Short title: tRNA\textsuperscript{Glu} in translation and chlorophyll synthesis

The Functions of Chloroplast Glutamyl-tRNA in Translation and Tetrpyrrole Biosynthesis

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S.A. performed research; S.A., D.K., S.R. and R.B. designed research and analyzed data; S.A. and R.B. wrote the paper with input from the other authors.

One-sentence summary:

The generation and characterization of transplastomic tobacco plants provide insight into the dual role of the chloroplast glutamyl-tRNA in protein biosynthesis and tetrpyrrole biosynthesis.
ABSTRACT

The chloroplast glutamyl-tRNA (tRNA\textsubscript{Glu}) is unique in that it has two entirely different functions. In addition to acting in translation, it serves as substrate of glutamyl-tRNA reductase, the enzyme catalyzing the committed step in the tetrapyrrole biosynthetic pathway. How the tRNA\textsubscript{Glu} pool is distributed between the two pathways and whether tRNA\textsubscript{Glu} allocation limits tetrapyrrole biosynthesis and/or protein biosynthesis remains poorly understood. We have generated a series of transplastomic tobacco (\textit{Nicotiana tabacum}) plants to alter tRNA\textsubscript{Glu} expression levels and also introduced a point mutation into the plastid \textit{trnE} gene, which was reported to uncouple protein biosynthesis from tetrapyrrole biosynthesis in chloroplasts of the protist \textit{Euglena gracilis}. We show that, rather than comparable uncoupling of the two pathways, the \textit{trnE} mutation is lethal in tobacco as it inhibits tRNA processing, thus preventing translation of glutamate codons. Ectopic expression of the mutated \textit{trnE} gene uncovered an unexpected inhibition of glutamyl-tRNA reductase by immature tRNA\textsubscript{Glu}. We further demonstrate that, whereas overexpression of tRNA\textsubscript{Glu} does not affect tetrapyrrole biosynthesis, reduction of GluTR activity through inhibition by tRNA\textsubscript{Glu} precursors causes tetrapyrrrole synthesis to become limiting in early plant development when active photosystem biogenesis provokes a high demand for \textit{de novo} chlorophyll biosynthesis. Taken together, our work provides insight into the roles of tRNA\textsubscript{Glu} at the intersection of protein biosynthesis and tetrapyrrole biosynthesis.

Keywords: translation; chlorophyll biosynthesis; plastid; chloroplast; tetrapyrrole biosynthesis; RNA processing; tRNA; plastid transformation; \textit{Nicotiana tabacum}
INTRODUCTION

The two DNA-containing organelles of plant cells, plastids (chloroplasts) and mitochondria, contain their own protein synthesis machinery. In agreement with their endosymbiotic origin from bacteria, both organelles possess bacterial-type 70S ribosomes consisting of a 30S and a 50S subunit (Bieri et al., 2017; Waltz et al., 2019; Tiller and Bock, 2014; Sun and Zerges, 2015; Zoschke and Bock, 2018). Plastids also encode a complete set of tRNAs that is sufficient to decode all 64 triplets of the genetic code (Cognat et al., 2013; Alkatib et al., 2012b). By contrast, plant mitochondria do not encode a full tRNA set in their genome and depend on the import of some tRNA species from the cytosol (Salinas et al., 2006; Duchêne et al., 2009; Vinogradova et al., 2009), a pathway that is likely absent from plastids (Rogalski et al., 2008a; Alkatib et al., 2012a).

The chloroplast glutamyl-tRNA (tRNA^{Glu}) is unique in that it has a second essential function. In addition to acting in plastid translation, it is also required for tetrapyrrole biosynthesis. Tetrapyrroles are macrocyclic molecules characterized by four pyrrole rings that are connected by methine bridges. Plants contain four classes of tetrapyrroles (heme, chlorophyll, siroheme, and phytochromobilin) that differ in conjugation state, side chains and/or the chelated ion. The universal precursor for the biosynthesis of all tetrapyrroles is 5-aminolevulinic acid (ALA). There are two alternative pathways for ALA synthesis: the C_4 pathways (or Shemin pathway) and the C_5 pathway. The C_4 pathway exists in animals, fungi, and certain groups of bacteria. It initiates with the condensation of succinyl-CoA and glycine, a reaction that is catalyzed by ALA synthetase (ALAS), to form ALA. In eukaryotes harboring the C_4 pathway, ALAS is typically localized in the mitochondrial compartment. The C_5 pathway depends on tRNA^{Glu} and exists in plants, archaea, and some groups of bacteria. In plants, the pathway is localized in plastids and utilizes charged plastid-encoded tRNA^{Glu} as substrate, from which the enzyme glutamyl-tRNA reductase (GluTR) forms glutamate-1-semialdehyde in the committed step of the tetrapyrrole biosynthetic pathway.
Glutamate-1-semialdehyde then undergoes an isomerization reaction catalyzed by the enzyme glutamate-1-semialdehyde aminotransferase (GSAT) to form ALA (Grimm, 1998; Brzezowski et al., 2015; Wang and Grimm, 2015). Thus, glutamyl-tRNA can enter one of two pathways in the chloroplast, namely protein biosynthesis or tetrapyrrole synthesis.

GluTR catalyzes the rate-limiting step in tetrapyrrole biosynthesis, and is a highly regulated enzyme (Richter et al., 2019). Tight regulation is essential, because many products and intermediates of the pathway are highly toxic when produced in excess and/or present as free compounds. For example, free chlorophylls and their precursors are extremely phototoxic. ALA synthesis from tRNA\textsuperscript{Glu} is regulated at multiple levels, including (i) biochemical feedback regulation by pathway products, especially heme, (ii) redox control of GluTR activity, (iii) interaction with a dedicated regulator protein, the GluTR-binding protein, GBP, (iv) protein turnover via recognition of the GluTR N-terminus by the chloroplast Clp protease, and (v) control of enzyme activity through interaction with the protein FLUORESCENT IN BLUE LIGHT (FLU; Meskauskiene et al., 2001; Richter et al., 2019).

A C-to-U point mutation in nucleotide position 56 of the mature tRNA\textsuperscript{Glu} (C56U) in plastids of the photosynthetic protist \textit{Euglena gracilis} has been reported to uncouple translation from tetrapyrrole biosynthesis. The mutant strain of \textit{E. gracilis} that harbored the corresponding C56T mutation in the plastid gene for tRNA\textsuperscript{Glu} (\textit{trnE} gene) was orange in color and incapable of chlorophyll biosynthesis, but was able to perform protein biosynthesis in plastids. Further investigation revealed that the point mutation in \textit{trnE} likely impairs tRNA\textsuperscript{Glu} substrate recognition by GluTR (Stange-Thomann et al., 1994).

Since the \textit{trnE} genes from \textit{E. gracilis} and seed plant plastids share a high degree of homology, we hypothesized that the C56U mutation might have similar effects in plants and also uncouple plastid translation from tetrapyrrole biosynthesis. An important difference between plants and \textit{E. gracilis} is that \textit{E. gracilis} possesses both the C\textsubscript{4} (Shemin pathway) and
the C₅ pathway for ALA synthesis. Consequently, even if the C₅ pathway is dysfunctional in
E. gracilis, the cells should still be able to synthesize some tetrapyrroles via the alternative C₄
pathway (especially heme which is essential for cell survival). By contrast, this is unlikely to
be the case with plants, which have only the C₅ pathway for synthesis of all tetrapyrroles.

The aim of this study was to assess the role of tRNA^{Glu} in plastid translation and
tetrapyrrole biosynthesis in seed plants. How the tRNA^{Glu} pool is distributed between the two
pathways and whether or not tRNA^{Glu} allocation limits tetrapyrrole biosynthesis and/or
protein biosynthesis in plastids is currently not known. We, therefore, used plastid
transformation in the model plant tobacco (Nicotiana tabacum) to (i) alter tRNA^{Glu} expression
levels, and (ii) introduce the C56T mutation in an attempt to uncouple plastid translation from
tetrapyrrole biosynthesis. We report that the C56T mutation, when introduced into the
endogenous trnE gene in the tobacco chloroplast genome, is lethal. We also show that ectopic
expression of a mutated trnE gene copy results in a pigment-deficient phenotype that,
surprisingly, is due to impaired tRNA maturation. Importantly, whereas accumulation of
immature tRNA^{Glu} has no detectable effect on plastid translation, ALA synthesis rates are
strongly diminished. Our findings indicate that immature tRNA^{Glu} inhibits GluTR at the level
of enzyme activity. By contrast, overexpression of the wild-type tRNA^{Glu} in chloroplasts
neither affects tetrapyrrole biosynthesis nor protein biosynthesis, suggesting that tetrapyrrole
biosynthesis in plants is not controlled at the level of tRNA^{Glu} provision.
RESULTS

Introduction of the C56T mutation and generation of transplastomic tobacco lines with altered expression levels of tRNA\textsuperscript{Glu}

To dissect the functions of tRNA\textsuperscript{Glu} in chloroplast translation and tetrapyrrole biosynthesis, four constructs for stable transformation of the tobacco plastid genome were designed (Figure 1). Vector pEndWt (Figure 1A) was constructed to test whether overexpression of tRNA\textsuperscript{Glu} is possible and how this would affect plastid translation and tetrapyrrole biosynthesis. To this end, the strongest known plastid promoter, specifically the ribosomal RNA operon promoter (Nt \textit{Prrn}), was placed upstream of the endogenous \textit{trnE} promoter (Hanaoka et al., 2003). Transformation vector pEndMut is similar to pEndWt, but replaces the endogenous copy of \textit{trnE} in the chloroplast genome with the mutated version harboring the C56T point mutation. Considering that (i) the point mutation abolishes the C\textsubscript{5} pathway in \textit{E. gracilis}, and (ii) plants do not have the alternative (Shemin) pathway for heme synthesis, this mutation could potentially lead to inviable plants. We, therefore, designed two additional constructs that left the endogenous \textit{trnE} gene unchanged, but introduced an additional gene copy into a neutral insertion site within a distant region of the plastid genome (Figure 1B; Ruf et al., 2001; Wurbs et al., 2007). Vector pEctWt introduces an additional wild-type copy of \textit{trnE}, whereas vector pEctMut introduces an additional \textit{trnE} gene copy that carries the C56T point mutation (Figure 1B).

Using biolistic transformation and selection for spectinomycin resistance conferred by the chimeric \textit{aadA} gene (Svab and Maliga, 1993), putative transplastomic lines were obtained with all four constructs. The transplastomic status of the lines was preliminarily confirmed by PCR assays amplifying the \textit{aadA} transgene, and positive lines were subjected to additional rounds of regeneration and selection to enrich the transplastome and isolate homoplasmic lines (Maliga, 2004; Bock, 2015). Transplastomic lines will subsequently be referred to as \textit{Nt}-
EndWt, Nt-EndMut, Nt-EctWt, and Nt-EctMut lines, respectively, with ‘End’ indicating that
the endogenous trnE gene copy was manipulated and ‘Ect’ indicating that an ectopic copy of
the trnE gene was introduced (Figure 1).

Lethality of the C56T mutation in tobacco

After three consecutive rounds of regeneration under stringent antibiotic selection, the
homoplasmic status of the transplastomic lines was assessed by DNA gel blot analyses
(Figure 2). To this end, total DNA was extracted from transplastomic plants and digested with
the restriction enzymes BamHI (for Nt-EndWt and Nt-EndMut) or BglII (for Nt-EctWt and
Nt-EctMut) for RFLP (restriction fragment length polymorphism) analysis. After separation
of DNA fragments by agarose gel electrophoresis, the blotted digestion products were
detected by hybridization using either a trnE-trnD-trnY locus-specific probe (expected
fragment sizes: 5.8 kb in the transplastomic Nt-EndWt and Nt-EndMut lines, and 4.5 kb in the
wild type; Figure 2A) or a psaB-specific probe (expected fragment sizes: 5.3 kb in the
transplastomic Nt-EctWt and Nt-EctMut lines, and 3.5 kb in the wild type; Figure 2B). Virtual
absence of the wild-type-size fragment from the transplastomic samples was taken as
preliminary evidence of homoplasmy. Several independent homoplasmic Nt-EctWt, Nt-
EctMut, and Nt-EndWt lines were identified, transferred to the greenhouse and grown to
maturity to obtain seeds.

By contrast, Nt-EndMut plants, in which replacement of the endogenous trnE with the
mutated version was attempted, were clearly not homoplasmic (Figure 2A). Stable
heteroplasmy in the presence of selection is typically associated with genetic changes that
inactivate an essential gene function in the plastid genome (Drescher et al., 2000; Shikanai et
al., 2001; Kode et al., 2005; Rogalski et al., 2006). Moreover, in addition to the expected
bands for the wild-type genome and the transformed genome, all lines displayed bands that presumable originated from undesired recombination events (Figure 2A). Such unexpected hybridization products are usually due to recombination between duplicated expression elements that drive transgene expression in addition to the expression of the endogenous gene they were taken from (Svab and Maliga, 1993; Rogalski et al., 2006; Gray et al., 2009; Li et al., 2011). After multiple rounds of plant regeneration in the presence of spectinomycin, a few homoplasmic lines were ultimately obtained. When these lines were tested for the presence of the C56T point mutation in the trnE gene by sequencing of amplified PCR products, all of them were found to lack the mutation, whereas the mutation was readily detectable in the heteroplasmic lines. Previous research had shown that deleterious mutations that are present in a heteroplasmic state are frequently eliminated by gene conversion (Khakhlova and Bock, 2006). Occurrence of gene conversion in conjunction with segregation to homoplasmy strongly suggests that the C56T mutation is lethal and remains heteroplasmic until the mutation is eliminated through gene conversion with a wild-type trnE copy.

Heteroplasmic Nt-EndMut plants displayed a characteristic leaf-loss phenotype, in that the leaves showed various deformations and often lacked parts of the leaf blade (Figure 3A). Very similar phenotypes were described previously for tobacco mutants with induced inactivation of plastid translation (Ahlert et al., 2003) and transplastomic plants in which essential components of the plastid gene expression machinery had been targeted by reverse genetics (Rogalski et al., 2008b; Fleischmann et al., 2011), including essential tRNA genes (Rogalski et al., 2008a; Alkatib et al., 2012a; Alkatib et al., 2012b). In these plants, segregation to homoplasmy leads to the loss of plastid gene expression, which in turn results in cell death. When this occurs early in leaf development, it can cause the loss of an entire cell lineage, which then results in misshapen leaves with large sectors of the leaf blade missing.

Lethality of the C56T mutation in tobacco suggests that it is incompatible with tRNA\textsuperscript{Glu} function in translation or tetrapyrrole biosynthesis (or both). Since the Nt-EndMut
transplastomic plants were genetically unstable, accumulated undesired recombination products, and tended to lose the C56T point mutation in trnE, they were excluded from further analyses.

**Phenotypes of transplastomic lines overexpressing wild-type or mutant trnE alleles**

When grown in soil under standard greenhouse conditions, transplastomic Nt-EctWt and Nt-EndWt plants were indistinguishable from wild-type tobacco plants (Figure 3B). By contrast, transplastomic Nt-EctMut plants were severely retarded in growth (by >2 weeks) compared to the wild type (Figure 3B). Moreover, leaves of Nt-EctMut plants that developed at early stages of plant growth displayed a pale or variegated phenotype. Interestingly, this phenotype was absent from leaves that developed at later stages of plant growth (Figure 3B, lower panel). The degree of chlorosis and the number of leaves showing the pale or variegated phenotype was quite variable, even among plants from the same transplastomic line. This suggests that, similar to previously described variegation mutants (Miura et al., 2007; Wang et al., 2018), a physiological threshold effect may be involved in the development of the phenotype.

Seeds were readily obtained from all three sets of transplastomic lines. In order to ultimately confirm homoplasmy of the lines and assess the phenotype at the seedling stage under heterotrophic conditions, seeds were surface sterilized and germinated on synthetic (sucrose-containing) culture media with or without spectinomycin (Figure 4). Whereas wild-type seedlings were clearly sensitive to spectinomycin (i.e., bleached out and ceased to grow), the progenies of all transplastomic lines showed uniform resistance to the antibiotic. Lack of segregation in the F1 generation is consistent with the uniparental inheritance of the plastid genome in tobacco (Greiner et al., 2014) and provides strong genetic evidence of
homoplasmy of the transplastome. *Nt*-EctMut seedlings were pale green, but their phenotype was clearly distinguishable from spectinomycin-sensitive wild-type seedlings that were completely white and showed arrested shoot and root growth. Also, the pale-green appearance of *Nt*-EctMut seedlings was independent of the presence of the antibiotic (Figure 4), confirming that these plants were also homoplasmic.

**Expression of tRNA\(^{\text{Glu}}\) in transplastomic tobacco plants**

All constructs introduced into the plastid genome share the strong constitutive *Nt Prrn* promoter upstream of *trnE* (Figure 1). To determine if the presence of this additional promoter (in the *Nt*-EndWt lines) and/or the ectopic expression of an extra copy of the *trnE* gene (in the *Nt*-EctWt and *Nt*-EctMut lines) results in overexpression of tRNA\(^{\text{Glu}}\) in transplastomic chloroplasts, northern blot experiments were conducted with total RNA samples extracted from two independent transplastomic lines for each of the constructs. A radiolabeled oligonucleotide derived from the sequence of the mature tRNA\(^{\text{Glu}}\) was used as the hybridization probe for detection of *trnE*-specific transcripts (Figure 5).

Interestingly, provision of the endogenous *trnE* with an additional *Nt Prrn* promoter (*Nt*-EndWt plants) did not lead to detectable overaccumulation of tRNA\(^{\text{Glu}}\) compared to the wild type (*Nt*-Wt; Figure 5). By contrast, insertion of an additional copy of the *trnE* gene into the plastid genome resulted in a strong increase in tRNA\(^{\text{Glu}}\) abundance in transplastomic *Nt*-EctWt plants (Figure 5). Surprisingly, transplastomic *Nt*-EctMut plants that ectopically express an additional *trnE* gene carrying the C56T mutation showed an additional hybridizing band in the northern blot analysis. This band was substantially larger (~150–200 nt) than that of the mature tRNA\(^{\text{Glu}}\) (76 nt, including the 3′ CCA end that is added enzymatically after 3′ processing by RNase Z). This band was absent from the wild type and all other transplastomic
lines (which only accumulated the mature tRNA) and could represent incompletely processed tRNA\textsuperscript{Glu} molecules (Figure 5). This observation raised the interesting possibility that the C at position 56 of \textit{trnE} plays a crucial role in the maturation of pre-tRNA\textsuperscript{Glu} to produce functional tRNA\textsuperscript{Glu}. The nucleotide at position 56, although being part of the loop region of the TΨC arm, is known to form a Watson-Crick base-pair with the nucleotide at position 19. This tertiary interaction plays an important role in tRNA elbow formation and is believed to be involved in the recognition of the tRNA by several protein factors, including the 5’ and 3’ processing enzymes RNase P and RNase Z (Zhang and Ferré-D’Amaré, 2016).

tRNA\textsuperscript{Glu} processing in transplastomic \textit{Nt}-EctMut plants

To test the idea that the C-to-U substitution at position 56 in tRNA\textsuperscript{Glu} results in impaired pre-tRNA processing, northern blot analyses were performed with hybridization probes specific to the 5’ leader and 3’ trailer sequences of the pre-tRNA\textsuperscript{Glu}. With both probes, bands were detected exclusively in the \textit{Nt}-EctMut plants (Figure 6A,B), suggesting that (i) tRNA processing in the wild-type and the transplastomic \textit{Nt}-EndWt and \textit{Nt}-EctWt plants is very efficient in that precursors and processing intermediates are undetectable, and (ii) the C56U exchange affects maturation of tRNA\textsuperscript{Glu} in transplastomic \textit{Nt}-EctMut plants at both the 5’ and the 3’ ends. The size of the incompletely processed tRNA\textsuperscript{Glu} molecules was in the same range as the larger band detected in northern blots with the \textit{trnE} probe (Figure 5), although the leader and trailer probes also detected additional presumptive precursor RNAs of even larger size but much lower abundance (Figure 6A,B).

To ultimately confirm the 5’ and 3’ processing defects in the \textit{Nt}-EctMut plants, RNA circularization experiments were undertaken. To this end, the regions corresponding to the bands seen in the RNA gel blot experiments were excised from urea-containing denaturing
polyacrylamide gels, self-ligated, and reverse transcribed into cDNA. Subsequent PCR amplification of the junction of the ligated termini precisely identified the 5′ and 3′ ends. When this assay was performed with RNA samples from the wild type, the Nt-EndWt line, and the Nt-EctWt line, only fully processed tRNA\textsubscript{Glu} molecules were detected (Figure 6C). Interestingly, none of the processed tRNA\textsubscript{Glu} molecules in the Nt-EctMut plants carried the point mutation, indicating that the faithfully processed tRNA molecules originate exclusively from the resident \textit{trnE} gene copy in the plastid genome. Conversely, all incompletely processed tRNA\textsubscript{Glu} molecules carried the point mutation (Figure 6C; Supplemental Figure 1). Whereas all immature tRNA molecules had 3′ extensions, only relatively few (2 out of 20 sequenced clones) carried 5′ leader sequences (Figure 6C; Supplemental Figure 1). This finding potentially indicates that the C56U mutation largely blocks precursor tRNA processing by the 3′ processing enzyme RNase Z (Stern et al., 2010; Stoppel and Meurer, 2012), whereas 5′ processing by the chloroplast form of RNase P (termed PRORP; Gutmann et al., 2012; Zhou et al., 2015) is less severely affected.

**Ectopic expression of mutated tRNA\textsubscript{Glu} affects neither aminoacylation of mature tRNA\textsubscript{Glu} nor processing of other plastid tRNAs**

Having uncovered processing defects in the mutated tRNA\textsubscript{Glu} (tRNA\textsubscript{Glu}_C56U), we next considered the possibility that accumulation of immature tRNA\textsubscript{Glu} molecules interferes with tRNA aminoacylation in the Nt-EctMut plants, for example, by inhibiting the corresponding aminoacyl-tRNA synthetase. We, therefore, determined the tRNA aminoacylation state by acidic urea polyacrylamide gel electrophoresis of acylated versus deacylated tRNAs. These experiments revealed that the aminoacylation state of tRNA\textsubscript{Glu} was unaffected in all
transplastomic lines, including the *Nt*-EctMut lines ectopically expressing the mutant tRNA\textsubscript{Glu} (Figure 7A).

Our sequencing analyses had indicated that the *Nt*-EctMut lines efficiently processed the tRNA\textsubscript{Glu} molecules transcribed from the wild-type *trnE* gene copy, whereas processing of the mutated tRNA\textsubscript{Glu}\_C56U was selectively impaired (Figure 6C; Supplemental Figure 1). This finding argues against the possibility that the mutated tRNA interferes with the general activity of PRORP and/or RNase Z in the chloroplast. To further verify this assumption, the processing status of two other chloroplast tRNAs, namely tRNA\textsubscript{Phe} and tRNA\textsubscript{Arg}, was investigated by northern blot analyses. As expected, only mature tRNAs could be detected in all transplastomic lines investigated (Figure 7B), confirming that accumulation of immature tRNA\textsubscript{Glu}\_C56U does not generally interfere with the activity of PRORP and/or RNase Z in the *Nt*-EctMut lines.

Accumulation of immature tRNA\textsubscript{Glu}\_C56U does not affect chloroplast protein biosynthesis

Our analyses of tRNA processing and aminoacylation suggested that processes upstream of tRNA\textsubscript{Glu} utilization in chloroplast translation and ALA synthesis are unlikely causally responsible for the mutant phenotype observed in *Nt*-EctMut plants. Therefore, we subsequently examined whether expression of the mutated *trnE* gene negatively affects chloroplast protein biosynthesis.

Polysome analyses were performed to determine if the pale-leaf phenotype of *Nt*-EctMut plants (Figure 3B) was the result of interference of tRNA\textsubscript{Glu}\_C56U with the translation by chloroplast ribosomes. Recent work has suggested that the abundance of plastid-encoded chlorophyll-binding proteins is adjusted to chlorophyll availability at the
level of proteolysis rather than de novo synthesis (Zoschke et al., 2017). Consequently, any observable effect on plastid translation rates should be a direct consequence of the interaction of mutated tRNA$^{\text{Glu}}$ molecules with translating ribosomes rather than a secondary effect from the pigment deficiency of Nt-EctMut plants. Northern blot analysis of RNAs extracted from fractionated polysome gradients revealed unaltered ribosome association of the mRNAs of two key genes involved in photosynthesis: rbcL (encoding the large subunit of Rubisco) and psbD (encoding the D2 protein of the photosystem II reaction center; Figure 8). These results indicate that accumulation of the mutated tRNA$^{\text{Glu}}_{\text{C56U}}$ does not affect protein synthesis rates in the chloroplast.

Presence of precursor tRNA$^{\text{Glu}}_{\text{C56U}}$ strongly reduces ALA synthesis rates

Having excluded tRNA metabolism, aminoacylation, and translation as possible causes of the mutant phenotype of Nt-EctMut lines, the possibility remained that the accumulation of mutated and incompletely processed tRNA$^{\text{Glu}}$ molecules inhibits tetrapyrrole biosynthesis. Such an inhibitory effect could come from binding of immature tRNA$^{\text{Glu}}_{\text{C56U}}$ to GluTR, the enzyme that uses tRNA$^{\text{Glu}}$ as substrate for ALA synthesis. As no mature tRNA$^{\text{Glu}}_{\text{C56U}}$ accumulates and 3’ processing is completely blocked (Figure 6C; Supplemental Figure 1), the tRNA$^{\text{Glu}}_{\text{C56U}}$ can neither undergo 3’ CCA addition nor be charged with glutamate. Thus, it seems clear that tRNA$^{\text{Glu}}_{\text{C56U}}$ cannot serve as substrate to form glutamate-1-semialdehyde in the committed step of tetrapyrrole biosynthesis. However, if the mutated and immature tRNA molecules would nonetheless bind to GluTR, they could either block the activity of the enzyme or, alternatively, destabilize the enzyme and condemn it to rapid degradation.

To investigate these possibilities, ALA synthesis rates were measured in wild-type, Nt-EctWt, and Nt-EctMut plants. Although the transplastomic Nt-EctWt plants strongly
overexpress tRNA\textsubscript{Glu} (Figure 5), tRNA overaccumulation did not result in a significant
stimulation of ALA synthesis (Figure 9A), indicating that tRNA\textsubscript{Glu} availability does not limit
tetrapyrrole biosynthesis. By contrast, ALA synthesis was strongly reduced in \textit{Nt}-EctMut
plants, with synthesis rates reaching only approximately 20% of those in wild-type and \textit{Nt}-
EctWt plants (Figure 9A). This finding raises the interesting possibility that the ectopically
expressed tRNA\textsubscript{Glu}_C56U inhibits ALA synthesis by GluTR.

To examine whether tRNA\textsubscript{Glu}_C56U affects GluTR accumulation (e.g., by forming a
dead-end complex that triggers proteolytic degradation of the enzyme), immunoblot analysis
with GluTR-specific antibodies was performed. These experiments revealed that GluTR
protein levels were very similar in all investigated plant lines (Figure 9B), indicating that
tRNA\textsubscript{Glu}_C56U does not exert its inhibitory action at the level of protein stability. Instead,
this finding suggests that inhibition occurs at the level of enzyme activity, presumably by the
immature and uncharged tRNA\textsubscript{Glu}_C56U molecules forming a stable dead-end complex with
GluTR.

Finally, we examined accumulation of two of the end products of tetrapyrrole
biosynthesis, chlorophyll a and chlorophyll b, in the transplastomic lines (Supplemental
Figure 2). We particularly wanted to know whether the green leaves appearing later in
development of \textit{Nt}-EctMut plants (Figure 3B) show full recovery at the level of chlorophyll
accumulation. This was indeed the case for both chlorophyll a and chlorophyll b
(Supplemental Figure 2), suggesting that rapid growth and high demand for chlorophylls early
in development cause the pale-leaf phenotype, whereas the reduced demands later in
development can be fully satisfied by the reduced synthesis capacity in \textit{Nt}-EctMut plants.
Also, none of the other transplastomic lines displayed any alteration in chlorophyll
accumulation (Supplemental Figure 2), consistent with our finding that ALA synthesis rates
are unaffected by overexpression of tRNA\textsubscript{Glu} (Figure 9A).
DISCUSSION

In the course of this work, we have generated a set of transplastomic tobacco plants to alter the expression of chloroplast tRNA\textsuperscript{Glu} and introduced a point mutation in the trnE gene, the latter of which had been described in a spontaneous mutant of Euglena gracilis and reported to uncouple plastid translation from tetrapyrrrole biosynthesis (Stange-Thomann et al., 1994). The C56T mutation in the trnE gene of the Euglena mutant led to reduced accumulation of tRNA\textsuperscript{Glu}, but the mutant tRNA appeared to be faithfully aminoacylated and, therefore, was suggested to participate in plastid protein biosynthesis. By contrast, the mutant tRNA\textsuperscript{Glu} did not support ALA synthesis by the C\textsubscript{5} pathway (Stange-Thomann et al., 1994).

Our work reported here in the seed plant tobacco did not confirm many of the findings reported for the Euglena mutant. Most importantly, when introduced into the trnE gene of tobacco plastids, the C56T mutation was incompatible with cell survival. Transplastomic lines remained heteroplasmic and displayed the malformed leaf phenotype that is typical of (heteroplasmic) loss-of-function mutants of essential genes in the plastid genome (Figure 2A; Figure 3A). Notably, this phenotype has been observed for all genes encoding essential components of the plastid translational machinery when targeted by reverse genetics, including ribosomal proteins and tRNAs (Ahlert et al., 2003; Alkatib et al., 2012b; Tiller and Bock, 2014; Zoschke and Bock, 2018). Our analysis of tRNA\textsuperscript{Glu}_C56U expression in transplastomic plants revealed defective tRNA processing and complete absence of correctly processed tRNA molecules that could be aminoacylated and serve as substrates in translation elongation (Figure 6C; Supplemental Figure 1). This finding suggests that the C56T mutation represents a trnE loss-of-function allele, thus providing a straightforward explanation for the phenotype of the Nt-EndMut plants (Figure 3A). However, since the C\textsubscript{5} pathway is likely also essential in seed plants (Czarnecki et al., 2011; Richter et al., 2019), the reason why Nt-
EndMut plants remain heteroplasmic and why segregation to homoplasmy causes cell death and leaf malformation could be twofold.

It is currently unclear why processed and aminoacylated tRNA$^\text{Glu}$ accumulated in the *Euglena* mutant. All sequenced *Euglena* strains contain a single copy of the *trnE* gene in their chloroplast genome. The tRNA processing machinery is highly conserved in that 5′ processing is conducted by RNase P and 3′ processing is performed by RNase Z in nearly all organisms. Also, the tertiary interaction nucleotide C$_{56}$ is involved in, its role in tRNA elbow formation, and its involvement in the interaction with the tRNA processing enzymes appear well conserved (Zhang and Ferré-D’Amaré, 2016). It, therefore, would be surprising if the same mutation completely blocks tRNA maturation in tobacco plastids, but allows for correct processing in *Euglena* plastids. In this context, it should be noted that the chloroplast genome of the *Euglena* mutant was not characterized beyond PCR amplification of the known genomic *trnE* locus. Thus, the presence of a second wild type-like copy of the *trnE* gene that provides the functional tRNA$^\text{Glu}$ identified in the aminoacylation assays (Stange-Thomann et al., 1994), along with an uncharacterized genetic defect that causes the chlorophyll deficiency, cannot be ruled out. Such gene duplication could be the result of a genomic rearrangement that went undetected in the studies conducted with the mutant. It is noteworthy in this regard that gene duplications in *Euglena* plastids are more stable than in seed-plant plastids. This is suggested, for example, by the presence of the rRNA operon as tandem repeats (Hallick et al., 1993), and may be due to absence (or very low activity) of homologous recombination (Doetsch et al., 2001). Alternatively, it is possible that the correctly processed tRNA$^\text{Glu}$ is encoded in the nuclear genome of *Euglena* and imported into plastids. However, whereas tRNA import is common in plant mitochondria (Duchêne et al., 2009), no direct evidence for the presence of it in plastids has been obtained to date, although some indirect evidence comes from the existence of highly reduced plastid genomes that do not encode a...
complete set of tRNAs (Morden et al., 1991; Delannoy et al., 2011). In view of all this, a thorough reinvestigation of the *Euglena* mutant, if still available, would be very worthwhile.

In this study, we also tested two different approaches towards overexpression of the chloroplast tRNA\textsubscript{Glu}. Since tRNA\textsubscript{Glu} serves as substrate of the rate-limiting initial reaction of tetrapyrrole biosynthesis (Brzezowski et al., 2015; Richter et al., 2019), it was conceivable that tRNA\textsubscript{Glu} provision limits flux through the pathway. In fact, expression of tRNA\textsubscript{Glu} has been shown to be developmentally regulated (Hanaoka et al., 2005). Moreover, tRNA\textsubscript{Glu} was proposed to feedback-regulate chloroplast transcription by directly binding to one of the two RNA polymerases in plastids (Hanaoka et al., 2005). The two overexpression strategies we pursued produced transplastomic plants that were indistinguishable from wild-type plants (Figure 3B). Whereas insertion of an additional strong promoter upstream of the trnE gene in its native genomic location did not result in appreciable overaccumulation of tRNA\textsubscript{Glu}, integration of an extra gene copy into a distant genomic location resulted in strong overexpression of the tRNA (Figure 5). However, this did not result in a measurable effect on ALA synthesis rates (and chlorophyll accumulation) in the transplastomic *Nt*-EctWt plants (Figure 9A; Supplemental Figure 2), suggesting that tRNA\textsubscript{Glu} does not limit tetrapyrrole biosynthesis, at least not under standard growth conditions.

Ectopic expression of tRNA\textsubscript{Glu}_C56U produced plants that exhibit a strong pigment-deficient phenotype at the seedling stage (Figure 3B). Interestingly, as the plants matured, the mutant phenotype became less severe and the leaves appearing later in development became progressively greener until they finally were nearly indistinguishable from wild-type leaves (young *Nt*-EctMut leaves in lower panel of Figure 3B). *Nt*-EctMut plants showed unaltered expression of the endogenous trnE gene and accumulated wild-type levels of fully processed and faithfully aminoacylated tRNA\textsubscript{Glu} (Figure 5; Figure 7A). In addition, the plants accumulated the mutant tRNA\textsubscript{Glu}_C56U to a similar level; however, this tRNA remained unprocessed and, therefore, cannot be aminoacylated. These results suggest that the pigment-
deficient mutant phenotype of the Nt-EctMut plants is the result of a dominant-negative effect exerted by the ectopic expression of tRNA\textsubscript{Glu}_C56U. We showed that GluTR, the enzyme that utilizes tRNA\textsubscript{Glu} to synthesize ALA, accumulates to unaltered levels in Nt-EctMut plants. However, ALA synthesis rates were drastically reduced in the transplastomic plants (Figure 9), indicating that the immature tRNA\textsubscript{Glu}_C56U molecules inhibits the enzyme, presumably by binding to it and forming a dead-end complex.

Our data suggest that inhibition of GluTR activity to approximately 20% of the wild-type levels causes ALA synthesis to limit biogenesis of the photosynthetic apparatus. This bottleneck is particularly severe in young developing plants that have high rates of photosystem biogenesis (Hojka et al., 2014; Schöttler and Tóth, 2014; Armarego-Marriott et al., 2019) and, therefore, a high demand for chlorophylls (Figure 3B). By contrast, when growth decelerates later in development, the low ALA synthesis rates are sufficient to satisfy the needs of the chloroplast, resulting in a striking developmental gradient in the leaf phenotypes. Interestingly, the pale leaves that developed under ALA limitation do not recover and never regreen (Figure 3B). This is likely due to loss of the photosystem assembly capacity that is known to occur as leaves mature (Schöttler et al., 2011; Hojka et al., 2014; Schöttler and Tóth, 2014).

The charging of tRNA\textsubscript{Glu} with the amino acid glutamate occurs by the two-step reaction typical of aminoacyl-tRNA synthetases. L-glutamate is first activated by ATP to form glutamyl-AMP and then transferred to the acceptor end (i.e., the 3’ CCA end) of the tRNA\textsubscript{Glu} molecule. The aminoacylation of mature tRNA\textsubscript{Glu} was virtually complete in all transplastomic lines generated in this study (Figure 7A), suggesting that even strong overexpression of the tRNA does not exceed the capacity of the plastid glutamyl-tRNA synthetase. Consistent with this finding, chloroplast translation was unaffected in all lines (Figure 8). As unprocessed and uncharged tRNAs are not taken into the ribosome, it is
unsurprising that accumulation of immature tRNA\textsubscript{Glu}_C56U does not interfere with translation elongation.

In summary, our work reported here shows that, unlike that previously reported for \textit{Euglena} (Stange-Thomann et al., 1994), the C56U substitution in chloroplast tRNA\textsubscript{Glu} does not uncouple plastid protein biosynthesis from tetrapyrrole biosynthesis in tobacco. Instead, the C56T substitution represents a lethal mutation. It inhibits tRNA processing and, in this way, prevents translation of glutamate codons. Moreover, our work has uncovered an unexpected inhibitory activity of unprocessed tRNA\textsubscript{Glu} on GluTR, the enzyme catalyzing the committed step of tetrapyrrole biosynthesis. Importantly, whereas overexpression of tRNA\textsubscript{Glu} does not stimulate ALA biosynthesis, reduction of GluTR activity through inhibition by tRNA\textsubscript{Glu} precursors makes ALA synthesis limiting to an extent that chlorophyll biosynthesis in early plant development cannot satisfy the demands of photosystem biogenesis.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

Sterile tobacco (\textit{Nicotiana tabacum} cv. Petit Havana) plants were grown on Murashige & Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose. The growth chambers had a light intensity of 50 μE m\textsuperscript{-2} s\textsuperscript{-1} and a photoperiod of 16-h light at 24°C and 8-h dark at 22°C. Soil-grown plants were raised in growth chambers with a light intensity of 350 μE m\textsuperscript{-2} s\textsuperscript{-1} and a photoperiod of 16-h light at 24°C and 8-h dark at 22°C.

**Construction of plastid transformation vectors**
For construction of transformation vector pNt-EctWt (Figure 1), PCR was performed to amplify (i) the Nt Prrn promoter using primer pair oSAA57_trnE_F3/oSAA58_trnE_R3, (ii) the trnE gene from the tobacco plastid genome and its promoter using primer pair oSAA59_trnE_F4/oSAA65_trnE_R8, and (iii) the 3' UTR from the plastid rbcL gene of Chlamydomonas reinhardtii (Cr TrbcL; Lu et al., 2017) with primer pair oSAA64_trnE_F7/oSAA66_trnE_R7 (Supplemental Table 1). Overlap extension PCR was performed to generate the Nt Prrn-trnE-Cr TrbcL fusion product. The resulting amplification product was digested with the restriction enzymes SacI and HindIII and cloned into a modified pRB96 vector (Ruf et al., 2001; Wurbs et al., 2007) cut with SacI and HindIII.

For construction of transformation vector pNt-EctMut, site-directed mutagenesis was performed on the fusion product generated for construction of pNt-EctWt to introduce the desired point mutation into trnE. To this end, PCRs were performed with the Nt Prrn-trnE-Cr TrbcL fusion product as template using the primer pairs oSAA57_trnE_F3/oSAA62_trnE_R5 and oSAA61_trnE_F5/oSAA66_trnE_R7 (Supplemental Table 1). Overlap extension PCR was performed to produce the Nt Prrn-trnE_C56U-Cr TrbcL fusion product. This amplification product was cloned in the pRB96-derived plastid transformation vector as described above.

For construction of vectors pNt-EndWt and pNt-EndMut, an intermediate vector (pSAA26) was generated. To this end, PCR was performed to amplify (i) a ~1 kb region upstream of trnE using tobacco total DNA as template and the primer pair oSAA53_trnE_F1 (introducing a SacI site) / oSAA54_trnE_R1, and (ii) an aadA expression cassette (Figure 1A) with the primer pair oSAA55_trnE_F2/oSAA56_trnE_R2 (introducing a HindIII site; Supplemental Table 1). Overlap extension PCR was performed to generate a fusion product, which was subsequently digested with SacI and HindIII and cloned into plasmid pBS KS(+) cut with SacI and HindIII.
For construction of pNt-EndWt, PCRs were performed to amplify (i) the Nt Prrn sequence with primer pair oSAA57_trnE_F3 (introducing a HindIII site) / oSAA58_trnE_R3, and (ii) the trnE-trnY-trnD region of the tobacco plastid genome and a ~500 bp region downstream with primer pair oSAA59_trnE_F4/oSAA60_trnE_R4 (introducing an XhoI site; Supplemental Table 1). Overlap extension PCR was performed to obtain a fusion product, which was then digested with HindIII and XhoI and cloned into pSAA26 cut with HindIII and XhoI. pNt-EndMut was constructed via site-directed mutagenesis performed on the fusion product produced for construction of pNt-EndWt to introduce the desired point mutation into the trnE gene sequence. To this end, PCRs were performed with the fusion product as template and the primer pairs oSAA57_trnE_F3 (introducing a HindIII site) / oSAA62_trnE_R5 and oSAA61_trnE_F5/oSAA60_trnE_R4 (introducing an XhoI site; Supplemental Table 1). Overlap extension PCR yielded a new fusion product, which was digested with HindIII and XhoI and cloned into pSAA26 cut with HindIII and XhoI.

Plastid transformation of tobacco and isolation of transplastomic lines

Biolistic transformation of plastids was conducted according to published protocols (Svab and Maliga, 1993). Briefly, young tobacco leaves from plants grown under sterile conditions were bombarded with plasmid DNA-coated gold particles using the DuPont PDS-1000/He biolistic gun (Bio-Rad, Munich, Germany). After bombardment, leaves were cut into small pieces of ~5 x 5 mm in size, placed onto MS-based plant regeneration medium containing 500 mg/L spectinomycin, and incubated for 2–3 months. Primary spectinomycin-resistant shoots or calli were subjected to two to three additional rounds of regeneration in the presence of the antibiotic to enrich the transformed plastid genome and isolate homoplasmic transplastomic lines (Bock, 2001). Finally, regenerated shoots were rooted and propagated on hormone-free MS medium containing 500 mg/L spectinomycin, then transferred to soil and grown to
maturity under standard greenhouse conditions with a photoperiod of 16-h light at 25°C and 8-h darkness at 20°C.

Seed assays

To test for maternal inheritance of the engineered plastid genomes, T1 generation seeds from the transplastomic lines were surface-sterilized and sown on MS medium containing 500 mg/L spectinomycin. Presence of a homogeneous antibiotic-resistance progeny confirmed homoplasmy of the transformed chloroplast genome (Bock, 2001).

Isolation of nucleic acids and gel blot analyses

Leaf tissue snap-frozen in liquid nitrogen was used for the isolation of nucleic acids. For extraction of total cellular DNA, a cetyltrimethylammoniumbromide (CTAB)-based method was used (Doyle and Doyle, 1990). Total plant RNA was extracted using the peqGOLD TriFast reagent (Peqlab GmbH, Erlangen, Germany). For RFLP analysis, samples of 3 μg total DNA were digested with appropriate restriction enzymes, separated in 1% (w/v) agarose gels by electrophoresis, and transferred onto Hybond nylon membranes (GE Healthcare, UK) by capillary blotting. For northern blot analysis, total cellular RNA was electrophoretically separated in 2% (w/v) agarose gels containing 5% (w/v) formaldehyde, and blotted onto Hybond nylon membranes (GE Healthcare).

Probes for RFLP analysis were amplified by PCR using primers listed in Supplemental Table 1, followed by agarose gel electrophoresis and purification of the PCR products from excised gel slices using the NucleoSpin® Extract II kit (Macherey-Nagel, Düren, Germany). Purified probes were radiolabeled with [α-32P]dCTP by random priming using the Multiprime DNA labeling system (GE Healthcare). Antisense oligonucleotides (synthesized
by Eurofins Genomics, Ebersberg, Germany, and listed in Supplemental Table 1) were used as probes for northern blot analyses. For radioactive labeling, 20 pmol of the oligonucleotide were incubated with 10 units of T4 polynucleotide kinase (New England Biolabs) and 30 µCi of [γ-32P]ATP for 30 min at 37°C to allow 5′ end labelling. All hybridizations, except those with the trnE 5′ leader and 3′ trailer sequences as probes, were performed at 65°C using standard protocols (Church and Gilbert, 1984). For probes derived from trnE 5′ leader and 3′ trailer sequences, 55°C was used as hybridization temperature. Signals were analyzed using a Typhoon Trio+ variable mode imager (GE Healthcare).

Circular RT-PCR

Samples of 8 µg total RNA were electrophoretically separated in 8% (w/v) urea-containing polyacrylamide gels. Bands corresponding to the sizes of processed and precursor tRNAs were cut out and treated with the enzyme RppH (New England Biolabs) to remove the pyrophosphate from the 5′ end of triphosphorylated RNAs. Subsequently, the monophosphorylated RNA molecules were circularized using T4 RNA ligase (New England Biolabs), followed by reverse transcription with primer oSAA105_tRNA-E_R that anneals specifically to the trnE gene sequence (Supplementary Table 1). RT-PCR was performed with cDNA as template and the primer pair oSAA101_tRNA-E_F/oSAA105_tRNA-E_R, followed by cloning of the amplification products using the TOPO® TA Cloning Kit (Invitrogen). The clones obtained were analyzed by colony PCR and DNA sequencing.

tRNA aminoacylation assays

Aminoacylation of tRNA^Glu was analyzed as described previously (Zhou et al., 2013). A specific hybridization probe for tRNA^Glu was prepared as described above.
Polysome analysis

Polysome analyses and puromycin controls were performed essentially as described previously (Barkan, 1998; Rogalski et al., 2008a). RNA was extracted from gradient fractions and the RNA pellet was resuspended in 30 µL RNase-free water. Aliquots of 5 µL per fraction were analyzed by northern blotting.

Determination of ALA synthesis rate and measurement of chlorophyll accumulation

ALA synthesis rates were determined according to published protocols (Czarnecki et al., 2011). Chlorophyll concentrations were measured in frozen tissue of defined fresh weight by extraction with 100% [v/v] acetone following published procedures (Porra et al., 1989; Lu et al., 2017).

Protein isolation and immunoblot analyses

Samples of 100 mg ground frozen leaf tissue were used for extraction of total cellular protein using a phenol-based method (Cahoon et al., 1992) with the minor modification that, instead of phenyl-methanesulfonyl fluoride (PMSF), cOmplete™ Protease Inhibitor Cocktail (Roche) was added. The protein pellets were dissolved in 1% (w/v) sodium dodecyl sulfate (SDS) and the protein concentration was determined with the BCA Protein Assay kit (Pierce, Rockford, USA). For immunoblot analysis, samples of 20 µg protein were separated by 10% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare). Membranes were treated with blocking buffer (1x Tris-buffered saline, TBS; 0.1% (v/v) Tween-20; 1% (w/v) bovine serum albumin, BSA) for 1 h,
and then incubated with a rabbit polyclonal anti-glutamyl-tRNA reductase antibody (Agrisera) for another hour. After incubation with anti-rabbit secondary antibody (Agrisera), immunodetection was performed with the ECL Prime system (GE Healthcare). For actin detection, the membranes were stripped using Thermo Scientific Restore™ Plus western blot stripping buffer (Fisher Scientific) according to the manufacturer’s instructions, followed by treatment with blocking buffer for 1 h, incubation in mouse polyclonal anti-actin antibody (Sigma) for 1 h, incubation in anti-mouse secondary antibody (Sigma), and detection using the ECL Prime system (GE Healthcare).

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NC_001879.2 (*Nicotiana tabacum*, complete plastid genome).

**Supplemental Data**

The following supplemental figures and tables are included separately as a single pdf file.

**Supplemental Figure S1.** Determination of the ends of incompletely processed *trnE* transcripts in the *Nt-EctMut* mutant.

**Supplemental Figure S2.** Quantification of chlorophyll a and b contents in mature transplastomic plants (12-leaf stage) grown under standard greenhouse conditions in a diurnal cycle of 16-h light at 25°C and 8-h darkness at 20°C.

**Supplemental Table S1.** List of synthetic oligonucleotides used as PCR primers in this study.

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Figure 1. Physical maps of targeting regions in the tobacco plastid genome (ptDNA) and the modified regions in the transplastomic mutants generated by stable transformation of the chloroplast genome. (A) Map of the trnE-encoding region in the tobacco plastid genome and changes introduced in Nt-EndWt and Nt-EndMut transplastomic lines. Genes above the line are transcribed from left to right, genes below the line are transcribed in the opposite direction. Relevant promoters are represented as green boxes, terminators (3′ UTRs) as brown boxes, and the coding regions of trnE and the selectable marker gene aadA as light-blue boxes. Note that, in both Nt-EndWt and Nt-EndMut transplastomic lines, trnE transcription is enhanced by the insertion of an additional strong promoter (the rRNA operon promoter, Nt Prrn) upstream of the endogenous trnE gene promoter (PtrnE). The Nt-EndMut transplastomic lines additionally carry the C56T mutation in trnE. (B) Maps of the targeting region in the wild type (ptDNA) and the engineered regions in the transplastomic Nt-EctWt and Nt-EctMut lines that ectopically overexpress the wild-type tRNA\textsubscript{Glu} or the mutated tRNA\textsubscript{Glu} sequence (harboring the C56T mutation), respectively. Cr PpsaA: psaA promoter from Chlamydomonas reinhardtii, Nt Prrn: plastid rRNA operon promoter from N. tabacum, Cr TatpB: 3′ UTR of the atpB gene from C. reinhardtii, PtrnE: promoter of trnE in N. tabacum. The recognition sites for digestion by restriction endonucleases used for RFLP analysis and the expected fragment sizes are indicated. The hybridization sites of radiolabeled probes for RFLP analyses are shown as black horizontal bars.

Figure 2. RFLP analysis of transplastomic plants generated for overexpression of the wild-type trnE gene or the mutated trnE gene (giving rise to tRNA\textsubscript{Glu}\_C56U) in tobacco chloroplasts. (A) Southern blot analysis of Nt-EndWt and Nt-EndMut lines. Total DNA was
digested with the restriction enzyme BamHI and fragments were detected by hybridization with a radiolabeled \textit{trnE-trnD-trnY} locus-specific probe (cf. Figure 1A). (B) Analysis of \textit{Nt-EctWt} and \textit{Nt-EctMut} lines. Total DNA was digested with BglIII and fragments were detected by hybridization with the radiolabeled \textit{psaB}-specific probe shown in Figure 1B. \textit{Nt-Wt}: wild-type tobacco, M: molecular weight marker.

**Figure 3.** Phenotypes of transplastomic plants generated in this study in comparison to wild-type plants. (A) Phenotype of \textit{Nt-EndMut} transplastomic plants in tissue culture in comparison to a control plant (\textit{Nt-Wt}: wild-type tobacco). The right panels show examples of misshapen leaves that are typical of mutations in chloroplast genes encoding essential components of the translation machinery (Ahlert et al., 2003; Rogalski et al., 2006; Rogalski et al., 2008b; Alkatib et al., 2012a; Alkatib et al., 2012b; reviewed in Tiller and Bock, 2014). Scale bar: 1 cm (B) Phenotype of \textit{Nt-EctWt}, \textit{Nt-EctMut}, and \textit{Nt-EndWt} transplastomic plants grown in soil. Upper row: 4-week-old plants (scale bar: 5 cm), lower row: 8-week-old plants (scale bar: 20 cm).

**Figure 4.** Seed assays to confirm homoplasmy of transplastomic plants. Wild-type (\textit{Nt-Wt}) seeds and T1 seeds from \textit{Nt-EctWt}, \textit{Nt-EctMut}, and \textit{Nt-EndWt} were germinated on synthetic medium in the presence of spectinomycin. Absence of antibiotic-sensitive progeny indicates the homoplastic state of all transplastomic lines. +Spec: 500 mg/L spectinomycin in the culture medium, -Spec: control with no spectinomycin in the culture medium. Scale bar: 2 cm.

**Figure 5.** Northern blot analysis to compare transcript accumulation levels and RNA processing of \textit{trnE} in transplastomic \textit{Nt-EctWt}, \textit{Nt-EctMut}, and \textit{Nt-EndWt} lines and wild-type...
(Nt-Wt) tobacco plants. 1 µg of total cellular RNA was electrophoretically separated under denaturing conditions and blotted. The blot was hybridized to a radiolabeled probe specific to the trnE gene (upper panel). The ethidium bromide (EtBr)-stained gel prior to blotting is shown as a control for equal loading (bottom panel). Dashes denote empty lanes. M: RNA size marker.

**Figure 6.** Northern blot analysis to detect incompletely processed precursors of tRNA\textsuperscript{Glu} in transplastomic Nt-EctWt, Nt-EctMut, and Nt-EndWt lines and wild-type (Nt-Wt) tobacco plants. (A) Detection of trnE transcripts that remain unprocessed at their 5′ end. The 5′ leader sequence of tRNA\textsuperscript{Glu} was used as radiolabeled probe. (B) Detection of trnE transcripts that remain unprocessed at their 3′ end. The 3′ trailer sequence of tRNA\textsuperscript{Glu} was used as probe. Samples of 3 µg total cellular RNA were electrophoretically separated under denaturing conditions and blotted. Blots were hybridized to radiolabeled probes (upper panels), and the ethidium bromide (EtBr)-stained gels prior to blotting are shown as controls for equal loading (lower panels). M: RNA size marker. (C) Schematic representation of the results from circular RT-PCR analysis to identify the termini of incompletely processed trnE transcripts. See text for details. For sequences of individual clones from Nt-EctMut, see Supplemental Figure 1.

**Figure 7.** Aminoacylation of processed tRNA\textsuperscript{Glu} and unaffected processing of other plastid tRNAs in transplastomic Nt-EctWt, Nt-EctMut, and Nt-EndWt plants. (A) Northern blot analysis to determine the aminoacylation status of tRNA\textsuperscript{Glu} in transplastomic Nt-EctWt, Nt-EctMut, and Nt-EndWt plants and wild-type tobacco (Nt-Wt). Samples of 2 µg total RNA isolated under acidic conditions were electrophoretically separated in a 14% urea-containing polyacrylamide gel and blotted. The blot was hybridized to a radiolabeled trnE-specific
probe. Deacylated tRNAs were obtained by subjecting total RNA to alkaline pH. (B) Northern blot analysis to investigate the processing status of two other plastid tRNAs. Samples of 1 µg total cellular RNA were electrophoretically separated under denaturing conditions and blotted. Blots were hybridized to radiolabeled probes specific to either trnF (top panel) or trnR (bottom panel). The ethidium bromide (EtBr)-stained gels prior to blotting are shown as control for equal loading. M: molecular weight marker.

**Figure 8.** Analysis of plastid translation in transplastomic *Nt*-EctMut and *Nt*-EctWt plants. Polysomes extracted from samples of 200 mg leaf tissue were separated by sucrose density gradient centrifugation. RNAs isolated from different gradient fractions were electrophoretically separated under denaturing conditions and blotted. The wedges above the blots indicate the increasing sucrose concentrations in the gradients. Blots were hybridized to radiolabeled probes specific to either *rbcL* or *psbD* transcripts. The ethidium bromide (EtBr)-stained gels prior to blotting are shown below each blot as control for equal loading. (A) Polysome loading analysis to compare translation rates in transplastomic *Nt*-EctWt and *Nt*-EctMut lines and wild-type (*Nt*-Wt) tobacco plants. (B) Identification of polysome-containing gradient fractions by analysis of puromycin-treated samples. Puromycin is an antibiotic that causes premature translation termination, thus revealing the gradient fractions that contain untranslated mRNAs (including mRNAs present in other ribonucleoprotein complexes but polysomes).

**Figure 9.** Analysis of ALA synthesis and GluTR accumulation levels in transplastomic *Nt*-EctWt and *Nt*-EctMut plants. (A) Comparison of ALA synthesis rates in *Nt*-EctWt, *Nt*-EctMut, and wild-type (*Nt*-Wt) tobacco plants. Error bars represent standard deviation (n=3, Student’s t-test, p<0.001) (B) Immunoblot analysis to determine GluTR levels in *Nt*-EctWt,
Nt-EctMut, and wild-type plants. Samples of 20 µg of extracted total cellular protein were separated by 10% SDS-PAGE, blotted, and hybridized to a GluTR-specific polyclonal antibody. Actin was analyzed as a control protein for equal loading.
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Figure 1. Physical maps of targeting regions in the tobacco plastid genome (ptDNA) and the modified regions in the transplastomic mutants generated by stable transformation of the chloroplast genome.

(A) Map of the trnE-encoding region in the tobacco plastid genome and changes introduced in Nt-EndWt and Nt-EndMut transplastomic lines. Genes above the line are transcribed from left to right, genes below the line are transcribed in the opposite direction. Relevant promoters are represented as green boxes, terminators (3' UTRs) as brown boxes, and the coding regions of trnE and the selectable marker gene aadA as light blue boxes. Note that, in both Nt-EndWt and Nt-EndMut transplastomic lines, trnE transcription is enhanced by insertion of an additional strong promoter (the rRNA operon promoter, Nt PrRN) upstream of the endogenous trnE gene promoter (PrRN). The Nt-EndMut transplastomic lines additionally carry the C56T mutation in trnE.

(B) Maps of the targeting region in the wild type (ptDNA) and the engineered regions in the transplastomic Nt-EctWt and Nt-EctMut lines that ectopically overexpress the wild-type tRNA^{psaB} or the mutated tRNA^{psaB} sequence (harboring the C56T mutation), respectively. Cr PpsaA: psaA promoter from Chlamydomonas reinhardtii, Nt PrRN: plastid rRNA operon promoter from N. tabacum, Cr TtpB: 3' UTR of the ttpB gene from C. reinhardtii, PrRN: promoter of trnE in N. tabacum. The oligonucleotides used for RFLP analysis and the expected fragment sizes are indicated. The hybridization sites of radiolabeled probes for RFLP analyses are shown as black horizontal bars.
Figure 2. RFLP analysis of transplastomic plants generated for overexpression of the wild-type *trnE* gene or the mutated *trnE* gene (giving rise to tRNA\(^{\text{Glu}}\)\(_{\text{C56U}}\)) in tobacco chloroplasts. (A) Southern blot analysis of *Nt-EndWt* and *Nt-EndMut* lines. Total DNA was digested with the restriction enzyme BamHI and fragments were detected by hybridization with a radiolabeled *trnE-trnD-trnY* locus-specific probe (cf. Figure 1A). (B) Analysis of *Nt-EctWt* and *Nt-EctMut* lines. Total DNA was digested with BamHI and fragments were detected by hybridization with the radiolabeled *psaB*-specific probe shown in Figure 1B. *Nt-Wt*: wild-type tobacco, M: molecular weight marker.
Figure 3. Phenotypes of transplastomic plants generated in this study in comparison to wild-type plants.
(A) Phenotype of Nt-EndMut transplastomic plants in tissue culture in comparison to a control plant (Nt-Wt: wild-type tobacco). The right panels show examples of misshapen leaves that are typical of mutations in chloroplast genes encoding essential components of the translation machinery (Ahlert et al., 2003; Rogalski et al., 2006; Rogalski et al., 2008b; Alkatib et al., 2012a; Alkatib et al., 2012b; reviewed in Tiller and Bock, 2014).
(B) Phenotype of Nt-EctWt, Nt-EctMut and Nt-EndWt transplastomic plants grown in soil. Upper row: 4 week-old plants (scale bar: 5 cm), lower row: 8 week-old plants (scale bar: 20 cm).
Figure 4. Seed assays to confirm homoplasmy of transplastomic plants. Wild-type (Nt-Wt) seeds and T1 seeds from Nt-EctWt, Nt-EctMut and Nt-EndWt were germinated on synthetic medium in the presence of spectinomycin. Absence of antibiotic-sensitive progeny indicates the homoplastic state of all transplastomic lines. +Spec: 500 mg/L spectinomycin in the culture medium, -Spec: control with no spectinomycin in the culture medium. Scale bar: 2 cm.
Figure 5. Northern blot analysis to compare transcript accumulation levels and RNA processing of trnE in transplastomic Nt-EctWt, Nt-EctMut and Nt-EndWt lines and wild-type (Nt-Wt) tobacco plants. 1 μg of total cellular RNA was electrophoretically separated under denaturing conditions and blotted. The blot was hybridized to a radiolabeled probe specific to the trnE gene (upper panel). The ethidium bromide (EtBr)-stained gel prior to blotting is shown as a control for equal loading (bottom panel). Dashes denote empty lanes. M, RNA size marker.
Figure 6. Northern blot analysis to detect incompletely processed precursors of tRNA$^{\text{Glu}}$ in transplastomic Nt-EctWt, Nt-EctMut and Nt-EndWt lines and wild-type (Nt-Wt) tobacco plants.

(A) Detection of trnE transcripts that remain unprocessed at their 5' end. The 5' leader sequence of tRNA$^{\text{Glu}}$ was used as radiolabeled probe.

(B) Detection of trnE transcripts that remain unprocessed at their 3' end. The 3' trailer sequence of tRNA$^{\text{Glu}}$ was used as probe. Samples of 3 μg total cellular RNA were electrophoretically separated under denaturing conditions and blotted. Blots were hybridized to radiolabeled probes (upper panels), and the ethidium bromide (EtBr)-stained gels prior to blotting are shown as controls for equal loading (lower panels). M: RNA size marker.

(C) Schematic representation of the results from similar RT-PCR analysis to identify the termini of incompletely processed trnE transcripts. See text for details. For sequences of individual clones from Nt-EctMut, see Supplemental Figure 1.
Figure 7. Aminoacylation of processed tRNA\(^{51}\) and unaffected processing of other plastid tRNAs in transplastomic \(Nt\)-EctWt, \(Nt\)-EctMut and \(Nt\)-EndWt plants.

(A) Northern blot analysis to determine the aminoacylation status of tRNA\(^{51}\) in transplastomic \(Nt\)-EctWt, \(Nt\)-EctMut and \(Nt\)-EndWt plants and wild-type tobacco (\(Nt\)-Wt). Samples of 2 \(\mu\)g total RNA isolated under acidic conditions were electrophoretically separated in a 14% urea-containing polyacrylamide gel and blotted. The blot was hybridized to a radiolabeled \(trnE\)-specific probe. Deacylated tRNAs were obtained by subjecting total RNA to alkaline pH.

(B) Northern blot analysis to investigate the processing status of two other plastid tRNAs. Samples of 1 \(\mu\)g total cellular RNA were electrophoretically separated under denaturing conditions and blotted. Blots were hybridized to \(trnE\) (top panel) or \(trnR\) (bottom panel). The ethidium bromide (EtBr)-stained gels prior to blotting are shown as control for equal loading. M: molecular weight marker.
**Figure 8.** Analysis of plastid translation in transplastomic *Nt-EctMut* and *Nt-EctWt* plants. Polysomes extracted from samples of 200 mg leaf tissue were separated by sucrose density gradient centrifugation. RNAs isolated from different gradient fractions were electrophoretically separated under denaturing conditions and blotted. The wedges above the blots indicate the increasing sucrose concentrations in the gradients. Blots were hybridized to radiolabeled probes specific to either *rbcL* or *psbD* transcripts. The ethidium bromide (EtBr)-stained gels prior to blotting are shown below each blot as control for equal loading.

(A) Polysome loading analysis to compare translation rates in transplastomic *Nt-EctWt* and *Nt-EctMut* lines and wild-type (*Nt-Wt*) tobacco plants.

(B) Identification of polysome-containing gradient fractions by analysis of puromycin-treated samples. Puromycin is an antibiotic that causes premature translation termination, thus revealing the gradient fractions that contain untranslated mRNAs (including mRNAs present in other ribonucleoprotein complexes but polysomes).
Figure 9. Analysis of ALA synthesis and GluTR accumulation levels in transplastomic Nt-EctWt and Nt-EctMut plants. (A) Comparison of ALA synthesis rate between Nt-EctWt, Nt-EctMut and wild-type (Nt-Wt) tobacco plants. Leaf extracts from leaf tissue incubated with levulinic acid were treated with modified Ehrlich's reagent and synthesis rate determined by colorimetric assay at 553 nm. (B) Western blot analysis to compare GluTR levels between Nt-EctWt, Nt-EctMut and wild-type (Nt-Wt) tobacco plants. The analysis was performed with 20 μg of total protein separated by 10% SDS-PAGE.
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