

High-frequency gene transfer from the chloroplast genome to the nucleus

Sandra Stegemann, Stefanie Hartmann, Stephanie Ruf, and Ralph Bock*

Westfälische Wilhelms-Universität Münster, Institut für Biochemie und Biotechnologie der Pflanzen, Hindenburgplatz 55, D-48143 Münster, Germany

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Eukaryotic cells arose through endosymbiotic uptake of free-living bacteria followed by massive gene transfer from the genome of the endosymbiont to the host nuclear genome. Because this gene transfer took place over a time scale of hundreds of millions of years, direct observation and analysis of primary transfer events has remained difficult. Hence, very little is known about the evolutionary frequency of gene transfer events, the size of transferred genome fragments, the molecular mechanisms of the transfer process, or the environmental conditions favoring its occurrence. We describe here a genetic system based on transgenic chloroplasts carrying a nuclear selectable marker gene that allows the efficient selection of plants with a nuclear genome that carries pieces transferred from the chloroplast genome. We can select such gene transfer events from a surprisingly small population of plant cells, indicating that the escape of genetic material from the chloroplast to the nuclear genome occurs much more frequently than generally believed and thus may contribute significantly to intraspecific and intraorganismic genetic variation.

The evolutionary origin of eukaryotic cells is characterized by the endosymbiotic uptake of bacteria and their gradual conversion into the DNA-containing cell organelles, mitochondria, and plastids (chloroplasts) (1–3). Genetically, the evolutionary optimization of endosymbiosis was accompanied by the loss of dispensable and redundant genetic information and the large-scale translocation of genetic information from the endosymbiont to the host genome (4–6). Consequently, contemporary organellar genomes are greatly reduced and contain only a small proportion of the genes that their free-living ancestors had possessed. By using molecular methods, the origins of organelles have been traced back to specific taxa of eubacteria: Whereas cyanobacteria were identified as presumptive ancestors of plastids, α -proteobacteria are related most closely to mitochondria (1).

Interspecific variation in the gene content of organellar genomes (7–11) suggests that gene transfer from organelles to the nucleus is an ongoing process. Moreover, pieces of chloroplast and mitochondrial DNA are often found in nuclear genomes (12–18) and commonly referred to as promiscuous DNA. These sequences lack any apparent function but may provide the raw material for converting organellar genes into functional nuclear genes, the products of which are reimported into the organelle, then allowing for subsequent loss of the genes from the organellar genome (10). In addition, promiscuous DNA of mitochondrial origin has been implicated recently in DNA repair in yeast by patching broken chromosomes (19, 20).

In the present study we developed an experimental system suitable for selecting and analyzing DNA transfer events from the chloroplast genome to the nuclear genome. We find that DNA escape out of the chloroplast and integration into the nuclear genome occurs much more frequently than generally believed and thus provides a mechanism not only causing intraspecific but also intraorganismic genetic variation.

Materials and Methods

Plant Material. Tobacco plants (*Nicotiana tabacum* cv. Petit Havana) were grown under sterile conditions on agar-solidified

MS medium (21) containing 30 g/liter sucrose. Homoplasmic transformed lines were rooted and propagated on the same medium.

Construction of Plastid Transformation Vectors. Plastid transformation vector pRB98 (Fig. 1A) is a derivative of the previously described vector pRB95 (22). A nuclear cauliflower mosaic virus 35S promoter-driven *nptII* expression cassette was excised from a plant transformation vector (23) and inserted into the polylinker of pRB95 yielding plasmid pRB98.

Plastid Transformation and Selection of Transplastomic Tobacco Lines.

Young leaves were harvested from sterile tobacco plants and bombarded with plasmid DNA-coated gold particles by using the DuPont PDS1000He biolistic gun (24). Spectinomycin-resistant shoots were selected on RMOP regeneration medium containing 500 mg/liter spectinomycin dihydrochloride (24). Primary plastid transformants were identified by a PCR strategy. Three independently generated transplastomic lines (designated Nt-pRB98-1, Nt-pRB98-11, and Nt-pRB98-12) were subjected to four additional rounds of regeneration on RMOP/spectinomycin to obtain homoplasmic tissue (24). Homoplasmy was verified by restriction fragment length polymorphism analysis and highly sensitive inheritance assays (see below).

Selection for Gene Transfer from the Chloroplast to the Nucleus.

Young leaves from Nt-pRB98 plastid transformants were harvested from sterile plants, cut into small pieces of ≈ 20 – 25 mm², and placed onto the surface of a selectable plant-regeneration medium containing kanamycin. Several independent screens were conducted with kanamycin concentrations ranging from 100 to 400 μ g/ml. With all concentrations, gene transfer plants were recovered. However, because the lower kanamycin concentrations produced a relatively high background of false-positive clones, the final screen was conducted with 400 μ g/ml kanamycin. All putative gene transfer plants were rescreened by placing small tissue pieces ($\approx 2 \times 2$ mm) on kanamycin-containing regeneration medium, a stringent selection step (because of the small explant size) that eliminated essentially all false-positive clones. Regenerated shoots then were transferred to sterile culture boxes and rooted on phytohormone-free MS medium in the presence of kanamycin. Finally, rooted plants were taken out of the sterile containers, planted into soil, and transferred to the greenhouse.

Isolation of Nucleic Acids. Total plant nucleic acids were isolated according to a miniprep procedure described by Doyle and Doyle (25).

PCR Assays. DNA templates were amplified according to standard protocols (45 s at 94°C, 1.5 min at 55–65°C, and 1.5 min at 72°C for 30 cycles) in an Eppendorf gradient thermocycler. Primers

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*To whom correspondence should be addressed. E-mail: rbock@uni-muenster.de.

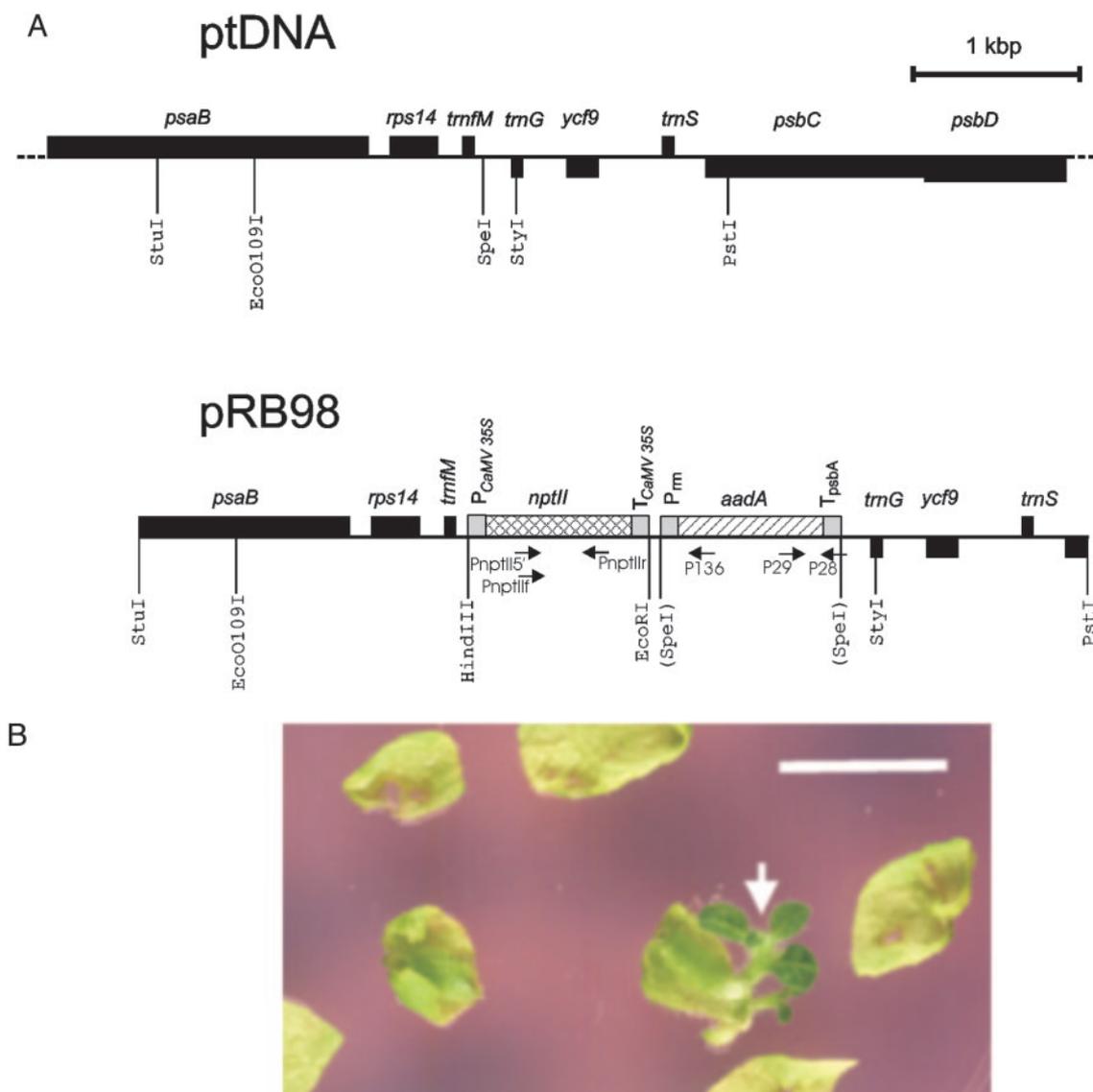


Fig. 1. A genetic screen for gene transfer from the chloroplast to the nucleus. (A) Physical map of the chloroplast transformation vector pRB98. (Upper) The region of the tobacco chloroplast genome chosen for insertion of the two foreign gene constructs: a chimeric *aadA* gene conferring spectinomycin resistance as plastid selectable marker gene (24) and a nuclear expression cassette containing the kanamycin-resistance gene *nptII*. (Lower) Homologous recombination targets the two linked transgenes from pRB98 to the intergenic region between two tRNA genes (*trnFM* and *trnG*) (22). Location and orientation of PCR primers are indicated by arrows. ptDNA, plastid DNA. (B) Selection of cell lines that have transferred the kanamycin-resistance gene from the chloroplast genome to the nuclear genome. Putative gene transfer plants (arrow) were selected on plant-regeneration medium containing 400 $\mu\text{g/ml}$ kanamycin and typically appeared after 3–6 weeks of selection. (Scale bar, 1 cm.)

specific for the *nptII* coding region (PnptIIr, 5'-GAG-GCAGCGGGCTATC-3'; PnptII5', 5'-GCGGTCGGCA-CACCCA-3') were used to assay for the presence of the transgene in individual seedlings resulting from crosses of wild-type plants and gene transfer plants, with the latter ones serving as the pollen donor. Presence of the *aadA* gene in the nuclear genome of the gene transfer plants was assayed with primers derived from the 3' part of the coding region (P29, 5'-CGCTATG-GAACTCGCGCC-3') and the downstream 3' untranslated region (P28, 5'-TAGCACCCTCTTGATAGAAC-3'). Linkage of *nptII* and *aadA* was analyzed by PCR by combining one primer derived from the *aadA* coding region (P136, 5'-TCGAT-GACGCCAACTACC-3') with a primer binding to the *nptII* coding region (PnptII5', 5'-GCTGCATACGCTTGATCC-3') (Fig. 1).

Crosses and Inheritance Tests of Transplastomic Lines. For confirmation of homoplasmy of transplastomic lines, wild-type and

transformed plants were transferred to soil and grown to maturity under greenhouse conditions. Seed pods were collected from selfed plants and reciprocal crosses of the transplastomic lines with wild-type plants. Surface-sterilized seeds were germinated on spectinomycin-containing (500 mg/liter) MS medium and analyzed for uniparental inheritance of the resistance trait. Selfed transformants and crosses with a chloroplast transformant as the maternal parent give rise to green (i.e., spectinomycin-resistant) progeny, whereas seeds collected from wild-type plants pollinated with pollen from transplastomic plants yield white (i.e., drug-sensitive) seedlings. Seeds from all crosses were also assayed for kanamycin resistance. Wild-type plants pollinated with pollen from transplastomic plants gave rise to progeny that were as kanamycin-sensitive as the wild type (assayed on 100 $\mu\text{g/ml}$). In contrast, transplastomic plants displayed an enhanced kanamycin tolerance (most likely caused by low-level *nptII* expression from the nuclear cauliflower mosaic

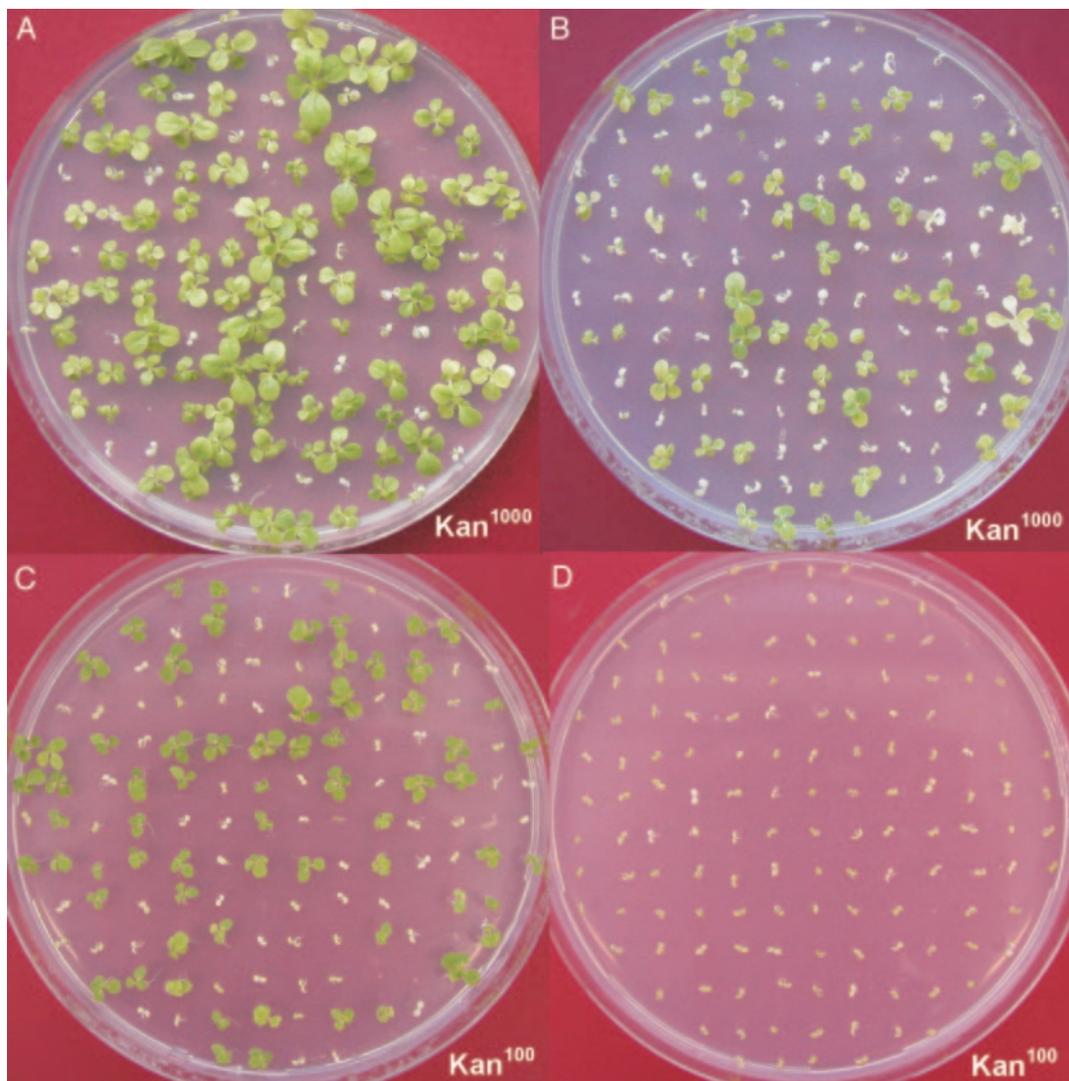


Fig. 2. Inheritance assays demonstrating gene transfer from the chloroplast to the nucleus. (A) Seed assay demonstrating Mendelian inheritance of the kanamycin resistance in a selected gene transfer line. Seeds from self-pollinated gene transfer plants were surface-sterilized and germinated on medium containing 1,000 $\mu\text{g/ml}$ kanamycin. Phenotypic segregation of the progeny in the expected 3:1 ratio (96 resistant:34 sensitive seedlings) indicates nuclear localization of the kanamycin-resistance gene. Note that approximately one-third of the resistant seedlings display particularly rapid growth, suggesting that they are homozygous and thus show enhanced kanamycin resistance. (B and C) Mendelian inheritance and 1:1 segregation of the kanamycin resistance in reciprocal crosses of gene transfer plants with wild-type plants. (B) Pollination of a gene transfer plant with pollen from a wild-type plant yields 1:1 segregating progeny (65 resistant:72 sensitive seedlings) and, because of the continued presence of the transgenic chloroplasts, requires selection at elevated kanamycin concentrations (1,000 $\mu\text{g/ml}$; see text). (C) Pollination of a wild-type plant with pollen from a gene transfer plant separates the cell nucleus with the transferred *nptII* gene from the transgenic chloroplasts and thus allows phenotypic selection already at low kanamycin concentrations (100 $\mu\text{g/ml}$). As in the reciprocal cross, a 1:1 segregation (68 resistant:68 sensitive seedlings) typical of nuclear-encoded traits is observed, ultimately confirming that the formerly chloroplast-located *nptII* gene has moved to the nucleus. (D) Wild-type control. Self-pollination of wild-type plants does not yield kanamycin-resistant progeny. Kanamycin (Kan) concentrations used for selection are indicated in micrograms per milliliter.

virus 35S promoter in the chloroplast), and seedlings from crosses with transplastomic plants as maternal parent therefore were assayed on 500 or 1,000 $\mu\text{g/ml}$ kanamycin.

Crosses and Inheritance Tests of Gene Transfer Lines. Surface-sterilized seeds from selfed gene transfer plants and reciprocal crosses of gene transfer plants with wild-type plants were phenotypically assayed by germination on kanamycin-containing MS medium. Seeds from crosses with gene transfer plants as maternal parent were assayed on 500 or 1,000 $\mu\text{g/ml}$ kanamycin, whereas seeds from crosses with wild-type plants as maternal parent were assayed on 100 $\mu\text{g/ml}$ kanamycin.

Results and Discussion

A Genetic Screen for Gene Transfer. To develop an experimental system suitable for selecting and analyzing DNA transfer events from the chloroplast genome to the nuclear genome, we constructed a chloroplast transformation vector in which we placed the kanamycin-resistance gene *nptII* under the control of strong nuclear expression signals (Fig. 1A). The cauliflower mosaic virus 35S promoter triggers more or less constitutive expression of nuclear genes and transgenes but is not expected to give rise to significant expression levels in the chloroplast. The nuclear kanamycin cassette was linked to a chloroplast-specific selectable marker gene (*aadA*; Fig. 1A), conferring spectinomycin resistance, and to two flanking sequence blocks to facilitate

chloroplast transformation by homologous recombination (22, 24). Chloroplast transformation experiments in tobacco yielded several transplastomic lines (designated Nt-pRB98), three of which were characterized further (Nt-pRB98-1, Nt-pRB98-11, and Nt-pRB98-12). PCR assays confirmed the presence of both transgenes (*aadA* and *nptII*), and reciprocal crosses and spectinomycin-resistance tests of the progeny established that, after four consecutive rounds of selection and regeneration, the plants were homoplasmic and lacked detectable levels of residual wild-type genome copies. Growth and regeneration tests on tissue culture medium containing 50 $\mu\text{g/ml}$ kanamycin revealed that the transplastomic lines displayed a somewhat higher kanamycin tolerance than the wild type, most probably because of some basic level of *nptII* expression from the eukaryotic-type cauliflower mosaic virus 35S promoter in the prokaryotic gene expression system of the chloroplast. However, this enhanced kanamycin tolerance could be suppressed by applying elevated kanamycin concentrations (100–400 $\mu\text{g/ml}$).

Selection of Gene Transfer Events. We next used these transgenic lines to set up a genetic screen for gene transfer events. Because it possesses strong nuclear expression signals, transfer of the *nptII* gene out of the chloroplast into the nuclear genome can be expected to result in high-level expression of kanamycin resistance and thus allow growth in the presence of the antibiotic. To this end, we subjected sterile leaf samples to selection for kanamycin resistance on a plant-regeneration medium (Fig. 1B). To eliminate false-positive clones, all primary lines were re-screened by exposing small tissue pieces to regeneration medium containing 400 $\mu\text{g/ml}$ kanamycin. From 89 primary selection plates (equaling 20 leaves cut into $\approx 1,200$ explants; Fig. 1B), 12 highly kanamycin-resistant clones were obtained after re-screening. Regenerated plantlets were rooted in the presence of kanamycin and transferred to the greenhouse.

Mendelian Inheritance of the Transferred *nptII* Gene. If kanamycin resistance indeed was caused by gene transfer to the nuclear genome, then the inheritance pattern of the resistance trait should change from uniparentally maternal inheritance, as is typical of organelle-encoded traits in tobacco and most other angiosperm plants (26–28), to Mendelian inheritance typical of nuclear-encoded traits. We therefore self-pollinated the plants and assayed the progeny for segregation of the kanamycin resistance. Resistance encoded by a nuclear gene is expected to yield a 3:1 segregation of resistant to sensitive progeny. Indeed, germinating seeds showed the expected 3:1 segregation ratio (Fig. 2A), strongly suggesting that the kanamycin-resistance gene is now located in the nuclear genome. To substantiate this conclusion further, we also pollinated the putative gene transfer plants with pollen from wild-type plants, a cross expected to give 1:1 segregation after Mendelian inheritance of the kanamycin resistance. The expected 1:1 segregation ratio was indeed observed (Fig. 2B), providing further genetic evidence for the formerly chloroplast-localized kanamycin-resistance gene now residing in the nucleus.

To facilitate the molecular analysis of the gene transfer events, we next wanted to separate the cell nucleus harboring the transferred kanamycin-resistance gene from the kanamycin-resistance gene-containing chloroplasts. To this end, we performed the reciprocal cross using the transplastomic plants as pollen donor and wild-type plants as maternal parent. Because of the maternal inheritance of tobacco chloroplasts, this cross transfers only the nuclear *nptII* gene into the next generation. Seeds now could be assayed on lower kanamycin concentrations (100 instead of 500 or 1,000 $\mu\text{g/ml}$; Fig. 2C), because in the absence of the transgenic chloroplasts seedlings not containing the transferred *nptII* can be expected to be as sensitive to kanamycin as the wild type (and will not display the enhanced

Table 1. Segregation of kanamycin resistance after crosses of 12 independently selected gene transfer plants with wild-type plants

Plant line	Segregation of kanamycin resistance (resistant:sensitive seedlings)	Segregation ratio
Nt-pRB98-1-29	88:86	1:1
Nt-pRB98-1-35	75:74	1:1
Nt-pRB98-1-38	60:57	1:1
Nt-pRB98-1-43	93:91	1:1
Nt-pRB98-1-55	105:108	1:1
Nt-pRB98-1-75	141:138	1:1
Nt-pRB98-1-81	84:80	1:1
Nt-pRB98-11-7	105:388	1:3.7*
Nt-pRB98-11-41	74:73	1:1
Nt-pRT98-12-16	430:432	1:1
Nt-pRB98-12-31	99:92	1:1
Nt-pRB98-12-36	245:230	1:1

*Drastic deviation from the expected 1:1 Mendelian segregation was confirmed by three independent crosses. Underrepresentation of kanamycin-resistant seedlings could be due to silencing of the *nptII* gene in part of the progeny (40).

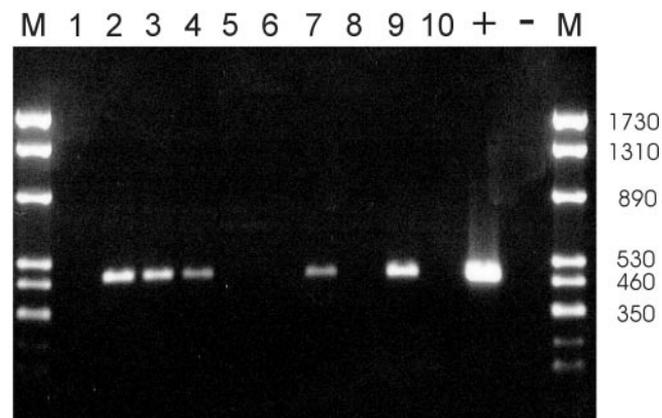
background levels of kanamycin tolerance observed in the transplastomic Nt-pRB98 plants; see above). Seed assays of the progeny from this reciprocal cross revealed that, as expected, a 1:1 segregation ratio was clearly seen already with 100 $\mu\text{g/ml}$ kanamycin (Fig. 2C; Table 1). PCR assays with individual seedlings with *nptII*-specific primers confirmed that the resistant seedlings all contained the *nptII* gene, whereas the sensitive seedlings did not (Fig. 3 and data not shown).

We considered the possibility that a nuclear copy of the *nptII* gene was already present in the transplastomic Nt-pRB98 plants due to simultaneous integration of the transforming DNA into the chloroplast and nuclear genomes (although cotransformation of two genetic compartments has not been observed to date). Such a nuclear copy might have been integrated in a transcriptionally silent genomic region, and in theory it could have been translocated into a new genomic location, permitting active expression in our screen for kanamycin resistance. To exclude this possibility, we performed control crosses of the initial Nt-pRB98 lines with wild-type plants (using the transplastomic plants as pollen donor) and tested the progeny for presence of the *nptII* gene. If a nuclear copy of the *nptII* were present in the transplastomic lines, then 50% of the progeny should test positive for *nptII* due to 1:1 Mendelian segregation. However, when we assayed 20 individual seedlings for the presence of *nptII* by PCR, all of them were negative (data not shown), confirming that the nuclear *nptII* copies in our selected kanamycin-resistant lines originate from gene transfer events out of the chloroplast.

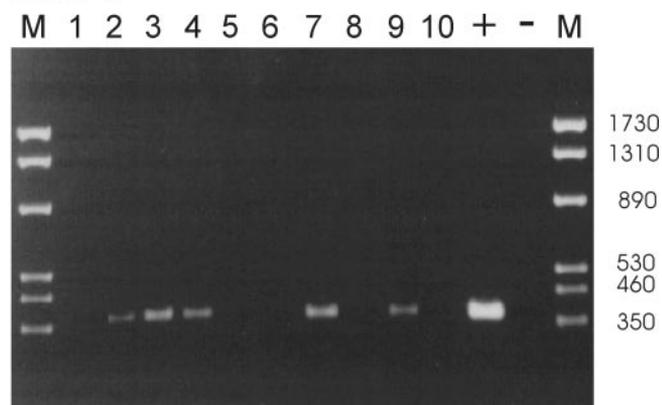
The 12 gene transfer lines obtained in our screen were selected from different leaves and, even more importantly, from three independently produced transplastomic plant lines (Table 1) clearly demonstrating that they represent independent transfer and integration events.

Cotransfer of *nptII* and *aadA*. Gene transfer from organelles to the nucleus can either occur by direct transfer of DNA sequences into the nuclear genome (10, 29) or, alternatively, be mediated by RNA that is reverse-transcribed and then integrated into the nuclear DNA (30–32). To distinguish between DNA-mediated and RNA-mediated transfer of the *nptII* gene from the chloroplast to the nucleus, we assayed for possible cotransfer of the spectinomycin-resistance gene *aadA*. Successful separation of the transgenic chloroplasts from the nucleus containing the transferred kanamycin-resistance gene (Fig. 2C) now allowed us

nptII



aadA



nptII + *aadA*

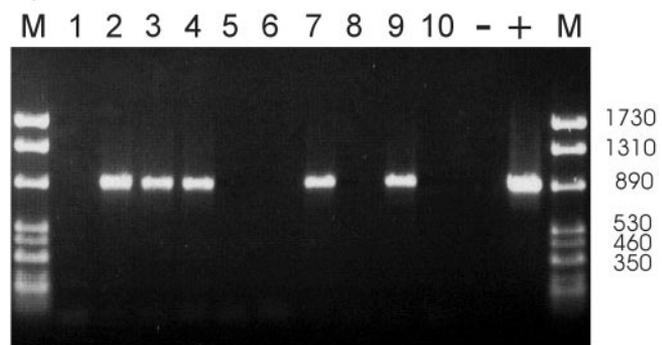


Fig. 3. Assay for cotransfer of the *aadA* gene with the selectable marker gene *nptII*. PCR with primers specific for the 3' region of the chimeric *aadA* gene (Fig. 1A) were performed to assay for transferred *aadA* sequences in individual seedlings from a cross of a wild-type plant (maternal parent) with a gene transfer plant (paternal parent). Although the five seedlings positive for *nptII* (samples 2, 3, 4, 7, and 9) also showed the *aadA*-specific 368-bp PCR product and displayed kanamycin resistance in growth assays on synthetic medium, the five PCR-negative seedlings (1, 5, 6, 8, and 10) are kanamycin-sensitive, indicating cotransfer of the two foreign genes and Mendelian 1:1 segregation also for the *aadA* gene. Cotransfer of the two genes and their physical linkage in the nuclear genome was ultimately confirmed by combining an *nptII*-specific with an *aadA*-specific primer (Fig. 1), yielding a 1-kb product for all *nptII*-positive seedlings. +, positive control (Nt-pRB98-12 chloroplast transformant harboring both *nptII* and *aadA* in the plastid genome; Fig. 1A); -, buffer control; M, molecular weight marker (fragment sizes in base pairs). Note much stronger PCR amplification in the chloroplast transformant (+) than in the gene transfer plants, which is due to the much higher copy number per cell of the chloroplast genome.

to test for the physical presence of the formerly plastid-localized *aadA* sequences inside the nuclear genome by molecular methods. Because *nptII* and *aadA* are transcribed from separate promoters (Fig. 1A), transfer of the *nptII* only would be indicative of an RNA-mediated transfer, whereas cotransfer of both genes would be more compatible with a DNA-mediated transfer. When we tested for the presence of *aadA* sequences (located ≈ 1 kb away from the *nptII* gene) in the nuclear genome of the segregating progeny from crosses of wild-type plants with gene transfer plants, we could readily detect them in all those seedlings that displayed kanamycin resistance (Fig. 3), demonstrating that the gene transfer event is not restricted to the kanamycin-resistance gene that was selected for but also involves flanking DNA sequences. This result might indicate that the transfer mechanism does not involve an mRNA/cDNA intermediate.

Frequency of Gene Transfer. When the approximate cell number (33) in the total leaf area exposed to selection for gene transfer events is divided by the number of events selected, we obtain a frequency of approximately 1 of 5 million cells where transfer of the chloroplast *nptII*-containing fragment into the nuclear genome has occurred. In reality, the frequency may be even higher, because our selection scheme missed all those events where the selectable marker gene was not included in the transferred chloroplast genome fragment. In addition, transfer events in cells that are not regeneration-competent as well as nuclear integration events not resulting in sufficiently high expression levels of the *nptII* transgene may have gone undetected in our rigorous selection scheme.

The estimated frequency of gene transfer from the chloroplast to the nucleus is remarkably similar to the transfer rate of yeast mitochondrial DNA to the nucleus, which has been estimated to be $\approx 2 \times 10^{-5}$ per cell per generation (15). Interestingly, the number of cells in a mature tobacco leaf (33) is at least 10 times higher than the average number of leaf cells required to select one chloroplast gene transfer event, which indicates that cells within a single leaf are not genetically identical but may differ in their nuclear genome with respect to the pattern of chloroplast DNA integrations. In addition, similar to movement of transposable elements (34, 35), high-frequency insertion of chloroplast DNA into the nuclear genome may be responsible for somatic mutations by integrating into functional nuclear genes.

The precise molecular mechanisms of DNA escape or transport out of the chloroplast into the nucleus currently remain unknown. Besides an active transport mechanism, several possibilities of a more passive transfer can be envisaged, e.g., (i) occasional slippage of DNA molecules out of the chloroplast during organelle division, (ii) release of plastid DNA after chloroplast degradation, or even (iii) accidental uptake of organelles by the nucleus as observed microscopically for plant mitochondria (36).

In sum, our results demonstrate a surprisingly high frequency of gene transfer out of the chloroplast into the nucleus, thus pointing to (i) an ongoing mechanism for nuclear genome evolution through frequent acquisition of organellar DNAs and (ii) a significant contribution of promiscuous DNA insertions to intraspecific and intraorganismic genetic variation in multicellular eukaryotes. The experimental reconstitution of organelle-to-nucleus gene transfer in our transgenic chloroplast system will facilitate the molecular analysis of the DNA movement process and, moreover, will allow the determination of frequency and copy number of gene transfer events as well as the sizes of transferred genome fragments (5). In addition, the system will facilitate the identification of environmental conditions favoring the occurrence of intracellular gene transfer and thus will help in reconstructing the evolutionary events that have shaped the genomes of eukaryotic cells after the uptake of endosymbiotic bacteria possibly >1.5 billion years ago (2, 37, 38).

Note. A very recent study has determined the frequency of plastid DNA escape during pollen development (and hence the paternal nuclear transmission rate of plastid transgenes) (39). The authors detected one escape event in $\approx 16,000$ pollen grains, a frequency that is ≈ 2 orders of magnitude higher than the somatic gene transfer frequency determined in this study. Because most angiosperm species inherit their chloroplasts maternally, paternal plastids are selectively eliminated or degraded during pollen grain maturation. To what extent

degradation of paternal plastids may contribute to the extremely high frequency of plastid DNA escape in pollen development remains to be investigated.

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