The Contributions of Wobbling and Superwobbling to the Reading of the Genetic Code

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Abstract
Reduced bacterial genomes and most genomes of cell organelles (chloroplasts and mitochondria) do not encode the full set of 32 tRNA species required to read all triplets of the genetic code according to the conventional wobble rules. Superwobbling, in which a single tRNA species that contains a uridine in the wobble position of the anticodon reads an entire four-fold degenerate codon box, has been suggested as a possible mechanism for how tRNA sets can be reduced. However, the general feasibility of superwobbling and its efficiency in the various codon boxes have remained unknown. Here we report a complete experimental assessment of the decoding rules in a typical prokaryotic genetic system, the plastid genome. By constructing a large set of transplastomic knock-out mutants for pairs of isoaccepting tRNA species, we show that superwobbling occurs in all codon boxes where it is theoretically possible. Phenotypic characterization of the transplastomic mutant plants revealed that the efficiency of superwobbling varies in a codon box-dependent manner, but—contrary to previous suggestions—it is independent of the number of hydrogen bonds engaged in codon-anticodon interaction. Finally, our data provide experimental evidence of the minimum tRNA set comprising 25 tRNA species, a number lower than previously suggested. Our results demonstrate that all triplets with pyrimidines in third codon position are dually decoded: by a tRNA species utilizing standard base pairing or wobbling and by a second tRNA species employing superwobbling. This has important implications for the interpretation of the genetic code and will aid the construction of synthetic genomes with a minimum-size translational apparatus.

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Introduction
32 tRNA species are needed to read all triplets of the genetic code according to the wobble rules proposed by Francis Crick [1]. However, reduced genomes, such as those of cell organelles (plastids and mitochondria) and some parasitic bacteria (e. g., mycoplasmas), contain fewer tRNA species than this minimal set [2]. In mitochondria of plants and of some lineages of protozoa, at least some of the missing tRNA species are imported from the cytosol [3,4] and the possibility of tRNA import from the cytosol has also been suggested for plastids of parasitic plants [5,6,7]. However, tRNA import is unlikely to account for the seemingly incomplete tRNA sets in human mitochondria (encoding only 22 tRNA species), plastids (encoding 30 tRNA species) and parasitic bacteria [8,9,10]. In these systems, extended wobbling is believed to facilitate translation with a reduced set of tRNA species [9]. Extended wobbling refers to the ability of a single tRNA species to read all four triplets in a codon family. For example, uridine 5'-oxyacetic acid at the wobble position enables a single tRNA to read all four triplets in a four-fold degenerate codon box [11]. Extended wobbling is also possible with an unmodified uridine in the wobble position of the anticodon and is also referred to as “four-way wobbling”, “hyperwobbling” or “superwobbling” [2,12,13,14]. Both theoretical considerations [1] and experimental data [10] support the idea that uridine in the wobble position of the anticodon can also engage in base-pairing interactions with U or G in the third codon position and, in this way promote reading of all four triplets in a codon family.

An alternative model of extended wobbling, referred to as the “two-out-of-three” reading hypothesis, was suggested by Lagerkvist [13,16]. This model defines “strong codons” as triplets with six hydrogen bonds formed by the first two bases of the codon in complementary base pairing with the anticodon. In contrast, the first two bases of “mixed codons” have five and the first two bases of “weak codons” have four hydrogen bonds participating in base pairing [16]. The “two-out-of-three” reading hypothesis proposes that “strong codons” (with only G-C interactions between the first two bases of the anticodon and the first two bases of the codon) can be read by relying on base pairing with the first two bases of the anticodon, without a significant contribution of the interaction between the third codon position and the wobble position of the anticodon [15]. Due to their lower number of hydrogen bonds, codon boxes with “mixed codons” would be less likely to be readable by a single tRNA species [16]. In contrast, if the U in the wobble position contributes to the stability of the codon-anticodon interaction, as implied by the superwobble hypothesis, boxes with “mixed codons” may be readable with similar efficiency as “strong codons”.

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Author Summary

Reduced genomes of parasitic bacteria, chloroplasts, and mitochondria do not encode the full set of 32 tRNAs required to read all triplets of the genetic code according to Francis Crick’s wobble rules. tRNAs with U in the wobble position of their anticodon might be able to make up for the deficit by pairing with any of the four bases at the third position of the codon via a mechanism called superwobbling. We have investigated the feasibility of superwobbling in the chloroplast genome of tobacco plants. We find that superwobbling occurs in all codon families where it is theoretically possible, demonstrating that all triplets with pyrimidines in third codon position are dually decoded: by a tRNA utilizing standard base pairing or wobbling and by a second tRNA employing superwobbling. We also show that the efficiency of superwobbling is variable in different codon families. Finally, our data reveal that the minimum number of tRNAs required to sustain protein biosynthesis is 25.

We have previously shown that the unmodified uridine in the wobble position of the plastid tRNA-Gly(UCC) allows decoding of all four glycine codons (GGA, GGC, GGG and GGU; [10]). Glycine codons are strong codons according to the “two-out-of-three” reading hypothesis. Here we have tested whether codon families of four mixed codons can be read by a single tRNA species. By systematically testing all tRNA species involved in reading four-fold degenerate codon boxes, we have established the complete set of decoding rules for the genetic system of the chloroplast. Moreover, we find that the efficiency of superwobbling varies in different codon boxes and is not directly correlated to the number of hydrogen bonds participating in the codon-anticodon interaction.

Results

Superwobbling in the ACN family of mixed codons

To examine the possibility of superwobbling in mixed codons, we investigated the pair of threonine tRNAs encoded in the plastid (chloroplast) genome of higher plants. According to the conventional wobble rules [1], the tRNA-Thr(GGU) encoded by the plastid trnT-UGU gene should decode the two threonine triplets with a cytosine in third codon position (ACC and ACU), whereas the tRNA-Thr(UGU) encoded by the trnT-UGU gene should read the two threonine triplets with a purine in third codon position (ACA and ACG). The tRNA-Thr(UGU) has an unmodified uridine in the wobble position of the anticodon in all organelles and bacterial species with reduced tRNA sets (mycoplasmas), where its sequence was determined (http://trnadb.bioinf.uni-leipzig.de/). The assumption that the tRNA-Thr(UGU) can superwobble, but the tRNA-Thr(GGU) cannot (because, for steric reasons, the purines A and G in third codon position should not be capable of base pairing with the purine base guanine in the wobble position of the anticodon), would lead to the following testable predictions: (i) the trnT-UGU gene should be non-essential, (ii) the trnT-UGU gene should be essential, and (iii) the tRNA-Thr(UGU) alone should be sufficient to sustain plastid translation, at least to some extent.

To test these predictions, we constructed knock-out alleles for both trnT genes in the plastid genome of the model plant tobacco (Nicotiana tabacum; Figure 1A–1D). The genes were disrupted by insertion of a selectable marker gene for chloroplast transformation (aadA) conferring spectinomycin resistance [17]. The knock-out alleles were then introduced into the plastid genome by particle gun-mediated transformation. Homologous recombination resulted in replacement of the wild-type allele with the knock-out allele. The resulting stably transformed plastid lines were subsequently referred to as ΔtrnT-UGU and ΔtrnT-GGU lines, respectively. Targeted disruption of the tRNA gene was confirmed by Southern blot analyses, which produced the expected restriction fragment length polymorphisms (Figure 1E, 1F).

Due to the polyploidy of the plastid genome, the knock-out of an essential gene results in balancing selection for two antagonistic genome types: the wild-type genome (expressing the essential gene but not the antibiotic resistance) and the transformed genome (expression the antibiotic resistance but not the essential gene). Consequently, a stable mix of both genome types (heteroplasmy) is observed under antibiotic selection [18,19,20]. This stable heteroplasmy was clearly seen in all ΔtrnT-UGU lines (Figure 1E), consistent with the prediction that this gene should be essential.

Growth in the absence of antibiotic selection releases the balancing selection in that it abrogates the selective pressure for maintenance of the transgenic plastid genome. This leads to predominant genome segregation towards the wild-type genome, which can be easily visualized by germinating seeds harvested from such plants on antibiotic-containing synthetic medium (where seedlings that lack the transgenic plastid genome bleach out; [21,22]). This was observed in all ΔtrnT-UGU lines (Figure 1G), providing further evidence of the essentiality of the trnT-UGU gene.

In contrast to trnT-UGU, the trnT-GGU gene turned out to be non-essential. Both DNA gel blot analyses and inheritance assays clearly demonstrated that homoplasmic knock-out plants had been obtained which lack residual wild-type copies of the plastid genome (Figure 1F, 1G). In addition, RNA gel blot analyses confirmed complete absence of tRNA-Thr(GGU) molecules from the ΔtrnT-GGU tobacco lines (Figure 1H), strongly suggesting that plastid translation can proceed in the absence of this tRNA species (and making it unlikely that this tRNA is imported from the cytosol). Thus, the tRNA-Thr(UGU) is necessary and sufficient to sustain translation, indicating that superwobbling in mixed codons is possible.

The single plastid alanine tRNA is essential

The plastid alanine tRNA-Ala(UCC) may exemplify obligatory superwobbling in that there is only a single alanine tRNA species encoded in the chloroplast genome and it has a uridine in the wobble position of the anticodon. The U remains unmodified in mitochondria of the fungus Neurospora crassa as well as in the reduced bacterial genomes of the genus Mycoplasma (http://trnadb.bioinf.uni-leipzig.de/), but may carry an unknown modification in plastids [23]. Given the essentiality of plastid translation [19,24] and the probable absence of tRNA import into plastids [8,10,22], this single alanine tRNA species is predicted to be encoded by an essential gene. To test this assumption, we constructed a knock-out allele for the trnA(UCC) gene and introduced it into the tobacco plastid genome (Figure S1). Molecular analysis of the generated ΔtrnA-UCC knock-out lines revealed stable heteroplasmy of the plastid genome under antibiotic selection (Figure S1D). Moreover, upon growth in soil, the heteroplasmic transplastomic plants displayed the characteristic leaf-loss phenotype (Figure S1F) caused by segregation into homoplasm for the knock-out of an essential gene. This leads to cessation of cell division and ultimately to cell death, which in turn produces misshapen leaves that lack parts of their blade [20,22,24]. Finally, the ΔtrnA-UCC knock-out lines also
showed a strong tendency to lose the transformed plastid genome in the absence of selective pressure, as evidenced by seed assays (Figure S1E). Taken together, these data suggest that the single plastid genome-encoded alanine tRNA is essential for translation and its loss cannot be complemented by tRNA import from the cytosol.

Superwobbling occurs in all four-fold degenerate codon boxes

To obtain a complete picture of the decoding rules in plastids and to determine the minimum tRNA set that is necessary and sufficient to facilitate translation, we next wanted to examine all cases in which wobbling and/or superwobbling theoretically

Figure 1. Targeted inactivation of the two plastid trnT genes. (A) Physical map of the trnT-UGU containing region in the tobacco plastid genome (ptDNA; [44]). Genes above the line are transcribed from the left to the right, genes below the line are transcribed in the opposite direction. Selected restriction sites used for cloning or RFLP analysis are indicated. The hybridization probe and the expected sizes of detected DNA fragments are also shown. Introns are represented by open boxes. (B) Map of the transformed plastid genome in ΔtrnT-UGU transplastomic plants. The selectable marker gene aadA (grey box) is inserted into the trnT-UGU gene in the same transcriptional orientation. (C) Physical map of the trnT-GGU containing region in the tobacco ptDNA. (D) Map of the transformed plastid genome in ΔtrnT-GGU plants. (E) RFLP analysis of ΔtrnT-UGU transplastomic lines. Independently generated transplastomic lines are designated by Arabic numerals following the tRNA gene name. All transplastomic lines remain heteroplasmic and show both the 1.9 kb wild type-specific hybridization band and the 3.1 kb band diagnostic of the transformed plastid genome. Wt: wild type. (F) RFLP analysis of ΔtrnT-GGU transplastomic plants. All lines are homoplasmic and show exclusively the 3.7 kb band diagnostic of the transgenic ptDNA. (G) Seed assays to confirm heteroplasmy of ΔtrnT-UGU plants and homoplasmy of ΔtrnT-GGU plants. Seeds were germinated in the absence or in the presence of spectinomycin. ΔtrnT-UGU plants produce mostly antibiotic-sensitive seedlings and a few antibiotic-resistant seedlings, as expected for a heteroplasmic situation. Moreover, many of the resistant seedlings are variegated indicating their composition of tissues possessing and tissues lacking the transgenic plastid genome. In contrast, the ΔtrnT-GGU lines produce homogeneous antibiotic-resistant progeny, confirming their homoplasmic status. (H) Analysis of tRNA-Thr(GGU) accumulation in the wild type, a heteroplasmic ΔtrnT-UGU line and a homoplasmic ΔtrnT-GGU line by northern blotting. Hybridization of electrophoretically separated RNA isolated from purified chloroplasts to a plastid trnT-GGU probe confirms complete lack of mature tRNA-Thr(GGU) in the ΔtrnT-GGU homoplasmic knock-out line, whereas its accumulation is unaltered in the heteroplasmic ΔtrnT-UGU line. Note accumulation of a ~1.5 kb hybridizing RNA species in the ΔtrnT-GGU line, which corresponds to the tRNA-Thr(GGU) disrupted with the aadA cassette. To control for RNA loading, part of the ethidium bromide-stained gel (containing the two largest 23S rRNA hidden break products) prior to blotting is also shown.

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allow a reduction of tRNA species. In addition to the GGN glycine box [10] and the ACN threonine box, this concerns two other four-fold degenerate boxes of mixed codons: the UCN serine box and the GUN valine box. For each of these boxes, two tRNA genes are present in the plastid genome and, if superwobbling occurs, the tRNA species with a U in the wobble position of the anticodon should suffice. Thus, the trnS-UGA and trnV-UAC genes should be essential and, if they are capable of superwobbling, the trnS-GGA and trnV-GAC genes should be non-essential. In addition to the UCN box, serine also has two codons in the AGN box (AGC and AGU). These triplets are most likely exclusively served by a third serine tRNA species, tRNA-Ser(GCU), and, therefore, the plastid gene encoding this tRNA species is also expected to be essential.

To test for decoding of serine and valine triplets by superwobbling, we analyzed the three plastid tRNA-Ser genes and the two tRNA-Val genes for their essentiality in knock-out experiments. Consistent with reading of UCU and UCC serine codons and GUU and GUC valine codons by superwobbling, both the trnS-GGA gene and the trnV-GAC gene were found to be non-essential (Figure 2 and Figure 3). The knock-out mutants were homoplasmic in Southern blot analyses (Figure 2C and Figure 3C), showed no detectable accumulation of the knocked-out tRNA species (Figure 2D, 2E and Figure 3D) and the seeds germinated as a homogeneous population of spectinomycin-resistant seedlings (Figure 2F, 2G and Figure 3E, 3F). Non-essentiality of the tRNA-Val(GAC) is in agreement with the existence of a deletion mutant in the plastid genome that comprises the trnV-GAC gene [25].

Figure 2. Targeted deletion of the plastid trnS-GGA gene. (A) Physical map of the region in the tobacco plastid genome harboring the trnS-GGA gene. Genes above the line are transcribed from the left to the right, genes below the line are transcribed in the opposite direction. The bent arrows indicate the borders of the transformation plasmid. Restriction sites used for RFLP analysis are indicated. The hybridization probe and the expected sizes of detected DNA fragments are also shown. Introns are represented by open boxes. (B) Map of the transformed plastid genome in ΔtrnS-GGA transplastomic lines. The aadA cassette is shown as grey box. (C) RFLP analysis of ΔtrnS-GGA plastid transformants. All lines are homoplasmic and show exclusively the 2.9 kb band diagnostic of the transplastome. Independently generated transplastomic lines are designated by Arabic numerals following the tRNA gene name, the following capital letter indicates an individual plant. Wt: wild type. (D) tRNA-Ser(GGA) accumulation in wild-type plants and ΔtrnS-GGA knock-out lines. Hybridization to the plastid trnS-GGA probe confirms complete absence of the tRNA from the transplastomic line. (F) Confirmation of homoplasy of the ΔtrnS-GGA lines by inheritance assays. Germination of seeds harvested from transplastomic plants on spectinomycin-containing medium results in a homogeneous population of green antibiotic-resistant seedlings. (G) Comparison with spectinomycin-sensitive wild-type seedlings. Antibiotic sensitivity is evidenced by the white phenotype of all seedlings.

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In contrast, the \textit{trnS-UGA}, \textit{trnS-GCU} and \textit{trnV-UAC} genes turned out to be essential (Figures S2, S3, S4). All mutant plants remained heteroplasmic, showed the typical leaf-loss phenotype upon growth in soil and only rarely transmitted the transformed plastid genome into the next generation (Figures S2, S3, S4).

Together, these results demonstrate that all four-fold degenerate codon boxes can be read by a single tRNA species. With a single exception (see analysis of the arginine tRNAs below), this tRNA species is always the one with uridine in the wobble position and, in all cases, is encoded by an essential gene. Consequently, the second tRNA species that, according to the classic wobble rules, should read the two triplets with a pyrimidine nucleotide in third codon position, is always non-essential.

Wobbling makes the \textit{trnL-CAA} gene dispensable

Organellar genomes make maximum use of Crick’s wobble rules [1] in that usually only a single tRNA species exists for each pair of codons (a pair being either the two triplets of a codon box with a purine in third position or the two triplets with a pyrimidine in third position). In the genomes of higher plant plastids, there is only a single exception: two distinct tRNA species exist for the two leucine codons UUA and UUG. These tRNAs are encoded by the \textit{trnL-UAA} and \textit{trnL-CAA} genes and the wobble positions of their anticodons are modified to 2'-O-methyluridine and 2'-O-methylcytidine, respectively [26]. Whether or not the base methylation in the wobble position enhances the specificity of decoding or perhaps even necessitates
two distinct tRNA species for the reading of UUA and UUG triplets, is not known.

To address these questions, we generated knock-out tobacco plants for the plastid \( \text{trnL-UAA} \) and \( \text{trnL-CAA} \) genes (Figure 4 and Figure S5). While homoplasmic knock-out lines were readily obtained for the \( \text{trnL-CAA} \) gene (Figure 4), the transplastomic \( \Delta \text{trnL-UAA} \) plants remained heteroplasmic and also fulfilled all other criteria of a mutant for an essential plastid gene (Figure S5). This indicates that the tRNA-Leu(UAA) can read both UUA and UUG triplets and suggests that 2'-O-methyluridine can wobble with G in the third codon position.

The four other leucine codons (in the CUN box) are most probably read by the third leucine tRNA species which is encoded by the plastid \( \text{trnL-UAG} \) gene. The tRNA-Leu(UAG) has an unmodified U in the wobble position (but an m\(^7\)G modification in position 36 of the anticodon loop; [26,23]). In the absence of a tRNA species with a GAG anticodon, the tRNA-Leu(UAG) should be capable of reading CUU and CUC triplets by superwobbling. As expected, transplastomic knock-out experiments confirmed essentiality of the \( \text{trnL-UAG} \) gene (Figure S6).

Previous work has demonstrated that 7-methylguanosine at the wobble position allows base pairing with all four nucleotides, A, C,
U and G [27]. In tRNA-Leu(UAG), the m7G modification is not in the wobble position, but in position 36, which base pairs with the first codon position. This raises the theoretical possibility that pairing of m7G with both C and U in first codon position could allow not only reading of CUN codons, but also of the two leucine codons in the UUN box, UUA and UUG. However, essentiality of the \( \Delta \text{trnL-UAG} \) (Figure S5) excludes this possibility and demonstrates that tRNA-Leu(UAG) cannot decode UUA and UUG triplets.

The two arginine tRNAs are essential

The plastid genome encodes two tRNAs for arginine, tRNA-Arg(UCC) and tRNA-Arg(AGC). The tRNA-Arg(UCC) should decode AGA and AGG triplets, whereas the four arginine triplets in the CGN box should be read by tRNA-Arg(AGC). The tRNA-Arg(AGC) carries an adenosine-to-inosine modification in the wobble position, which is thought to enable reading of all four nucleotides in the third position of CGN codons [possibly by “two-out-of-three” reading; [28,29]]. Considering the presence of only two tRNA species for the six arginine codons and the properties of their anticodons, there should be no possibility for further tRNA reduction by employing superwobbling. To confirm this assumption, we analyzed the two genes for plastid arginine tRNAs, \( \text{trnR-ACG} \) and \( \text{trnR-UCC} \), by reverse genetics (Figures S7 and S8). Molecular, and phenotypic analyses as well as inheritance assays revealed heteroplasmy of all transplastomic lines (Figures S7 and S8), thus suggesting essentiality of both arginine tRNA genes.

Anticodon-dependent efficiency of wobbling and superwobbling

In the course of this work, we identified four non-essential plastid tRNA genes: \( \text{trnT-GGU} \), \( \text{trnL-CAI} \), \( \text{trnS-GGA} \) and \( \text{trnV-GAC} \). To assess the phenotypic consequences of the lack of these tRNAs, we compared transplastomic mutant plants with wild-type plants under a variety of growth conditions. The \( \Delta \text{trnT-GGU} \) plants, initially generated to test whether or not reading of an entire fourfold degenerate codon box by a single tRNA species is also possible in boxes with “mixed codons”, displayed a strong mutant phenotype characterized by reduced leaf pigmentation and severely retarded growth (Figure 5A–5C). This is consistent with the conclusion drawn from a \( \text{trnG-GCC} \) knock-out that superwobbling is less efficient than classic wobbling [10]. Interestingly, while \( \Delta \text{trnG-GCC} \) seedlings were nearly white and showed strongly delayed greening both in sterile culture and in soil [10], \( \Delta \text{trnT-GGU} \) seedlings were light-green in both synthetic medium and soil (Figure 6A). Analysis of electrophoretically separated total soluble protein by Coomassie staining confirmed the underrepresentation of the (usually highly abundant) plastid-encoded proteins in the \( \Delta \text{trnT-GGU} \) and \( \Delta \text{trnS-GGA} \) plants, as evidenced by the high intensity background staining, which largely comes from lowly abundant cytosolic proteins (Figure 6A).

As the vast majority of plastid-encoded gene products is either directly or indirectly involved in photosynthesis, impaired plastid gene expression is usually well correlated with reduced photosynthetic activity [10,20,21,30]. We, therefore, measured chlorophyll content, chlorophyll a:b ratio and the maximum quantum efficiency of photosystem II (\( F_{v}/F_{m} \)) in the homoplasmic tRNA knock-out mutants. As expected, these analyses revealed a good correlation between the severity of the mutant phenotype (Figure 5) and the degree of impairment in these key photosynthetic parameters. When young developing leaves were examined (where the demand for chloroplast protein biosynthesis is highest due to the costly \textit{de novo} synthesis of the thyakoidal protein complexes; [20,21]), the \( \Delta \text{trnT-GGU} \) plants were most strongly affected in all three parameters, followed by the \( \Delta \text{trnS-GGA} \) plants. This was also the case in mature plants, with the notable exception that the chlorophyll content was higher in the \( \Delta \text{trnT-GGU} \) plants than in the \( \Delta \text{trnS-GGA} \) plants (Figure 6B). Taken together, these results confirm that the synthesis rate of photosynthetic protein complexes is significantly reduced in the chloroplasts of two mutants exhibiting pronounced mutant phenotypes. Our data also suggest that the effects of enforced superwobbling can be variable during development in a codon box-dependent manner.

To confirm that superwobbling results in incorporation of the correct amino acid into the polypeptide chain, we determined peptide sequences from the plastid genome-encoded large subunit of Rubisco (\( \text{RbcL} \)) in the mutant with the strongest phenotype, the \( \Delta \text{trnT-GGU} \) plants, using mass spectrometric methods [10]. The detected peptides covered 19 out of 23 threonine triplets in \( \text{RbcL} \), whose decoding is dependent on superwobbling in the \( \Delta \text{trnT-GGU} \) mutant (Figure S9). For 17 residues, correct incorporation of threonine was additionally verified by \textit{de novo} amino acid sequencing. At all positions, only threonine was found to be incorporated into the Rubisco protein, suggesting a high accuracy of codon reading by superwobbling.

Discussion

In the course of this work, we have generated a set of wobbling and superwobbling mutants that allowed us to derive the complete set of decoding rules for a prokaryotic system, the chloroplast of higher plants (Figure 7). The genetic system of higher plant chloroplasts has the important advantage over bacterial systems that reduced translational activity results in readily visible phenotypes that are easily quantifiable (Figure 5 and Figure 6). We have shown here that (i) superwobbling is not restricted to
strong codons (in the sense of the Lagerkvist hypothesis [15,16]), (ii) superwobbling in mixed codons can be even more efficient than superwobbling in strong codons, and (iii) the efficiency of superwobbling varies greatly in a codon box-dependent manner (and apparently does not correlate with the number of hydrogen bonds established between codon and anticodon).

The functional and evolutionary significance of tRNA gene reduction facilitated by superwobbling is exemplified by the decoding in the threonine (ACN) box (Figure 1 and Figure 5). The trnT-GGU gene shown to be non-essential in this study is missing from the plastid genomes of presumably all species in the family Geraniaceae [31]. Our data suggest that the loss of this gene can be largely compensated by superwobbling of the second threonine isoacceptor, the tRNA-Thr(UGU). However, although our results provide a mechanistic framework for the dispensability of tRNA-Thr(GGU), the reduced fitness of the ΔtrnT-GGU plants indicates that additional genetic adaptations were likely required in Geraniaceae evolution to make the loss of the trnT-GGU gene neutral. Whether these additional changes lie in the codon usage, the structure of the chloroplast ribosome or the structure and/or modification pattern of the tRNA-Thr(UGU), remains to be determined. It is noteworthy in this respect that, in addition to the anticodon itself, sequence features in the anticodon loop and stem (the so-called “extended anticodon”) have been suggested to contribute to the efficiency of decoding [32].

We have also investigated the curious case of the two leucine codons UUA and UUG, which represent the only codon pair that is read by two distinct tRNA species, tRNA-Leu(CAA) and tRNA-Leu(UAA). Thus, this appeared to be the only case of avoided wobbling and preferred decoding by standard Watson-Crick base pairing. However, non-essentiality of the trnL-CAA gene and lack of a discernible phenotype of the ΔtrnL-CAA plants suggests that

Figure 5. Phenotypes of transplastomic plants generated with knock-out constructs for the tRNA genes trnT-UGU, trnT-GGU, trnL-CAA, trnS-GGA, and trnV-GAC. (A) Growth phenotype of a ΔtrnT-GGU plant in comparison with a wild-type plant. Plants were grow from seeds in soil (with nitrogen-rich fertilizer) under ~100 μE m⁻² s⁻¹ light intensity and photographed after 12 weeks. The red arrow points to the ΔtrnT-GGU plant. (B) Flowering and seed set of a ΔtrnT-GGU plant after 50 weeks of growth under ~20 μE m⁻² s⁻¹ light intensity. (C) Growth of ΔtrnT-GGU and ΔtrnT-UGU plants in comparison with a wild-type plant after 6 weeks of growth on sucrose-containing synthetic medium (left pictures) and a subsequent 16 day growth period in soil (right pictures). Note the typical leaf-loss phenotype in the ΔtrnT-UGU plant indicating essentiality of the tRNA gene [19,20]. (D) Phenotypes of ΔtrnL-CAA, ΔtrnS-GGA and ΔtrnV-GAC transplastomic plants in comparison with a wild-type plant after growth for 13 weeks in soil under standard greenhouse conditions (average light intensity: 200 μE m⁻² s⁻¹). (E) Phenotype of ΔtrnL-CAA, ΔtrnS-GGA, ΔtrnT-GGU and ΔtrnV-GAC transplastomic plants in comparison with a wild-type plant after growth for 20 weeks under low-light conditions (~80 μE m⁻² s⁻¹).

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there is neither a requirement nor an obvious selective advantage of decoding UUA and UUG triplets by standard base pairing. This finding raises the question why a gene for tRNA-Leu(CAA) was retained in the plastid genome. We propose here that this is due to the location of the trnL-CAA gene in the inverted repeat region of the plastid genome. Due to copy correction by gene conversion [33,34], the inverted repeat region is known to have a much lower mutation rate than the two single-copy regions of the plastid genome. This effect makes gene losses by mutational degeneration less probable and may be responsible for the retention of the trnL-CAA gene. It could also explain retention of the trnV-GAC gene, despite its seeming expendability due to efficient superwobbling by the tRNA-Val(UAC) isoacceptor (Figure 5 and Figure 6). The trnV-GAC gene is also located in the inverted repeat region of the genome and, thus, copy correction by gene conversion may have prevented its evolutionary loss.

Figure 6. Analysis of plastid protein synthesis and photosynthetic parameters in ΔtrnL-CAA, ΔtrnS-GGA, ΔtrnT-GGU, and ΔtrnV-GAC plants. (A) Assessment of RbcL protein accumulation by western blotting using a specific anti-RbcL antibody. For semiquantitatitive analysis, a dilution series of wild-type protein was loaded. Consistent with the differences in the severity of the growth phenotypes (cf. Figure 5), the ΔtrnV-GAC and ΔtrnL-CAA mutants show the smallest reduction in RbcL accumulation, whereas the ΔtrnS-GGA mutant and especially the ΔtrnT-GGU mutant are more strongly affected, with RbcL levels being in the range of the 25% dilution of the wild-type sample in the ΔtrnT-GGU mutant. Reduced synthesis of chloroplast proteins is also apparent, when total plant protein samples are separated by gel electrophoresis and stained with Coomassie (lower panel). The two most abundant proteins (representing the large and small subunits of Rubisco, RbcL and RbcS) are indicated. Reduced abundance of chloroplast proteins in the ΔtrnS-GGA and ΔtrnT-GGU mutants also becomes evident by a stronger background staining (coming from a large number of lower abundant nuclear-encoded proteins). (B) Analysis of chlorophyll content, chlorophyll a:b ratio and the maximum quantum efficiency of photosystem II (Fv/Fm) in wild-type plants and homoplasmic transplastomic tRNA knock-out mutants. Datasets are shown for plants grown under ~80 μE m⁻² s⁻¹ light intensity. Young ΔtrnL-CAA, ΔtrnS-GGA and ΔtrnV-GAC plants were measured after 7 weeks of growth, ΔtrnT-GGU plants after 30 weeks (when they had reached a similar size as the other lines after 7 weeks). Mature ΔtrnL-CAA, ΔtrnS-GGA and ΔtrnV-GAC plants were measured after 20 weeks of growth, ΔtrnT-GGU plants were raised at ~20 μE m⁻² s⁻¹ for 40 weeks and then grown for 4 weeks at ~80 μE m⁻² s⁻¹. The fourth leaf from the top was analyzed. For each plant line, three different plants were measured. Fv/Fm represents the maximum quantum efficiency of PSII in the dark adapted state. The error bars indicate the standard deviation, statistically significant differences from the wild type (p<0.05; Student’s t-test) are indicated by asterisks.

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loss. Alternatively, these seemingly dispensable tRNA species could be maintained because they confer a selective advantage under specific environmental conditions, such as abiotic stress conditions known to impact plastid translation [20].

Our results reported here show that superwobbling can participate in the decoding of all four-fold degenerate codon boxes (Figure 7). Although the phenotypes of some of our tRNA knock-out mutants indicate that superwobbling is, at least in some codon boxes, less efficient than standard and wobble base pairing, our data suggest that superwobbling is also used in the presence of the tRNA isoacceptor that reads the codons with a pyrimidine in third position by Watson-Crick base pairing and wobbling. Thus, the tRNA isoacceptor with uridine in the wobble position significantly contributes to the reading of triplets with pyrimidines in third codon position in vivo. Based on these findings and previous analyses of essential plastid tRNA genes [35,10,22], we have compiled a revised table of the decoding rules in plastids (Figure 7). It shows the codon reading by Watson-Crick base pairing, wobbling and superwobbling, which is now supported by a complete set of experimental data for all codon boxes, in which superwobbling is theoretically possible. Essentiality of both tRNA-Ile species (encoded by the plastid trnI-GAU and trnI-CAU genes) for reading of the three isoleucine codons (AUU, AUC and AUA) was demonstrated previously [22]. Figure 7 also includes information on tRNA essentiality and relates the decoding rules to the codon usage in the plastid genome [36]. The latter analysis shows that the codon usage is much stronger correlated with the (generally high) AT content in the plastid DNA than with the mode of triplet reading. For example, in the three codon boxes read by a single tRNA species with a uridine in the wobble position of the anticodon (the CCN proline box, the CUN leucine box and the GCN alanine box), the triplets with U and A in third codon position are most frequently used, even though the triplet with U in third position can only be read by superwobbling. In all four-fold degenerate codon boxes, the tRNA species with uridine in the wobble position turned out to be essential (Figure 7; Figures S1, S2, S3, S4, S5, S6, S7, S8), indicating that two-out-of-three reading [15,16] by the isoacceptor with guanosine in the

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**Figure 7. Decoding of the 64 triplets of the genetic code in plastids.** Codon recognition by standard Watson-Crick base pairing, wobbling and/or superwobbling is indicated by the nucleotide in the wobble position of the anticodon of the tRNA species that can decode the triplet. Essential tRNA species are indicated in bold, non-essential tRNAs in normal font. The codon usage in plastids of *Nicotiana tabacum* is shown on a greyscale. Superscript numbers and indices indicate nucleoside modifications in the wobble position (\(N_3\)) of the anticodon of the tRNA species. 1: 2' - O-methyluridine [26]; 2: O-methylcytidine [26]; 3: unknown modification [23,45,46,47]; 4: inosine [28]; 5: lysidine [48,49]; 6: 5-carboxymethylaminonucleoside (cmnm5U; [50]; 7: 5-methylaminomethyl-2-thiouridine (mam5s2U; [51]; http://trnadb.bioinf.uni-leipzig.de/); *: modification status of the wobble uridine unknown (RNA sequence not determined); -: stop codon.

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wobble position is not possible. This is in agreement with previous reasoning that, for steric reasons, G-G and G-A base pairing in the wobble position is unlikely to occur [1]. Finally, our experimental data suggest that the minimum set of tRNAs comprises 25 tRNA species (Figure 7; additionally including the initiator tRNA^Met), rather than 26 as proposed recently [9]. This minimum number holds under the assumptions that the standard genetic code is used and no unorthodox tRNA modifications occur, while maximum use is made of wobbling and superwobbling.

### Materials and Methods

#### Plant material, growth conditions, and phenotypic analyses

To generate leaf material for chloroplast transformation experiments, tobacco (*Nicotiana tabacum* cv. Petit Havana) plants were raised under aseptic conditions on agar-solidified Murashige and Skoog medium (MS medium) containing 30 g/L sucrose [37]. Transplastomic lines were rooted and propagated on MS medium supplemented with 500 mg/L spectinomycin. For seed production and analysis of plant phenotypes, transplastomic plants were grown in soil under standard greenhouse conditions (relative humidity: 55%; day temperature: 25°C; night temperature: 20°C; diurnal cycle: 16 h light and 8 h darkness; light intensity ~200 μE m⁻² s⁻¹). For growth assays under low-light conditions, light intensities of 9,206, 2,060, and 200 μE m⁻² s⁻¹ were used. Inheritance patterns and seedling phenotypes were analyzed by germination of surface-sterilized seeds on MS medium with 500 mg/L spectinomycin.

#### Construction of plastid transformation vectors

Vectors for the targeted knock-out of tRNA genes were constructed by inserting or replacing the respective genes with an *aadA* cassette in cloned plastid DNA fragments. For knock-out of the *trnL*, *trnR* and *trnT* genes, a cassette comprising the *aadA* coding region from *Escherichia coli*, the *Prrn* promoter and the *psbA* 3’ UTR from *Nicotiana tabacum* [17] was used. For inactivation of the *trnL*, *trnS* and *trnV* genes, an *aadA* cassette derived from plasmid pLS1 (comprising the *aadA* coding region from *E. coli*, the *psbA* promoter and the 19S 3’ UTR from *Chlamydomonas reinhardtii* [21]) was used. All plastid transformation vectors constructed for this study were confirmed by restriction analysis and DNA sequencing.

For construction of vector p*Atm*-*UCG*, the genomic region surrounding the *tmL*-UCG gene (corresponding to nucleotide positions 135,448 to 138,542 of the *N. tabacum* genome) was subcloned as PstI/SalI fragment and inserted into the cloning vector pUC18 digested with the same enzymes. The resulting plasmid clone was digested with *AccI* (to linearize it within the *trnL*-UAG gene), treated with T4 DNA polymerase to generate blunt ends and ligated to the *aadA* cassette excised from pLS1 with SmaI. Vector p*AtmL*-*UAG* was constructed by amplifying the *tmL*-UAG containing region (corresponding to nucleotide positions 49,624 to 50,543) with primers P5-*tmUAAmod1* and P2-*tmUAAmot2* and the obtained PCR fragment was cloned into pUC18 digested with *HincII*. To replace the *tmL*-UAG gene with the *aadA* cassette, a PCR strategy was used. An initial PCR amplification was done with primers P3-*tmUAAmot1* and P3-*tmUAAmot2* resulting in amplification using the primer pair P5-*tmUAAaadAaaKas5* and P3-*tmUAAmot2*, resulting in a knock-out allele in which *tmL*-UAG is substituted by the *aadA* cassette. The amplification product was cloned into pUC18 digested with *HincII*. For construction of vector p*AtmR*-UCU, the respective genomic region (corresponding to nucleotide positions 7,924 to 10,206 of the *N. tabacum* plastid genome) was subcloned as StuI/Sall fragment and inserted into the cloning vector pBS KS+ digested with EcoRV and Sall. The resulting plasmid clone was digested with PstI and Sall and ligated to the genomic region surrounding the *tmR*-UCU gene (corresponding to nucleotide positions 10,207 to 12,066 and excised from a library clone with the same two enzymes). The resulting vector was linearized with *PstI* and ligated to the *aadA* cassette [17] excised from a plasmid clone with Ecl136I and DraI. For construction of the vector p*AtmR*-ACG, a fragment from the *N. tabacum* plastid genome (corresponding to nucleotide positions 131,485 to 133,119) was subcloned with *PstI* and *Sall* and inserted into the cloning vector pBS KS+ digested with *PstI* and EcoRV. The resulting plasmid clone was digested with *PwmI* to linearize it within the *tmR*-ACG gene, followed by insertion of the *aadA* cassette [17] at Ecl136I and DraI. For construction of the vector p*AtmS*-UGA, a plasmid clone containing the *tnG*-GCC and *tmS*-UGA genes (corresponding to nucleotide positions 36,834 to 38,889; [10]) was cut with *BsaI*, treated with T4 DNA polymerase to produce blunt ends and ligated to the *aadA* cassette excised from pLS1 with *SmaI*, resulting in disruption of the *tmS*-UGA gene. Vector p*AtmS*-*UGA* was constructed as follows: the region upstream of *tmS*-UGA (corresponding to nucleotide positions 46,432 to 47,125) was amplified with primers P5*-5’GlaAaS* and P3*-3’UAAmodS* (with overhangs that are complementary to the *aadA* cassette) using tobacco genomic DNA as template. A second PCR amplified the *aadA* cassette using the primers P5*-5’aadbAaS* and P3*-3’aadbAaS* (with overhangs that are complementary to the plastid DNA sequences flanking *tmS*-UGA) and pLS1 as template. A third PCR amplified the region downstream of *tmS*-UGA (corresponding to nucleotides 47,205 to 47,498) with primers P5*-5’tmS* and P3*-3’Sbu36*AaS* and tobacco genomic DNA as template. In a final PCR, the three generated PCR products were combined and used as templates for amplification with primers P5*-5’ClAaS* and P3*-3’Sbu36* to produce a knock-out allele of *tmS*-UGA, in which the trNA gene was replaced by the *aadA* cassette. The resulting amplification product was cloned into the pCR®2.1-TOPO vector (Invitrogen,
Darmstadt, Germany) via TA cloning. For construction of vector pΔnms-GCU, a plasmid containing the sequences flanking tmG-UAC and tmG-GCU (corresponding to nucleotide positions 7,839 to 9,994; [10]) was cut with PstI, then treated with T4 DNA polymerase to generate blunt ends and ligated to the aadA cassette excised from pLS1 with SmaI to disrupt the tmG-GCU gene. For construction of vector pΔnmt-T-UGU, the genomic region surrounding the tmn-T-UGU gene (corresponding to nucleotide positions 46,536 to 49,222) was subcloned as Xhol/EcoRI fragment and inserted into the similarly digested cloning vector PBS KS+.

The resulting plasmid was linearized with BplI, treated with T4 DNA polymerase to produce blunt ends and ligated to the aadA cassette excised as EagI/DraI fragment [17]. Vector pΔnmt-T-UGU was constructed by subcloning the genomic region surrounding the tmn-T-UGU gene (corresponding to nucleotide positions 32,276 to 33,823) as EcoRI/XbaI fragment into the similarly cut cloning vector PBS KS+. The resulting plasmid was linearized with NcoI, the overhanging ends were filled in with T4 DNA polymerase and ligated to the aadA cassette (as EagI/DraI fragment; [17]).

Vector pΔnmt-GAC was constructed by amplifying the genomic region containing tmV-GAC (corresponding to nucleotide positions 101,426 to 103,540) with the primer pair P5_trnVfor and P3_trnVrev using tobacco total DNA as template. The resulting PCR fragment was cloned into the SmaI site of pUC18. To excise the aadA cassette from plasmid pΔnms-GCU with BamHI and EcoRI. For the Δnmt-UAI1 lines, the probe (corresponding to positions 49,891 to 50,503) was excised from plasmid pΔnmt-UAI1 using PstI and XcmI. For RFLP analysis of Δnmt-UAI4 lines, the plastid genome sequence from positions 93,336 to 95,947 was excised from plasmid pΔnmt-UAI4 with PstI and EcoRI and used as probe. A probe for analysis of Δnms-GCU4 lines (covering the genomic region from nucleotide positions 34,311 to 34,769) was prepared by PCR amplification with primers P5_nmtS and P3_antisense (Table S2). To produce a probe for analysis of the Δnms-GCU4 lines, the sequence corresponding to nucleotide positions 8,725 to 9,750 was excised from plasmid pΔnms-GCU4 with MclI. For RFLP analysis of the Δnms-GCU4 lines, the probe corresponded to nucleotide positions 47,205 to 47,498 in the tobacco plastid genome and was prepared by PCR amplification with primers P5_5trnS and P3_Bsm36ItrnS. The probe for analysis of Δnmt-GAC4 lines (covering the genomic region from nucleotide positions 10,2769 to 10,4234) was generated by PCR amplification with primers P5_trnV-UAC4 and P3_trnV-UAC4-rev using genomic DNA as template. A third PCR amplified the region downstream of tmV-GAC with primers P5_trnV-GAC-aadA-hin and P3_trnV-GAC-aadA-rev (carrying overhangs complementary to the plastome sequences surrounding tmV-GAC). In a second PCR, the region upstream of tmV-GAC was amplified with primers P3_trnV-GAC-MfiI-hin and P3_trnV-GAC-MfiI-rev using genomic DNA as template. A PCR product was digested with MfeI and BseCI and inserted into the similarly cut pUC18 clone of the tmV-GAC genomic region. For construction of vector pΔnmt-V-UAC, a fragment from the N. tabacum plastid genome (corresponding to nucleotide positions 53,421 to 55,429) was amplified with primers P5_trnV-UAC-for and P3_trnV-UAC-rev and cloned into vector pUC18 digested with HincII. The resulting plasmid was digested with AccI (to linearize it within the tmV-UAC gene), treated with T4 DNA polymerase to produce blunt ends and ligated to the aadA cassette excised from pLS1 with SmaI.

Isolation of nucleic acids and hybridization procedures

Total plant DNA was extracted from plants grown under spectinomycin selection in vitro by a cetyltrimethylammoniumbromide-based method [41]. For RFLP analysis, DNA samples were treated with restriction enzymes, separated in 0.8–1% agarose gels, and blotted onto Hybond N nylon membranes (GE Healthcare, Buckinghamshire, UK). Total plant RNA for northern blot experiments was extracted using the peqGOLD TriFast reagent (Peqlab) following the manufacturer’s protocol. RNA samples were denatured, separated in denaturing formaldehyde-containing agarose gels (2%) and blotted onto Hybond N nylon membranes (GE Healthcare). For hybridization, [γ-32P]dCTP-labeled probes were produced by random priming [Multiprime DNA labeling kit; GE Healthcare]. Prior to labeling, DNA fragments were purified by agarose gel electrophoresis followed by extraction from excised gel slices using the Nucleospin Extract II kit (Macherey-Nagel). Hybridizations were performed at 65°C using standard protocols.

The probes for RFLP analysis of putative plastomonic lines were prepared from restriction fragments or amplified PCR products. For analysis of the Δnmt-UAI4 lines, the probe (corresponding to nucleotides 116,280 to 117,079 in the plastid genome of N. tabacum) was excised from plasmid pΔnmt-UAI4 with BamHI and EcoRI. For the Δnmt-UAI1 lines, the probe (corresponding to positions 49,891 to 50,503) was excised from pΔnmt-UAI1 using PstI and XcmI. For RFLP analysis of Δnmt-UAI4 lines, the plastid genome sequence from positions 93,336 to 95,947 was excised from plasmid pΔnmt-UAI4 with PstI and EcoRI and used as probe. A probe for analysis of Δnms-GCU4 lines (covering the genomic region from nucleotide positions 34,311 to 34,769) was prepared by PCR amplification with primers P5_nmtS and P3_antisense (Table S2). To produce a probe for analysis of the Δnms-GCU4 lines, the sequence corresponding to nucleotide positions 8,725 to 9,750 was excised from plasmid pΔnms-GCU4 with MclI. For RFLP analysis of the Δnms-GCU4 lines, the probe corresponded to nucleotide positions 47,205 to 47,498 in the tobacco plastid genome and was prepared by PCR amplification with primers P5_5trnS and P3_Bsm36ItrnS. The probe for analysis of Δnmt-V-GAC lines (covering the genomic region from nucleotide positions 10,2769 to 10,4234) was generated by PCR amplification with primers P5_trnV-UAC4 and P3_trnV-UAC4-rev using genomic DNA as template. A third PCR amplified the region downstream of tmV-GAC with primers P5_trnV-GAC-aadA-hin and P3_trnV-GAC-aadA-rev (carrying overhangs complementary to the plastome sequences surrounding tmV-GAC). In a second PCR, the region upstream of tmV-GAC was amplified with primers P3_trnV-GAC-MfiI-hin and P3_trnV-GAC-MfiI-rev using genomic DNA as template. A PCR product was digested with MfeI and BseCI and inserted into the similarly cut pUC18 clone of the tmV-GAC genomic region. For construction of vector pΔnmt-V-UAC, a fragment from the N. tabacum plastid genome (corresponding to nucleotide positions 53,421 to 55,429) was amplified with primers P5_trnV-UAC-for and P3_trnV-UAC-rev and cloned into vector pUC18 digested with HincII. The resulting plasmid was digested with AccI (to linearize it within the tmV-UAC gene), treated with T4 DNA polymerase to produce blunt ends and ligated to the aadA cassette excised from pLS1 with SmaI.

Plastid transformation and selection of transplastomic lines

For plastid transformation, young leaves from asceptically grown tobacco plants were bombarded with plasmid-coated 0.6-μm gold particles using a helium-driven biolistic gun (PDS1000He; BioRad). Primary spectinomycin-resistant lines were selected on plant regeneration medium containing 500 mg/L spectinomycin [17,39]. Spontaneous spectinomycin-resistant plants were eliminated by double selection tests on medium containing both spectinomycin and streptomycin (500 mg/L each; [17,40]). Several independent transplastomic lines were produced for each construct and subsequently subjected to three to four additional rounds of plant regeneration on spectinomycin-containing medium to enrich the transformed plastid genome and select for homoplasmy [20].
Chloroplast isolation and RNA extraction from purified chloroplasts

30–40 g tobacco leaves (harvested from 4–5 week old plants) were homogenized in 300 ml Grinding Buffer (GB; 0.4 M sorbitol, 50 mM HEPES, 2 mM EDTA, 0.1% BSA, 0.1% isoascorbat, pH 8.0) in a Waring Blender. The homogenate was filtrated through four layers of absorbent gauze and one layer of Miracloth (Calbiochem, San Diego, CA). The filtrate was centrifuged at 2,500 × g for 3 min and the chloroplast pellet was dissolved in 1–2 ml GB, loaded onto Percoll gradients (40%–80% v/v in GB; Percoll®; GE Healthcare) and centrifuged at 13,300 × g for 14 min. The intact chloroplasts were collected, washed with GB and pelleted by centrifugation at 2,000 × g for 2 min. RNA was extracted with peqGOLD TriFast (Peqlab) following the manufacturer’s instructions.

Protein extraction, Western blot analysis, and mass spectrometric analysis

Total soluble protein was extracted from leaf samples using published procedures [42]. The protein concentration of the extracts was determined using the Bradford assay (Roth, Karlsruhe, Germany) and known concentrations of bovine serum albumin as standard. Protein samples were separated by electrophoresis in 15% SDS-containing polyacrylamide gels, and the proteins in the gel were directly visualized by Coomassie blue staining. For immunoblot analysis, protein gels were blotted onto polyvinylidene fluoride (PVDF) membranes (Hybond-P; GE Healthcare). For detection of the large subunit of Rubisco, a polyclonal antibody (raised in rabbits; Agrisera, Vännäs, Sweden) was used. The chemiluminescence detection reaction was carried out with the ECL Plus western blotting detection system (GE Healthcare). For detection of the large subunit of Rubisco, a polyclonal antibody (raised in rabbits; Agrisera, Vännäs, Sweden) was used. The chemiluminescence detection reaction was carried out with the ECL Plus western blotting detection system (GE Healthcare). Mass spectrometric determination of Rubisco peptide sequences was performed as described previously [10].

Physiological measurements

Chlorophyll contents were determined in 80% (v/v) acetone using published methods [43]. PSII quantum efficiency (Fv/Fm) was determined using a pulse-amplitude modulated fluorimeter (DUAL-PAM-100; Heinz Walz GmbH). Prior to the measurements, plants were dark adapted for 20 min.

Supporting Information

Figure S1  Targeted disruption of the plastid trnA-UGC gene. (A) Physical map of the trnA-UGC-containing region in the tobacco plastid genome (pDNA). Wt: wild type. (B) Map of the transformation product in ΔtrnA-UGC. The aadA cassette is shown as grey box. (C) Map of the recombination product in ΔtrnA-UGC-Rc transplastomic lines. As shown in earlier studies, recombination occurs between the 3′ UTR of the aadA cassette and that of the endogenous psbA gene [19,20]. Genes above the line are transcribed from the left to the right, genes below the line are transcribed in the opposite direction. Selected restriction sites used for cloning and RFLP analysis are indicated. The hybridization probe and the expected sizes of detected DNA fragments are also shown. Introns are represented by open boxes. (D) RFLP analysis of ΔtrnA-UGC plastid transplants. The plastomic lines remain heteroplasmic and show both the 3.9 kb restriction fragment and the S.6 kb band diagnostic of the transplastome. Wt: wild type. (E) Leaf phenotype of a typical transplastomic plant. Arrows point to misshapen leaves. (F) Segregation analysis of a ΔtrnS-UGA plant. Seeds from a selfed transplastomic plant were sown on spectinomycin-containing synthetic medium. Spectinomycin sensitivity of most seedlings suggests a strong tendency to lose the transgenic plastid genome in the absence of antibiotic selection. A single spectinomycin-resistant seedling (that has retained the transplastome) is indicated by the arrow.

Figure S2  Targeted disruption of the plastid trnS-UGA gene. (A) Physical map of the region in the tobacco plastid DNA containing the trnS-UGA gene. Genes above the line are transcribed from the left to the right, genes below the line are transcribed in the opposite direction. The bent arrows indicate the borders of the transformation plasmid. Restriction sites used for RFLP analysis are indicated. The hybridization probe and the expected size of the detected restriction fragment are also shown. (B) Map of the transplastome produced with plastid transformation vector pΔtrnS-UGA. The aadA marker gene is shown as grey box. (C) RFLP analysis of ΔtrnS-UGA transplastomic plants. The plastomic lines remain heteroplasmic and show both the wild type-specific 5 kb restriction fragment and the 6.6 kb band diagnostic of the transplastome. Wt: wild type. (D) Leaf phenotype of a typical heteroplasmic ΔtrnS-UGA plant. Arrows point to misshapen leaves.

Figure S3  Targeted inactivation of the plastid trnS-GCU gene. (A) Physical map of the region in the tobacco plastid genome containing trnS-GCU. Genes above the line are transcribed from the left to the right, genes below the line are transcribed in the opposite direction. The bent arrows indicate the borders of the transformation vector. The restriction sites used for RFLP analysis are indicated. The hybridization probe and the expected size of detected DNA fragment are also shown. Introns are represented by open boxes. (B) Map of the transformed plastid genome obtained with plastid transformation vector pΔtrnS-GCU. The aadA selectable marker gene is shown in grey. (C) RFLP analysis of ΔtrnS-GCU plastid transplants. The plastomic lines remain heteroplasmic and show both the 3.9 kb hybridization band diagnostic of the wild-type plastid genome and the 5.5 kb band diagnostic of the transplastome. Wt: wild type. (D) Leaf-los plante phenotype of a typical heteroplasmic ΔtrnS-GCU plant. A misshapen leaf is indicated by the arrow. (E) Inheritance assay of a ΔtrnS-GCU plant. An example of a spectinomycin-resistance seedling that has retained copies of the transplastome is indicated by the arrow.

Figure S4  Targeted inactivation of the plastid tmV-UAC gene. (A) Physical map of the region in the tobacco plastid genome containing tmV-UAC. Genes above the line are transcribed from the left to the right, genes below the line are transcribed in the opposite direction. The bent arrows indicate the borders of the transformation plasmid. The restriction sites used for RFLP analysis are indicated. The hybridization probe and the expected size of the detected DNA fragment are also shown. The introns in tmV-UAC are represented by open boxes. (B) Map of the transformed plastid genome (transplastome) produced with plastid transformation vector pΔtmV-UAC. The aadA marker is shown as
grey box. (C) RFLP analysis of ΔtrnV-UAC plastid transformants. The transplastomic lines remain heteroplasmic and show a stable ratio of the wild-type-specific 1.8 kb band and the 3.3 kb band diagnostic of the transplastome. Wt: wild type. (D) Phenotype of a typical heteroplasmic ΔtrnV-UAC plant. The arrow points to an example of a misshapen leaf that lacks part of the leaf blade. (E) Inheritance assay of a ΔtrnV-UAC plant. Spectinomycin-resistance seedlings that have retained copies of the transplastome are green, seedlings that have lost all copies of the transgenic plastid genome are white.

**Figure S5** Targeted inactivation of the plastid trnL-UAA gene. (A) Physical map of the region in the tobacco plastid genome (ptDNA) containing the trnL-UAA gene. Genes above the line are transcribed from the left to the right, genes below the line are transcribed in the opposite direction. The bent arrows indicate the borders of the transformation plasmid. Restriction sites used for RFLP analysis are indicated. The hybridization probe and the expected size of the detected DNA fragment are also shown. The intron in the trnL-UAA gene is represented by an open box. (B) Map of the transformed plastid genome (transplastome) produced with plastid transformation vector pΔtrnL-UAA. The aadA cassette is shown as grey box. (C) RFLP analysis of ΔtrnL-UAA plastid transformants. The transplastomic lines remain heteroplasmic and show a stable ratio of the 1.6 kb hybridizing fragment diagnostic of the wild-type ptDNA and the 3.1 kb band diagnostic of the transformed ptDNA. Wt: wild type. (D) Phenotype of a typical heteroplasmic ΔtrnL-UAA plant. The arrow points to an example of a misshapen leaf. (E) Segregation analysis of a ΔtrnL-UAA plant. Seeds from a selfed transplastomic plant were sown on spectinomycin-containing synthetic medium. The transplastome is lost from most seedlings as evidenced by their white phenotype upon germination in the absence of antibiotic selection. An example of a selfed transplastomic plant was sown on spectinomycin-containing synthetic medium. Spectinomycin-resistant seedlings that have retained copies of the transplastome are green, seedlings that have lost all copies of the transgenic plastid genome are white.

**Figure S6** Targeted disruption of the plastid trnL-UAG gene. (A) Physical map of the region in the tobacco plastid genome containing the trnL-UAG gene. Genes above the line are transcribed from the left to the right, genes below the line are transcribed in the opposite direction. The bent arrows indicate the borders of the transformation plasmid. Restriction sites used for RFLP analysis are indicated. The hybridization probe and the expected size of the detected DNA fragment are also shown. (B) Map of the transformed plastid genome (transplastome) produced with plastid transformation vector pΔtrnL-UAG. The aadA selectable marker cassette is shown as grey box. (C) RFLP analysis of ΔtrnL-UAG plastid transformants. The transplastomic lines remain heteroplasmic and show a stable ratio of the 1.6 kb hybridizing fragment diagnostic of the wild-type ptDNA and the 3.1 kb band diagnostic of the transformed ptDNA. Wt: wild type. (D) Phenotype of a typical heteroplasmic ΔtrnL-UAG plant. The arrow points to an example of a misshapen leaf. (E) Segregation analysis of a ΔtrnL-UAG plant. Seeds from a selfed transplastomic plant were sown on spectinomycin-containing synthetic medium. Spectinomycin-resistance seedlings that have retained copies of the transplastome are green, seedlings that have lost all copies of the transgenic plastid genome are white.

**Figure S7** Targeted disruption of the plastid trnR-UCU gene. (A) Physical map of the region in the tobacco plastid genome containing the trnR-UCU gene. Genes above the line are transcribed from the left to the right, genes below the line are transcribed in the opposite direction. Selected restriction sites used for cloning and RFLP analysis are indicated. The hybridization probe and the expected sizes of detected DNA fragments are also shown. Introns are represented by open boxes. (B) Map of the transformed plastid genome (transplastome) produced with plastid transformation vector pΔtrnR-UCU. The aadA cassette is shown as grey box. (C) RFLP analysis of ΔtrnR-UCU plastid transformants. The transplastome is lost from most seedlings as evidenced by their white phenotype upon germination in the absence of antibiotic selection. An example of a selfed transplastomic plant was sown on spectinomycin-containing synthetic medium. The transplastome is lost from most seedlings as evidenced by their white phenotype upon germination in the absence of antibiotic selection. The 7.3 kb band appearing in addition to or instead of the expected 4.7 kb transplastomic fragment in the transformants is the result of flip-flop recombination between the 3’ UTR of the aadA and that of the endogenous psbD gene [19]. Wt: wild type. (E) Inheritance assay of a ΔtrnR-ACG plant. Spectinomycin-resistance seedlings that have retained the transplastome are green on antibiotic-containing medium. The phenotype of heteroplasmatic ΔtrnR-ACG lines is shown in Figure S8E.

**Figure S8** Targeted disruption of the plastid trnR-UCU gene. (A) Physical map of the region in the tobacco plastid genome containing the trnR-UCU gene. Genes above the line are transcribed from the left to the right, genes below the line are transcribed in the opposite direction. Selected restriction sites used for cloning and RFLP analysis are indicated. The hybridization probe and the expected size of detected DNA fragments are also shown. Introns are represented by open boxes. (B) Map of the transformed plastid genome (transplastome) produced with plastid transformation vector pΔtrnR-UCU. The aadA marker cassette is shown as grey box. (C) RFLP analysis of ΔtrnR-UCU plastid transformants. The transplastomic lines remain heteroplasmic and show both the 5.4 kb wild-type-specific hybridization band and the 6.6 kb band diagnostic of the transplastome. Wt: wild type. (D) Inheritance assay of a ΔtrnR-UCU plant. Spectinomycin-resistance seedlings that have retained the transplastome are green on antibiotic-containing medium. (E) Phenotype of heteroplasmatic ΔtrnR-UCU and ΔtrnR-ACG plants. A wild-type plant (left), two ΔtrnR-UCU plants (middle) and two ΔtrnR-ACG plants grown under greenhouse conditions are shown. Misshapen leaves with missing sectors indicate essentiality of the trnR-UCU and ΔtrnR-ACG genes.

**Figure S9** Verification of threonine incorporation into the large subunit of Rubisco (RbcL) in the ΔtrnT-GGU plants by MS/MS. (A) Detected RbcL peptides and threonine residues dependent on superwobbling in the ΔtrnT-GGU mutant. The RbcL peptides detected by mass spectrometry are marked in bold. The threonines encoded by ACU and ACC codons are indicated in blue and green, respectively. These threonines are encoded by tRNA-Thr(GGU) in the wild type, but are read by tRNA-Thr(UGU) in the ΔtrnT-GGU transplastomic lines using superwobbling. In all detected peptides, threonine was correctly incorporated. (B) Confirmation of threonine incorporation into RbcL by de novo sequencing. Example of a y-ion series from a peptide containing an ACC-encoded threonine from the wild-type sample. (C) Example of a y-ion series from a peptide containing an ACC-encoded threonine from a ΔtrnT-GGU plant.

**Table S1** Numbers of codons in the plastid genome affected by the knock-out of the four non-essential tRNA genes trnL-CUA, trnS-
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