (Kellogg and Juliano, 1997; Parry et al., 2003) should also benefit the mechanistic understanding of higher plant Rubisco catalysis. Indeed, replicating the cyanobacterial L335V mutation (Leu332 in the *Synechococcus* PCC6301 L-subunit; Lee et al., 1993) in tobacco Rubisco unveiled novel aspects of the higher plant catalytic mechanism (Pearce and Andrews, 2003). The large database of available Rubisco sequences (>3200; Kapralov and Filatov, 2007) and the resolution of >20 high resolution Rubisco crystal structures (Andersson and Taylor, 2003) should greatly assist in identifying candidate residues for mutation, though undoubtedly such an undertaking would greatly benefit from more comprehensive kinetic surveys of sequenced Rubiscos.

The \( \text{cm}^{\text{tr}}\text{L} \) plants also provide a means to improve our understanding of \( rbc \) regulation and \( L \) subunit production. For plastid genes, protein expression is primarily regulated post-transcriptionally (Somanchi and Mayfield, 1999) and involves features in the 5′- and 3′-UTRs of the mRNA which are requisite for the binding of auxiliary trans-acting factors (many nucleus encoded) that directly or indirectly influence transcript stability; mRNA processing, mRNA editing; and translation of the mRNA (Monde et al., 2000; Nickelsen, 2003; Bollenbach et al., 2004). By transforming into \( \text{cm}^{\text{tr}}\text{L} \) it should now be possible to augment the previous study of Shinya et al. (1998) and use Rubisco as the reporter protein to pinpoint sequence elements in the \( rbc \) regulatory sequences that are pertinent for transcription and translation, and possibly identify auxiliary factors that are involved in regulating these processes.

As highlighted with the LEVUbS transformants, an appropriate means for the engineering changes to the native (or foreign) S subunit in tobacco remains an elusive challenge. The pervasive influence of the S subunit on the kinetics of form I Rubisco suggests engineering kinetic improvements may necessitate complementary engineering of both the \( L \) and \( S \) subunits (Spreitzer, 2003). The paucity of plastid-synthesized S subunits incorporated into Rubisco hexadecamers may arise from problems with their translation in the stroma or result from limitations in their post-translational folding and assembly with the \( L \) subunits that hinder them in effectively competing with the cytosolically synthesized S subunits for assembly (Whitney and Andrews, 2001b; Andrews and Whitney, 2003). Clearly a concerted effort is required to address whether this limitation to Rubisco engineering in tobacco can be surmounted by reducing/eliminating the nuclear \( \text{RbcS} \) mRNA levels in \( \text{cm}^{\text{tr}}\text{L} \) by antisense/RNAi techniques and/or modifying the transformation strategy and...
introducing the tailored rbcL and rbcS genes either together or separately elsewhere in the plastome, such as the reported transcriptional hot spot in the trnL–trnA intergenic region (Dhingra et al., 2004).

Examining issues related to assembly of foreign Rubiscos in higher plant plastids is likely to be simplified using a cmtrL master line. The inability of tobacco plastids to meet the folding and assembly requirements of the ‘red’-type Rubisco subunits from the non-green algae Phaeodacylum tricornutum (diatom) and Galdieria sulphuraia (red alga) has deterred the pursuit of such endeavours with other ‘red’-type Rubiscos. However, the recent finding that a single amino acid substitution improved the assembly of Synechococcus PCC6301 Rubisco in E. coli >5-fold (Greene et al., 2007) suggests there is merit to exploiting the high transformation and LαS8 screening capabilities of cmtrL to survey more thoroughly the capabilities of other ‘red’-type Rubisco subunits to assemble functionally in tobacco chloroplasts and examine whether or not the tobacco S subunits are excluded from assembly. Broadening this survey to include replacement of cmrbcM with other higher plant rbcL genes without, and in conjunction with, one of their cognate RbcS gene copies is also paramount for, first, ascertaining to what extent the sustained kinetics and stability of the hybrid Rubisco comprising sunflower L and tobacco S subunits (Sharwood et al., 2008) are emulated in hybrid enzymes comprising other higher plant L subunits and, secondly, to examine the capacity of the complementary S subunit to outcompete the tobacco S subunit for assembly. Of further benefit to performing the studies in tobacco plastids is the capacity to examine in vivo using light transient gas exchange measurements (Hammond et al., 1998) the capacity of (or requirement for) the tobacco Rubisco activase to regulate the transplanted Rubisco.

Supplementary data
Supplementary data mentioned in this article are available at JXB online.

Acknowledgements
This research was supported by an Australian Research Council Discovery grant (DP0450564) awarded to SW. We thank Pal Maliga for supplying the pKO27 and pNK30 plasmids and thank Jeff Wilson for his photography.

References
Hammond ET, Andrews TJ, Mott KA, Woodrow IE. 1998. Regulation of Rubisco activation in antisense plants of tobacco containing reduced levels of Rubisco activase. The Plant Journal 14, 101–110.
Genetic manipulation of Rubisco in tobacco plastids


