Transfer of Plastid DNA to the Nucleus Is Elevated during Male Gametogenesis in Tobacco¹[OA]

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In eukaryotes, many genes were transferred to the nucleus from prokaryotic ancestors of the cytoplasmic organelles during endosymbiotic evolution. In plants, the transfer of genetic material from the plastid (chloroplast) and mitochondrion to the nucleus is a continuing process. The cellular location of a kanamycin resistance gene tailored for nuclear expression (35SneoSTLS2) was monitored in the progeny of reciprocal crosses of tobacco (Nicotiana tabacum) in which, at the start of the experiments, the reporter gene was confined either to the male or the female parental plastid genome. Among 146,000 progeny from crosses where the transplastomic parent was male, 13 transposition events were identified, whereas only one atypical transposition was identified in a screen of 273,000 transplastomic ovules. In a second experiment, a transplastomic β-glucuronidase reporter gene, tailored to be expressed only in the nucleus, showed frequent stochastic expression that was confined to the cytoplasm in the somatic cells of several plant tissues. This gene was stably transferred in two out of 98,000 seedlings derived from a male transplastomic line crossed with a female wild type. These data demonstrate relocation of plastid DNA to the nucleus in both somatic and gametophytic tissue and reveal a large elevation of the frequency of transposition in the male germline. The results suggest a new explanation for the occurrence of uniparental inheritance in eukaryotes.

The plastid (chloroplast) genome of higher plants has been reduced to approximately 130 genes, while its cyanobacterial ancestor is estimated to have contained more than 3,000 genes (Timmis et al., 2004). Most erstwhile prokaryotic genes of the ancestor were either lost or transferred to the nucleus during more than a billion years of endosymbiotic evolution (Timmis et al., 2004) such that plastid biogenesis is heavily dependent upon nuclear genes. Thousands of nuclear genes in Arabidopsis (Arabidopsis thaliana) are derived from the endosymbiont genome (Martin et al., 2002), and, while many of these make products that enter the plastids, many others support cellular processes that are unrelated to plastid biogenesis and biochemistry. A similar scenario underlies mitochondrial evolution (Timmis et al., 2004). Gene transfer is initiated by the transposition of endosymbiotic nucleic acid sequences to the nucleus where they may be retained in large numbers as nonfunctional numts and nupts (nuclear integrants of mitochondrial and plastid DNA [ptDNA], respectively) or, much more rarely, they evolve into novel nuclear genes concerned with organelle biogenesis or with new cellular or extracellular functions (Martin et al., 2002).

Two independent experimental estimates (Huang et al., 2003a; Stegemann et al., 2003) of the frequency of de novo nupt formation in tobacco (Nicotiana tabacum) differ by more than two orders of magnitude. Both used a similar experimental approach involving transposition and recombinant DNA in tobacco (Nicotiana tabacum) that required nuclear relocation to confer kanamycin resistance. A significant difference between these reports is that the first experiments (Huang et al., 2003a) measured transposition in a male transplastomic parent by screening the progeny of crosses to wild-type females for kanamycin resistance and did not involve selection pressure at the time of transposition. The frequency of transposition was measured as the proportion of kanamycin-resistant progeny, and one in approximately 16,000 pollen grains carried an active nuclear copy of neo. The second experiments (Stegemann et al., 2003) regenerated kanamycin-resistant plants from somatic cells of transplastomic plants that were screened under antibiotic selection. The frequency of transposition was calculated using an estimate of the total number of cells in the screened leaf explants and the number of kanamycin-resistant plants recovered. A transposition event was estimated to occur once in every five million cells screened by this procedure.

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These two experimental measurements were derived from different tissues, raising the possibility of tissue-specific rates of ptDNA relocation. Male gametes of most angiosperm species undergo a programmed elimination of plastids during pollen development (Yu and Russell, 1994; Nagata et al., 1999), a process that underpins maternal inheritance of organellar genes (Mogensen, 1996). Here, we show that transfer of ptDNA to the nucleus takes place far more frequently in the male germline than in the female germline. We also demonstrate a high frequency of transfer in somatic tissues.

RESULTS

The Frequency of Plastid-to-Nucleus DNA Transposition Is Lower in the Female Germline Than in the Male Germline

In a previous study (Huang et al., 2003a), the frequency of ptDNA transfer to the tobacco nucleus was determined by analysis of progeny derived from fertilization of wild-type plants with pollen from a plastomastic line (tp7). Contained within the tp7 plastid genome was a 35SneoSTLS2 transgene tailored specifically for nuclear expression (Fig. 1, A and C). This transgene consisted of a region encoding neomycin phosphotransferase under the regulatory control of the cauliflower mosaic virus 35S promoter and terminator (Benfrey and Chua, 1990), which allowed selection of nuclear integration events by screening for kanamycin resistance during early seedling growth. A nuclear intron (STLS2), designed to prevent translation of a functional protein even if unexpected transcription occurred in the plastid, was inserted into the neo reading frame (Fig. 1A). When tp7 was used as a male parent in a cross to wild-type tobacco, one in approximately 16,000 progeny were resistant to kanamycin, indicative of multiple independent transfers of this plastid transgene to the nucleus and subsequent nuclear expression (Huang et al., 2003a). The selected kanamycin-resistant plants each carried a copy, or sometimes several copies, of 35SneoSTLS2 integrated into chromosomal DNA that, with some exceptions (Huang et al., 2003a), behaved as single Mendelian loci in genetic studies.

To determine whether the frequency of maternal plastid-to-nucleus DNA transfer was similar to that in the male germline, crosses were performed using the tp7 transplastomic as the female parent (tp7♂ × NtBAR/GUS♀), i.e., the reverse direction of that used by Huang et al. (2003a). The male parent used in this reciprocal cross was homozygous for a BAR/GUS nuclear transgene, which acted as a nuclear marker to confirm that any emerging kanamycin-resistant progeny were derived from the cross. A complication in screening progeny from the cross in which tp7 was used as the female parent was that 35SneoSTLS2 in the tp7 transplastome conferred partial kanamycin resistance (Huang et al., 2003a; Stegemann et al., 2003), and this was maternally inherited by all the progeny, thus precluding an identical screen to that used previously (Huang et al., 2003a). To overcome this background resistance, the concentration of kanamycin used in screening the reciprocal cross progeny was increased from 150 μg mL⁻¹ to 300 μg mL⁻¹ (Stegemann et al., 2003). To validate effective selection at this antibiotic concentration, emasculated tp7 flowers were fertilized with pollen from plants hemizygous for newly transposed 35SneoSTLS2 in their nuclear genomes (kr18 described previously [Huang et al., 2003a], and kr2.2, kr2.3, kr2.7, kr2.9, and kr2.10 described below; kr, kanamycin resistant). In each cross, all progeny contained the maternal tp7 transplastome, while one-half of the progeny were expected to also contain a nuclear 35SneoSTLS2 copy inherited from the hemizygous paternal kanamycin-resistant parent. For each of these crosses, 13 seeds were plated at defined positions among a high density of tp7 seeds on medium containing 300 μg mL⁻¹ kanamycin. After 3 months, progeny containing both plastid and nuclear copies of 35SneoSTLS2 from all genotypes except kr2.3 were clearly distinguished at this antibiotic concentration from seedlings exhibiting the background resistance conferred by the transplastome alone (Fig. 2A). While it is possible that the kr2.3 nuclear genotype is not detectable above the background resistance of the transplastome, a more likely explanation is that none of the progeny contained a nuclear copy of 35SneoSTLS2, because this genotype is known to be highly unstable (A.E. Sheppard and J.N. Timmis, unpublished data). Nevertheless, it can be concluded that this screen is very close in sensitivity to that used previously for crosses where the transplastomic parent was male.
Having established the ability of the selection regime to recognize transposition events efficiently, approximately 273,000 seeds from \( \text{tp7} \) \( \varphi \times \text{NtBAR/GUS} \) crosses were screened under these conditions. After 3 months, no kanamycin-resistant seedlings similar to the positive controls were observed. However, in contrast to the progeny of the reverse cross (Huang et al., 2003a), some of the plants were not killed by the antibiotic, though they showed severe growth restriction (Fig. 2A). After a further 6 to 9 months, a number of seedlings were still alive, but these lacked the clear phenotype associated with a nuclear 35S\( \text{neo}^{STLS2} \) in the transplastomic background (Fig. 2A). The most promising candidates for true kanamycin resistance (green seedlings that were clearly larger than other seedlings on the same plate) were tested by back-crossing to female wild type to remove plastid-localized 35S\( \text{neo}^{STLS2} \) genes. Of these, only a single plant (kr3.1) produced a proportion of kanamycin-resistant progeny, and these appeared to be chimeric for kanamycin resistance, containing both resistant and susceptible sectors on their cotyledons (Fig. 2C), suggesting unstable expression of \( \text{neo} \) in the nucleus. Consequently, it was not possible to determine unequivocally the proportion of kanamycin-resistant progeny, because seedlings with only very small resistant sectors could not be clearly distinguished from fully sensitive seedlings. Positive GUS expression in progeny of kr3.1 confirmed that it had resulted from a cross with the homozygous NtBAR/GUS parent (data not shown). PCR analysis confirmed the presence of \( \text{neo} \) in kanamycin-resistant plants (data not shown), indicating that kr3.1 arose from transposition of the plastid-encoded \( \text{neo} \) to the nucleus. Nevertheless, the phenotype of the progeny of this plant was atypical compared with those reported earlier (Huang et al., 2003a), and the antibiotic selection time required for its identification was much greater than that necessary for the positive controls (Fig. 2A).

Given the remarkably low frequency of transplastomic 35S\( \text{neo}^{STLS2} \) transposition in the female germ-line (one atypically resistant plant in 273,000), screening of the original cross undertaken by Huang et al. (2003a) was repeated using the parental lines described above to confirm elevated transfer rates in pollen. That is, pollen from the \( \text{tp7} \) transplastomic line was used to fertilize NtBAR/GUS plants, and seedlings derived from this cross were screened for kanamycin resistance using 150 \( \mu \text{g mL}^{-1} \) kanamycin. Ten

\[ \text{Figure 2. Detection and analysis of ptDNA transfer to the nucleus.} \]
\[ \text{A, Four kanamycin-resistant plants from the segregating progeny of } \text{tp7} \] \( \varphi \times \text{kr2.7} \) among 2,000 progeny of self-fertilized \( \text{tp7} \) grown for 3 months on medium containing 300 \( \mu \text{g mL}^{-1} \) kanamycin. B, One kanamycin-resistant plant (kr2.4) among 2,000 progeny of NtBAR/GUS \( \varphi \times \text{tp7} \) grown for 11 weeks on medium containing 150 \( \mu \text{g mL}^{-1} \) kanamycin. C, Progeny of wild-type \( \varphi \times \text{kr3.1} \) grown for 6 weeks on medium containing 150 \( \mu \text{g mL}^{-1} \) kanamycin. D, Progeny of wild-type \( \varphi \times \text{kr4.1} \) grown for 3 weeks on medium containing 150 \( \mu \text{g mL}^{-1} \) kanamycin. E to I, Leaf (E–G), anther (H), and root (I) tissues from \( \text{tp7/GUS} \) plants that have been histochemically stained for GUS expression. The arrow in F indicates a stained trichome. J, A 3.5-cm leaf of NtBAR/GUS that has been histochemically stained for GUS expression. The arrow in F indicates a stained trichome. K, A 6-cm leaf of \( \text{gs1.1} \) that has been histochemically stained for GUS expression. L, An 8-cm leaf of \( \text{gs1.2} \) that has been histochemically stained for GUS expression. Scale bars = 20 mm (A and B), 5 mm (C and D), 2 mm (E), 0.3 mm (F and G), and 0.5 mm (H–L).
kanamycin-resistant seedlings (kr2.1–kr2.10) were isolated from 126,000 seedlings (Fig. 2B), and all except kr2.8 survived to maturity after transplanting to soil. To ensure that the elevated levels of kanamycin (300 μg mL⁻¹) used for screening progeny from the reciprocal crosses did not affect the assay, a further 20,000 seedlings from progeny of the NtBAR/GUS δ × tp7 δ crosses were screened at the higher kanamycin concentration, resulting in the isolation of three additional kanamycin-resistant plants.

Positive GUS expression in kr2.1 to kr2.10 (except kr2.8, which did not survive to maturity and was therefore not tested) confirmed that each had resulted from a cross with the homozygous NtBAR/GUS parent (data not shown). The independent origin of these kanamycin-resistant plants was demonstrated by DNA blotting of total cellular DNA restricted with XbaI (Fig. 3). DNA from each line showed a unique combination of restriction patterns when hybridized with aadA- and neo-specific probes. The NtBAR/GUS transgenotype DNA showed weak cross hybridization at high molecular size to the aadA probe. DNA of tp7 showed the expected hybridization at 10.9 kb to the neo probe and at 11.4 kb and 18.5 kb to the aadA probe (Huang et al., 2003a). An unexpected band was also seen at approximately 2.5 kb with the aadA probe, which is most likely due to a rearrangement of the transplastome arising from recombination between the native psbA gene and the plastid-derived psbA terminator sequence that regulates aadA.

An overall frequency of one kanamycin resistance event in approximately 11,000 pollen grains was obtained from the two experiments that used seedlings derived from NtBAR/GUS δ × tp7 δ crosses. This is consistent with the previously reported transposition frequency of one event in 16,000 pollen grains (Huang et al., 2003a). In marked contrast, only a single atypical transposition event could be detected among 273,000 progeny of the reciprocal cross. These data demonstrate an approximately 25-fold greater frequency of transposition and stable integration of the plastid-encoded 35SneoSTLS2 gene into the plant nuclear genome in the male germline compared with the female germline. Even if we include only those kanamycin-resistant lines where transposition was confirmed at the molecular level, which gives a more conservative estimate of nine events out of 126,000 progeny from the cross where the transplastomic parent was male, the reciprocal difference described here is highly significant (P < 0.001; Fisher’s Exact Test). As a final confirmation of the reliability of the selection regime used for screening tp7 δ × NtBAR/GUS δ crosses, 40,000 seedlings (sufficient to give >97% chance of recovering an event assuming a male germline transposition frequency of one in 11,000) of self-fertilized tp7 were screened at 300 μg mL⁻¹ kanamycin. Similarly to the tp7 δ × NtBAR/GUS δ cross described above, no resistant seedlings could be observed after 3 months, but some were still alive after a further 3.5 months. The most promising candidates for true kanamycin resistance were tested by backcrossing to female wild type, and a single plant (kr4.1) was identified that produced Mendelian ratios of kanamycin-resistant progeny (Fig. 2D).

Monitoring Plastid-to-Nucleus DNA Transposition
Using a gus Reporter Gene

To investigate the timing and frequency of ptDNA transposition in somatic cells, a second transplastomic line (tpGUS) analogous to tp7 but containing a gus reporter gene in place of neoSTLS2 was generated (Fig. 1B). The tpGUS line is homoplasmic (data not shown) for a single 35SgusSTLS2 and aadA cassette inserted into the plastid genome near rbcL (which encodes the large subunit of ribulose bisphosphate carboxylase-oxygenase; Fig. 1D). Due to the presence of a nuclear promoter (35S) and intron (STLS2), the gus gene was expected to be expressed only upon transfer to the nucleus.

Histochemical staining of tpGUS plants identified sectors of GUS-positive tissue in leaves, cotyledons, roots, vasculature, anther walls, and trichomes. Staining appeared as small, discrete foci of expression in these tissues surrounded by areas where staining was not detectable (Fig. 2, E–I). To verify that these sectors were the result of transfer of 35SgusSTLS2 to the nucleus rather than activation of the gene in the plastid genome, the cellular localization of the GUS protein was examined. After transfer of 35SgusSTLS2 from the plastid to the nucleus, the resulting GUS enzyme, which does not contain any organelle targeting signals, would be expected to accumulate in the cytosol where the gus mRNA is translated. Nonlocalized and uniform staining of cells is diagnostic of GUS located in the cytosol, and this was observed in all the blue sectors examined in tpGUS plants. Figure 4 shows
examples of sectors composed of one (Fig. 4, A, C and E), two (Fig. 4, B and D), or more (Fig. 4, F and G) blue cells in *tpGUS* plants. Leakage of cytosolic contents results in patchy staining of adjacent cells and intercellular spaces (Fig. 4, C–G). These results are clearly distinguishable from expression in the chloroplasts of a control transplastomic line containing the *gus* gene driven by a plastid promoter (pUM79; Kode et al., 2006) in which GUS activity is confined to chloroplasts (Fig. 4H).

In a single 18-cm leaf, 228 GUS-stained sectors were observed. Using the same estimation of cell number (Hannam, 1968) as Stegemann et al. (2003), this implies transfer and expression of 35S*gusSTLS2* in at least one in 200,000 somatic cells, if each sector is assumed to result from a single transfer event. This frequency is approximately 25 times higher than that measured by Stegemann et al. (2003) where the observed transposition events required stable integration and subsequent cell division and plant regeneration.

Due to the significant discrepancy between transfer frequencies, we sought to provide a more detailed analysis of the DNA transfer frequency. Seeds were germinated in vitro and GUS sectors scored when cotyledons and the first true leaves had reached a length of 3 to 4 mm (Fig. 5). Large variations in sector numbers were found in different cotyledons and leaves, which is reflected in large SDs. No sectors were found in the cotyledons and leaves of a transplastomic line lacking the *gus* gene (negative control).

All four *tpGUS* transplastomic lines (*tpGUS*5.3, 5.6, 8.4, and 9.4), which were derived from independent transformation events, gave a similar range of sector numbers in cotyledons and leaves. This consistency between transplastomic lines supports plastid-to-nucleus transfer of 35S*gusSTLS2* as an explanation for the origin of sectors. The possibility that the sectors arise from activation of a silenced *gus* gene, inadvertently introduced into the nucleus during transformation, is unlikely given the comparable sector frequencies in the four transplastomic lines. An average of between five and six sectors per cotyledon or leaf was found when the results from all four transplastomic *tpGUS* lines were combined (111 cotyledons and 89 leaves). The total number of cells per cotyledon or leaf was estimated to be 100,000 (see “Materials and Methods”), which corresponds to a DNA transfer frequency of approximately one event per 18,000 cells, if each sector is assumed to result from an independent transfer event. The correspondence in sector frequencies between cotyledons and leaves probably reflects the similar numbers of cells present in the organs when sectors were scored and a similar frequency of transfer.

GUS-expressing sectors were found in all types of leaf cells, and their appearance appeared to be random. The variations in sector sizes probably reflect the timing of plastid-to-nucleus DNA transfer during leaf development. Among 225 sectors examined, we found 121 single-cell sectors, 60 two-cell sectors, 23 three-cell sectors, and 12 four-cell sectors. The remaining nine sectors contained five to 10 cells. Guard cells that are derived from a common guard mother cell (Pillitteri et al., 2007) provide a clear example of DNA transfer in progenitor and terminally differentiated cells. DNA transfer in a terminally differentiated cell gives rise to a single GUS-positive guard cell (Fig. 4A).

Figure 4. Microscopic analysis of GUS expression in *tpGUS* transplastomic plants. A and B, Single (A) and double (B) guard cells (labeled GC) expressing GUS. C, Transverse section showing a palisade cell expressing GUS. Labels indicate epidermis (Ep), palisade mesophyll (Pm), and spongy mesophyll (Sm). D, Two mesophyll cells expressing GUS. E, Epidermal cell expressing GUS. F and G, Multiple cells expressing GUS. H, Mesophyll cells in a previously isolated pUM79 transplastomic plant (see Kode et al., 2006) showing GUS localized to chloroplasts (cp). Scale bars = 20 or 100 microns.

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whereas two GUS-positive sister guard cells probably result from DNA transfer in their shared mother cell (Fig. 4B).

The Effect of Gene Copy Number and Location in the Transplastome on Transposition Frequency

Potentially, both copy number and location of a reporter gene within the plastid genome could affect the frequency of its transposition to the nucleus. In tp7, the 35SneoSTLS2 transgene was inserted into the inverted repeat region of the plastid genome between the 16S rRNA and rps7/12 genes and is therefore present as two gene copies per plastid genome (Fig. 1C). In contrast, the 35SgusSTLS2 reporter gene is located adjacent to rbcL in the large single-copy region of the plastid genome (Fig. 1D). Therefore, these different transplastomic types may show different frequencies of plastid-to-nucleus DNA transfer because of the copy number difference of the reporter gene in the transplastome or different transposition frequencies existing among plastid sequences.

This hypothesis was tested by performing histochemical GUS staining to identify progeny with widespread GUS-positive staining from self-fertilized tpGUS plants. Two GUS-positive plants, gs1.1 and gs1.2, were identified in a nondestructive screen of 98,000 seedlings, giving a frequency of one recoverable transposition event in approximately 49,000 progeny. The histochemical staining phenotypes of these two lines were similar to nuclear 35Sgus-positive control plants (Fig. 2, J–L). Spliced gus mRNA, lacking the STLS2 intron, was amplified from gs1.1 and gs1.2, verifying that these two lines were the result of nuclear transposition of 35SgusSTLS2 (Fig. 6). In contrast, the plastid-localized 35SgusSTLS2 gene remained unspliced. Furthermore, after backcrossing gs1.1 to female wild type, GUS staining was present in approximately one-half of the progeny (data not shown), consistent with the expected segregation pattern for a nuclear gene. Therefore, prima facie, the frequency of plastid-to-nucleus DNA transfer observed in tpGUS appears to be approximately 4-fold lower than that observed for 35SneoSTLS2 in tp7. This relatively small difference in transposition frequencies could be due to sampling error arising from the rarity of the events or could be due to kanamycin selection being more efficient than vital GUS staining in identifying seedlings resulting from transfer events in pollen. However, from these experiments, it may be concluded that the insertion of transgenes in these two very different transplastomic locations with two quite different reporter genes does not appear to have a large effect on transposition frequency.

Figure 5. Analysis of sector frequency in cotyledons (3–4 mm) and first true leaves (3–4 mm) in tpGUS lines and a control transplastomic line lacking the gus gene. SDs are shown. n = 24 cotyledons for tpGUS lines and 16 cotyledons for the control line. n = 15 to 21 leaves for tpGUS lines and 12 leaves for the control line.

Figure 6. RT-PCR analysis. RT-PCR was performed using gus primers spanning the STLS2 intron. The higher band (450 bp) represents unspliced transcript, and the lower band (250 bp) represents spliced transcript. In the NtBAR/GUS control, the gus gene does not contain an intron. Control RT-PCRs with ribosomal protein L25 primers are also shown, giving a single 370-bp band. Lanes marked − indicate no RT.
DISCUSSION

The results from reciprocal crosses demonstrate a large difference between the male and female germlines in the frequency of DNA transposition from the plastid to the nuclear genome. Crosses where the male parent was transplastomic gave a transposition frequency of one stable event per 11,000 pollen grains, while crosses where the female parent was transplastomic showed one stable transposition in 273,000 ovules, but the phenotype of the plant recovered from this cross was atypical compared with all others isolated. Hence, the frequency of a newly transposed fragment of ptDNA in male gametes of tobacco is at least an order of magnitude higher than in those of the female.

The elevated frequency of transposition observed within the male germline may be associated with mechanisms that prevent paternal inheritance of the plastid genome, because degradation of the plastid genome in male gametes may result in DNA fragments that could enter and transform the nucleus. We attempted to detect this directly in microspores, pollen grains, and growing pollen tubes by two methods. First, we used quantitative real-time PCR to target spliced neo transcripts in tp7, and, second, we used histochemical and quantitative GUS assays in tpGUS (data not shown). Neither of these approaches were successful in detecting elevated ptDNA transfer in any particular cell type, which is consistent with other reports indicating that the 35S promoter has very poor expression in these tissues (Wilkinson et al., 1997; Custers et al., 1999). Therefore, with the transplastomic lines available, current methods appear to be inappropriate for the identification of those cells that are prone to integrate ptDNA in their nuclei and then transmit this insertion to subsequent generations. A pollen-specific promoter could be used to provide more efficient transcription than the 35S promoter in the male germline, but this would still be confounded by the possibility that transposition is elevated in a cell type that is not part of the expression repertoire of the chosen promoter.

Histochemical analysis of the transplastomic line tpGUS indicates that plastid nucleic acid enters the nucleus in a variety of somatic tissues at a high frequency. These data are the first demonstration of somatic transposition of a plastid transgene to the nucleus in the absence of antibiotic selection and plant regeneration. Analysis of a single 18-cm leaf indicated a transfer frequency of approximately one event per 200,000 cells. A more thorough analysis of 3- to 4-mm cotyledons and leaves indicated a transfer frequency of one event per 18,000 cells. There are several experimental differences that could explain this discrepancy in calculated transposition frequencies. First, the tissues analyzed were at different stages of development; second, the former experiment was performed with a soil-grown plant while the latter experiments used seedlings grown in vitro; and finally, different methods were used for the calculation of cell number. The frequency of transposition we observed in leaves is 25 to 300 times higher than a previous estimate based on regenerative selection (kanamycin) of somatic cells containing stable integrants of ptDNA in the nucleus (Stegemann et al., 2003). Potentially, the histochemical staining of tpGUS tissues identified both stable and transient expression, which could account for the difference. Therefore, it is likely that ptDNA enters the nucleus and is expressed far more frequently than it integrates into a chromosome. In addition, not all kanamycin-resistant cells would be expected to survive selection and be capable of regenerating into plants.

The frequency of transposition varied greatly between replicate plants and tissues, implying that it is not tightly regulated and is mainly the result of chance plastid degradation and nucleic acid escape. A rare stochastic process such as this might be expected to give rise to a predominance of small sectors due to the larger number of cells present at later stages of leaf development compared with those present in the leaf initials. Indeed, large sectors of GUS-stained tissue were not found in older leaves, reflecting the rarity of transfer events early in leaf development and also suggesting that any stable integration events that occur at earlier stages of leaf development very rarely involve cells that proceed to further divisions. Therefore, because the 35SneoSTLS2 and 35SgusSTLS2 systems are likely to be comparable, the regeneration-based selection procedures applied by Stegemann et al. (2003) must have induced some cells to propagate when their fate would have been nonproliferative in normal leaf development.

The reciprocal difference between the frequencies of plastid-to-nucleus DNA transfer in the male and female germlines may have arisen under the influence of the selective pressures that maintain uniparental organelle inheritance. It is clear that transfer of plastid genes to the nuclear genome (either to replace the original gene or to take on a new function) has been selected for, because this has been such a widespread phenomenon throughout eukaryotic evolution (Martin et al., 2002, 2003; Timmis et al., 2004). In fact, recent results indicate that smaller fragments of noncoding organellar DNA can also be incorporated as functional exons in the nucleus (Noutsos et al., 2007). Therefore, transposition of ptDNA to the nucleus must confer a selective advantage at the species level, even though most ptDNA transpositions are likely to be nonfunctional or detrimental. However, inheritance and replication of a functional chloroplast genome is essential for survival. Processes that allow transposition in the male germline, while suppressing transposition in the female germline, would maximize the benefits associated with transposition while maintaining the essential functions of the plastid genome, and hence would become a characteristic of successful species. There is ample evidence that both loss of plastids and degradation of ptDNA occur during pollen development, particularly in species that show uniparental inheri-
Some plant biotechnologists have advocated the placement of transgenes in the plastid genome to ensure their containment in the maternal parent and prevent their escape through pollen dispersal. This study demonstrates that the frequency of plastid transgene relocation to the nucleus in the male germline is an order of magnitude higher than in the female germline. Hence, plastid transgenesis alone does not provide complete transgene containment in tobacco, and additional safeguards will be necessary to eliminate all possibility of transgene escape.

**MATERIALS AND METHODS**

**Plant Growth Conditions**

Tobacco (*Nicotiana tabacum*) plants grown in soil were kept in a controlled environment chamber with a 14-h-light/10-h-dark and 25°C-day/18°C-night growth regime.

**Analysis of Kanamycin Resistance**

Kanamycin selection was performed using 0.5× Murashige and Skoog salt medium (Murashige and Skoog, 1962) containing 150 or 300 μg mL⁻¹ kanamycin for plants with *tp*² plasts. Screened were performed by plating surface-sterilized seeds on 150-mm plates containing 80 mL of the above medium at a density of 2,000 seeds/plate. Progeny testing of kanamycin-resistant plants was performed by plating surface-sterilized seeds on 90-mm plates containing 20 mL of the above medium. All plates were incubated at 25°C with 16 h light/8 h dark.

**Analysis of GUS Activity**

For histochemical GUS assays, tissues were fixed by vacuum infiltration in 100 mM sodium phosphate buffer, pH 7.0, 0.12% formaldehyde, 0.1% β-mercaptoethanol, 0.1% Triton X-100 for 10 min, washed three times with 100 mM sodium phosphate buffer, pH 7.0, and stained in 45 mM sodium phosphate buffer, pH 7.0, 0.45 mM potassium ferricyanide, 0.45 mM potassium ferrocyanide, 0.1% Triton X-100, 0.05% chloramphenicol, 0.1% β-mercaptoethanol, 10% dimethyl sulfoxide, 0.1% X-Gluc overnight at 37°C. After staining, tissues were cleared in 70% ethanol.

Viable GUS staining was performed in tissue culture as described (Martin et al., 1992).

For the analysis of GUS sectors in seedlings, seeds were germinated on Murashige and Skoog medium as described (Kode et al., 2006). Whole seedlings were incubated in buffer containing X-Gluc at 37°C, then chlorophyll was removed with ethanol before mounting on slides to examine sectors using a Leica S8APO Stereo Zoom microscope. For sections, leaf pieces were fixed (0.3% formaldehyde, 30 min) and stained in X-Gluc buffer as described (Klösgen and Weil, 1991). Leaf pieces were embedded in wax (Thermoshandon Histocentre 3), sectioned into 5-μm slices (Microm HM350), cleared (Histo-Clear; National Diagnostics), and mounted on slides for microscopy (Leica DMR microscope). The average number of cells per leaf or cotyledon was estimated to be 100,000 cells based on counting the average number of cells in a 100-μm × 100-μm square (20 cells), the average cotyledon area (9.2 mm²), or leaf area (9.6 mm²) and the observation of six cell layers in the cotyledons and leaves examined (e.g., see Fig. 4C).

**Southern Blotting**

DNA blot analyses were carried out as described (Ayliffe and Timmis, 1992; Huang et al., 2003b).

**Generation of *tpGUS***

A *gus* gene containing the second intron of the potato (*Solanum tuberosum*) STLS-T1 gene inserted into the open reading frame, with 35S promoter and terminator sequences, was amplified from p35S GUS INT (Vancanneyt et al., 1990) using the following primers (NotI and SacII sites underlined): 5′-ATCGTACCCGGGCAACATGCGACAGCACCTCTGCTAC-3′ and 5′-TCACTACGCCAGGGCAGGAGCATTCGTTTTC-3′. The resulting PCR product was cloned into pGEM-T Easy (Promega). The SacII/NotI fragment of this vector containing *gus* and the Apal/SacII fragment of pUM35 containing adaA flanked by the Brassica napus 16S rnm promoter and pS1C terminator regions (Zubko et al., 2004) were then ligated into Apal/NotI-digested pATB27-link (Zubko et al., 2004) to generate the transformation vector. Transplastomic plants were isolated by selecting for dark-green spectinomycin-resistant shoots following particle bombardment of pale-green ΔrbcL leaves as described (Kode et al., 2006).

**Reverse Transcription-PCR Analysis**

RNA extraction was performed using an RNeasy Plant Mini kit (Qiagen) and genomic DNA contamination removed using a TURBO DNA-free kit (Ambion). Reverse transcription (RT) was then performed using an Advan-tage RT-for-PCR kit (CLONTECH) with oligo(dT) primer. All kits were used in accordance with the manufacturers’ instructions. PCR amplification was performed using Taq DNA Polymerase (New England Biolabs) according to standard protocols. Primers used were 5′-TCATACGCCAGAAATGGTGCTC-3′ and 5′-TAGAGCTACCCGCTGCGATGG-3′ for *gus* PCRs and 5′-AAAATCT-GACCCAGGGCCAC-3′ and 5′-GGTTCCTGCTGCCATCAGG-3′ for L25 PCRs.

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