Determining the transgene containment level provided by chloroplast transformation

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Edited by Maarten Koornneef, Wageningen University and Research Centre, Wageningen, The Netherlands, and approved February 28, 2007 (received for review January 2, 2007)

Plastids (chloroplasts) are maternally inherited in most crops. Maternal inheritance excludes plastid genes and transgenes from pollen transmission. Therefore, plastid transformation is considered a superb tool for ensuring transgene containment and improving the biosafety of transgenic plants. Here, we have assessed the strictness of maternal inheritance and the extent to which plastid transformation technology confers an increase in transgene confinement. We describe an experimental system facilitating stringent selection for occasional paternal plastid transmission. In a large screen, we detected low-level paternal inheritance of transgenic plastids in tobacco. Whereas the frequency of transmission into the cotyledons of F_1 seedlings was $\approx 1.58 \times 10^{-5}$ (on 100% cross-fertilization), transmission into the shoot apical meristem was significantly lower (2.86×10^{-6}). Our data demonstrate that plastid transformation provides an effective tool to increase the biosafety of transgenic plants. However, in cases where pollen transmission must be prevented altogether, stacking with other containment methods will be necessary to eliminate the residual outcrossing risk.

biosafety | *Nicotiana tabacum* | plastid transformation | pollen transmission | paternal leakage

Plants with transgenic plastid genomes ("transplastomic" plants) offer an attractive alternative to conventional transgenic plants. Besides several technological attractions, such as high-level transgene expression, convenient transgene pyramiding in operons, and absence of epigenetic transgene instability (1, 2), the increased biosafety provided by transplastomic plants is of particular relevance to future applications of genetic engineering in agriculture and biotechnology. In most crops, plastids are maternally inherited (3–5), largely excluding plastid-localized transgenes from pollen transmission and thus greatly reducing the risk of unwanted transgene transfer from fields with genetically modified (GM) plants to neighboring fields with non-GM plants. How strict maternal inheritance can be has been a highly controversial issue. Whereas cytological studies indicate that paternal chloroplast DNA may be completely absent from male gametes and pollen tubes of many angiosperm species (6, 7), genetic data have suggested that low-level paternal chloroplast leakage can occur even in species believed to inherit their plastids maternally. Unfortunately, published values for occasional paternal chloroplast transmission differ widely even for one and the same species (8–10), with tobacco providing a particularly striking example of this confusion: Whereas cytological and early genetic analyses had suggested a strictly maternal mode of plastid inheritance (reviewed, e.g., in ref. 11), two other genetic studies suggested low-level paternal plastid transmission (8, 9) and one even reported very high values of up to 2% biparental plastid inheritance (10). One of the underlying problems may be that all previous studies were restricted to the analysis of spontaneous point mutations in the chloroplast genome that confer herbicide tolerance, antibiotic resistance, or chlorophyll deficiency, traits that could be used to visualize paternal chloroplast transmission in crosses. Also, most of these studies analyzed plastid inheritance in interspecific hybrids or cybrids (8, 9), which has been suggested to contribute substantially to the observed paternal leakage (2). However, to what extent hybrid effects and/or differences in the plastid mutations and markers used in the different studies account for the different findings is unknown, and thus, reliable quantitative data on occasional paternal plastid transmission are largely lacking. Because such data are of outstanding importance to the critical evaluation of the biosafety of the transplastomic technology as well as to the mathematical modeling of outcrossing scenarios, we set out to develop an experimental system suitable for determining the frequency of occasional paternal transmission of plastid transgenes.

We have set up the system for tobacco (*Nicotiana tabacum*) for several reasons: (*i*) tobacco is currently the only species in which plastid transformation is a routine technique; (*ii*) tobacco plants produce huge numbers of seeds per plant, as is required for detection of even very low frequencies of paternal transmission; (*iii*) existing data (from studies in which spontaneous mutants were used) on chloroplast inheritance in tobacco are largely contradictory; and (*iv*) tobacco is one of the prime candidate species in agricultural biotechnology with numerous pharmaceutical-producing transgenic lines being already grown in large-scale field trials.

Results

A Genetic Screen for Paternal Leakage. To facilitate large-scale selection for occasional paternal transmission of transgenic chloroplasts, we devised an experimental system consisting of (*i*) male sterile plants as maternal parents and (*ii*) plastid-transformed (transplastomic) lines as pollen donors that carry a plastid-localized antibiotic resistance gene and a reporter gene for visualization of paternally transmitted plastids (Fig. 1). Male sterile tobacco plants were selected by tissue culture procedures employing somaclonal variation (see *Materials and Methods*). Male sterility was evidenced by the lack of fertile pollen from greenhouse-grown plants. Four independently generated male sterile lines were selected as maternal parents for our genetic screen for paternal transmission of transgenic plastids (Table 1). To generate the paternal parent, we transformed tobacco chloroplasts with a vector containing (*i*) a chimeric *aadA* gene (12) serving as a plastid-specific selectable marker gene and conferring resistance to the aminoglycoside antibiotics spectinomycin and streptomycin and (*ii*) a chimeric *gfp* gene providing a fluorescence marker for the paternal plastids (Fig. 1*A*). The transgenic construct was introduced into the plastid genome by

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Author contributions: R.B. designed research; S.R. and D.K. performed research; S.R. and R.B. analyzed data; and R.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: GM, genetically modified; RFLP, restriction fragment length polymorphism.

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www.pnas.org/cgi/doi/10.1073/pnas.0700008104

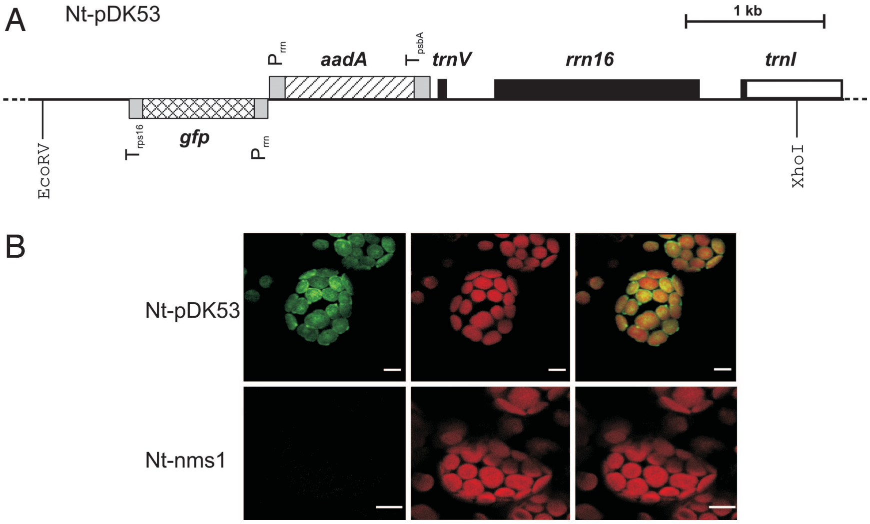

Fig. 1. Generation of parental lines for the screen for occasional paternal transmission of transgenic chloroplasts. (*A*) Physical map of the transgenic plastid genome in the paternal tobacco line Nt-pDK53. Genes above the line are transcribed from left to right, and genes below the line are transcribed in the opposite direction. Two transgenes are integrated into the intergenic spacer upstream of the tRNA-Val gene (*trnV*): the selectable marker gene *aadA* conferring resistance to spectinomycin and streptomycin (12), and the *gfp* gene serving as a reporter gene. Location of the EcoRV and XhoI restriction sites used for RFLP analysis is indicated. Prrn, chimeric rRNA operon promoter; TpsbA, 3′ untranslated region from the *psbA* gene; Trps16, 3′ untranslated region from the *rps16* gene. (*B*) GFP accumulation in transgenic chloroplasts. (*Left*) GFP. (*Center*) Chlorophyll. (*Right*) Overlay. Green GFP fluorescence and red autofluorescence of chlorophyll were detected by confocal laser scanning microscopy. The green GFP fluorescence is only visible in chloroplasts of the transplastomic paternal line Nt-pDK53, and not in the male sterile maternal line Nt-nms1. (Scale bars: 8 μm.)

biolistic transformation followed by selection for spectinomycin resistance and isolation of homoplasmic transplastomic lines. Homoplasmy was verified by DNA gel blot analysis and seed assays (data not shown). Strong GFP fluorescence localized to plastids confirmed high expression of the plastid *gfp* gene construct (Fig. 1*B*).

Having generated the two parental lines, we performed large-scale genetic crosses by pollinating male sterile lines with pollen obtained from the transplastomic lines. In this way, >2.5 million seeds were generated. To screen for occasional transmission of paternal plastids, seeds were surface-sterilized and sown on synthetic medium containing spectinomycin.

Identification of F_1 Progeny with Paternal Chloroplasts. More than two million germinating seedlings were screened for paternally inherited chloroplasts by searching for green tissue sectors in otherwise bleached-out white seedlings (Fig. 2 *A* and *B*). To be able to also detect small groups of green cells, visual inspection of all seedlings was followed by close inspection under a stereomicroscope. The presence of green sectors indicates resistance to spectinomycin and thus could be due to paternal inheritance of *aadA*-containing transgenic plastids. However, the appearance of spectinomycin-resistant green sectors is not necessarily indicative of paternal plastid inheritance because resistance can also be acquired through specific point mutations in the target molecule of the antibiotic, the 16S ribosomal RNA (13). To preliminarily distinguish between pollen transfer of transgenic plastids and spontaneous antibiotic resistance, green sectors were analyzed under a fluorescence stereomicroscope and assayed for GFP fluorescence (Fig. 2 *C* and *D*). Alternatively, cross-sections were inspected under a fluorescence microscope. These analyses identified several candidate lines (designated F1-spec lines) and proved to be a reliable indicator of paternal plastid transmission versus spontaneous resistance mutations (Table 1).

We found two types of partially spectinomycin-resistant seedlings, seedlings with green sectors in the cotyledons only (Figs. 2 *A* and *B* and 3*A*) and, less frequently, seedlings that also had green cells in their apical meristem (Fig. 3*B*). Whereas the former did not pass the spectinomycin-resistant chloroplasts into the growing shoot (and eventually died on spectinomycin-containing medium), the latter survived and continued to grow in the presence of the antibiotic (Fig. 3 *C* and *D*).

From all identified candidate seedlings (Table 1), tissue

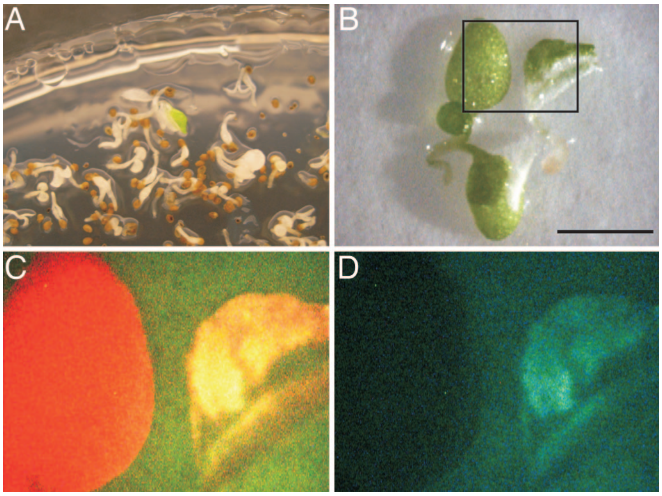

Fig. 2. Selection for paternal transmission of transgenic plastids. (*A*) Identification of a seedling displaying green cotyledon sectors on spectinomycin-containing medium. (*B*) Close-up of the seedling (line F1-spec1) in comparison to a green wild-type seedling (left) grown in the absence of spectinomycin. The frame marks the window observed under the UV stereomicroscope in *C* and *D*. (Scale bar: 0.5 cm.) (*C*) Detection of GFP fluorescence by using long pass filter GFP2. Whereas the wild-type seedling shows the red autofluorescence of the chlorophyll, the F1-spec1 seedling shows both red chlorophyll and green GFP fluorescence resulting in yellow overall fluorescence. (*D*) Detection of GFP fluorescence by using bandpass filter GFP3 (which excludes chlorophyll autofluorescence from detection). Only the green sectors in the F1-spec1 seedling show GFP fluorescence indicating that they contain paternally inherited transgenic chloroplasts.

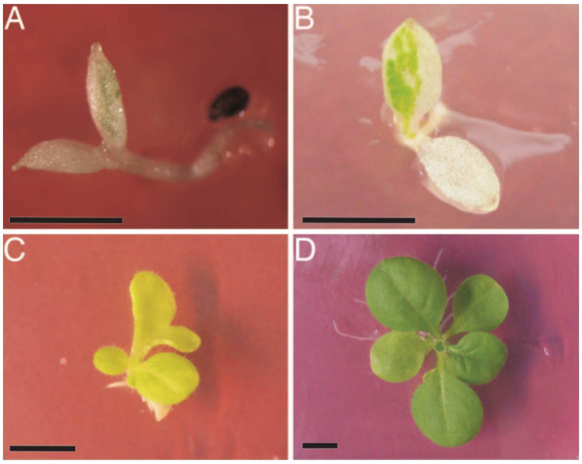

Fig. 3. Phenotypes of seedlings selected for paternal transmission of transgenic chloroplasts as evidenced by the presence of green tissue in F_1 seedlings. (*A*) Seedling with small green islands in one cotyledon (line F1-spec81). (*B*) Seedling with green sectors in a cotyledon and a green meristem (line F1-spec7). (*C* and *D*) Same seedling after 3 (*C*) and 5 (*D*) weeks of continued growth in the presence of spectinomycin. (Scale bars: 0.5 cm.)

samples were taken and exposed to spectinomycin-containing regeneration medium to select for homoplasmic spectinomycin-resistant cell lines. After two to three additional rounds of regeneration in the presence of the antibiotic, tissue samples were reanalyzed for GFP fluorescence by using UV and confocal laser scanning microscopy. These tests confirmed the results from the initial fluorescence stereomicroscopy in all cases and also demonstrated that GFP fluorescence localizes to plastids (data not shown).

We next wanted to confirm the presence of transgenic chloroplast genomes in the lines displaying GFP fluorescence. We therefore performed DNA gel blot analyses suitable to visualize wild-type and transgenic plastid genomes as restriction fragment length polymorphism (RFLP). Whereas all GFP-positive lines showed the restriction pattern of the paternal transgenic chloroplasts, all GFP-negative spectinomycin-resistant lines displayed the wild-type restriction pattern (Fig. 4*A* and Table 1). Interestingly, when different regeneration rounds were assayed, homoplasmy appeared to already be present in the first round of regeneration (Fig. 4*A*), suggesting that wild-type chloroplasts are effectively eliminated from the initially chimeric tissue by spectinomycin selection. To confirm homoplasmy of the paternally inherited transgenic plastids, crosses of F_1-spec lines with wild-type plants were performed. Seed assays revealed a homogeneous population of antibiotic-resistant F_1 seedlings, demonstrating that the resistance trait is maternally inherited, as expected (Fig. 4*B*).

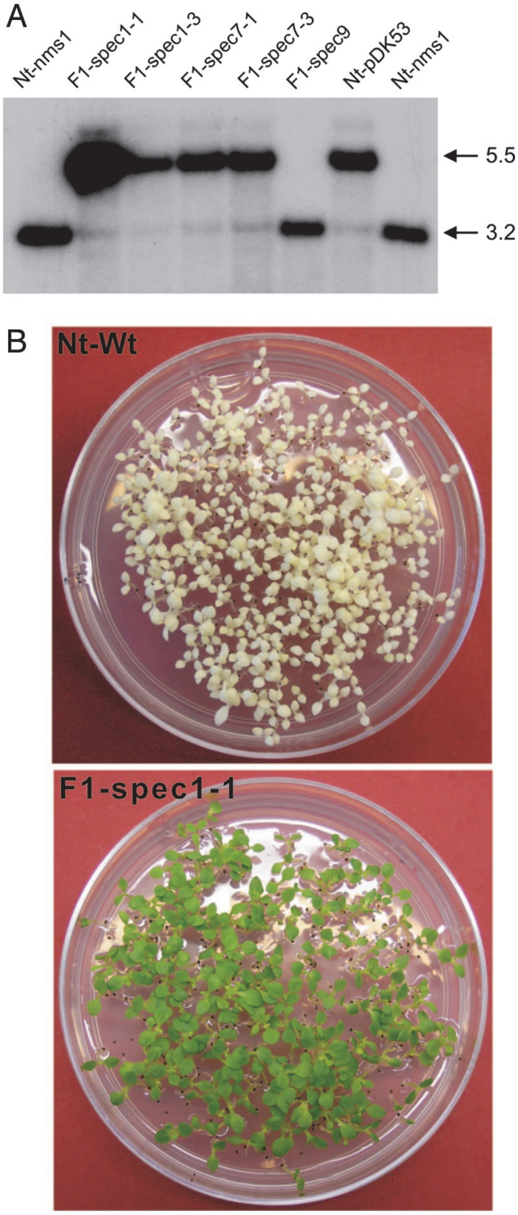

Fig. 4. Test for presence of transgenic plastids and their maternal inheritance after purification to homoplasmy. (*A*) RFLP analysis to confirm the presence of paternally inherited transgenic plastids in the selected lines. Two lines with paternal plastids (F1-spec1 and F1-spec7) are shown in comparison to a spontaneous spectinomycin-resistant mutant (F1-spec9), the paternal parent (Nt-pDK53), and a maternal parent (Nt-nms1) containing wild-type plastids. For the two lines with paternally inherited plastids, tissue samples from the first and third regeneration rounds were analyzed (indicated by the final number in the line designation). Weak bands at wild-type size correspond to promiscuous plastid DNA in the nuclear genome that is frequently seen in such RFLP analyses (e.g., see refs. 23 and 24). (*B*) Seed assays confirming homoplasmy of plants regenerated from green seedling tissue. The lower Petri dish shows the progeny from line F1-spec1 germinated on spectinomycin-containing medium. Antibiotic resistance of all seedlings confirms homoplasmy for the paternally inherited transgenic chloroplasts. The upper Petri dish displays the wild-type control, which is sensitive to the antibiotic.

Frequency of Paternal Plastid Transmission. Having demonstrated the occurrence of occasional paternal transmission of transgenic plastids, we were interested in quantitatively assessing the implications of our findings for plastid inheritance and transgene containment. Among 2.1 million seedlings, 118 seedlings with green tissue sectors on spectinomycin-containing medium were identified. Of those, 79 seedlings proved to represent spontaneous-resistance mutants (Table 1), as demonstrated by three lines of evidence. First, when tissue samples were exposed to plant regeneration medium containing streptomycin, they lacked resistance to streptomycin (data not shown). Though the *aadA* gene present in the transgenic chloroplasts confers resistance to both spectinomycin and streptomycin, all resistance-conferring point mutations are strictly antibiotic-specific and thus do not confer cross-resistance (1, 13). Second, lack of green GFP fluorescence also indicated absence of the second transgene, *gfp*. Third, the lines showed a wild-type-like restriction pattern in our RFLP analyses (Fig. 4). The remaining 39 lines with spectinomycin-resistant sectors clearly originated from paternal plastid

Table 1. Statistics of the selection experiments for paternal transmission of transgenic plastids

Maternal line	Seeds germinated	Seedlings with green sectors in cotyledons		Seedlings with green meristem	
		GFP-negative	GFP-positive	GFP-negative	GFP-positive
Nt-nms1	942,992	30	16	27	4
Nt-nms2	238,423	4	6	0	1
Nt-nms3	637,379	11	10	7	1
Nt-nms5	275,794	0	1	0	0
Total	2,094,588	45	33	34	6

The presence of transgenic (paternal) plastids in the shoot apical meristem was evidenced by continuing growth of the seedlings in the presence of spectinomycin.

transmission. This number is equivalent to a frequency of occasional paternal plastid transmission of $\approx 1.58 \times 10^{-5}$. It should be noted that, in all of these cases, the mode of plastid inheritance was biparental and not purely paternal, as evidenced by the presence of green sectors in otherwise white seedlings (Figs. 2 and 3 *A* and *B*). Interestingly, a significant number of lines contained the paternal transgenic chloroplasts only in their cotyledons. In these cases, the transgenic plastids are no longer present in the mature plants (because the cotyledons are subject to early senescence). Thus, the frequency of potentially stable paternal transmission of transgenic plastids is equivalent to the number of seedlings that contain paternal chloroplasts in their apical meristem. In our screen, we identified six such seedlings, amounting to a frequency of $\approx 2.86 \times 10^{-6}$.

Discussion

In this work, we have shown that plastid genome-encoded transgenes can be transmitted by pollen at low frequencies in the *N. tabacum* cultivar Petit Havana. Transmission into the shoot apical meristem was $\approx 2.86 \times 10^{-6}$ (on 100% cross-fertilization) and thus significantly lower than the overall frequency of transmission into the F_1 seedlings (1.58×10^{-5}). For transgene containment, only transmission into the meristem of the seedling is relevant, because the cotyledons senesce early and thus are not part of the harvest and do not contribute to the "germline."

What are the implications of these frequencies at the field level? Clearly, containment levels in the field would be significantly higher than the frequencies of occasional paternal transgene transmission measured in our experiments. This is because, like many crop species, tobacco has a strong tendency toward self-fertilization. Even if transgenic and wild-type tobacco plants are grown together in one plot, the rate of cross-fertilization is relatively low, usually $<10\%$ (14). If genetically modified and unmodified plants are separated by a corridor of 10 m, the outcrossing rate decreases by another order of magnitude and is well below 1% (14). Thus, it is reasonable to assume that, under field conditions, where transgenic and nontransgenic plants are grown separately, the frequency of occasional paternal transmission of transgenic plastids is, at most, in the range of 10^{-8}. This frequency makes plastid transformation an excellent tool for the prevention of unwanted transgene dispersal via pollen. Another factor that may additionally decrease the rate at which paternal plastids are present in the next generation is the absence of selection for paternal plastid transmission under field conditions. Although paternal plastids can be present in the meristem of F_1 seedlings (as shown in our study), these seedlings contain a mixed population of maternal and paternal plastids and thus are heteroplasmic. Heteroplasmy is unstable and, in the absence of selective pressure, one of the two plastid types is usually rapidly lost due to random somatic segregation of maternal and paternal plastids. It is therefore reasonable to assume that not all F_1 seedlings that initially contain paternal chloroplasts in their shoot apical meristem would transmit the paternal plastids into their floral meristem and thus into their germline.

These considerations and the very low frequency of paternal leakage notwithstanding, it has become clear from our study that plastid transformation does not ensure absolute transgene confinement. Although the level of containment conferred by the transplastomic technology is by far sufficient to facilitate coexistence of GM and non-GM crops in agriculture, it is conceivable that, for a subset of applications, it may be desirable to reduce the outcrossing risk to zero (e.g., for the production of certain pharmaceuticals or chemicals in plants). To achieve this, stacking of plastid transformation with other containment measures could provide a viable strategy. Containment methods that plastid transformation could be combined with include, for example, cytoplasmic male sterility, genetic use restriction technology, and transgene mitigation strategies (15, 16).

It also should be noted that the data obtained in this study determined the containment level of plastid transformation for tobacco, one of the most preferred species in molecular farming and the only species in which plastid transformation is currently routine. It remains to be investigated whether the frequencies measured here are directly transferable to other crop species that transmit their plastids (predominantly) maternally. Based on cytological investigations, three types of maternal inheritance have been defined (referred to as Lycopersicon type, Solanum type, and Triticum type) which differ in the mechanisms of plastid elimination during microsporogenesis, pollen development, and fertilization (5). Like most angiosperms investigated to date, tobacco belongs to the Lycopersicon type, in which unequal plastid distribution during the first pollen mitosis places all plastids in the vegetative cell and thus excludes them from the generative cell (17). Though it may be reasonable to assume that other species of the Lycopersicon type will show a similar frequency of paternal leakage as tobacco, this cannot necessarily be assumed for species belonging to the Solanum or Triticum types, in which plastids are regularly transmitted into the generative cell but eliminated before or during fertilization (17).

It is also important to realize that the frequency of cross-fertilization under field conditions varies greatly among species and is another factor that must be taken into account when assessing transgene containment levels in crops. Finally, it seems possible that environmental factors influence the rate of occasional paternal plastid transmission. The experimental system described here will also be suitable to quantitatively determine the influence of environmental factors (e.g., abiotic and biotic stresses) on plastid transgene inheritance.

Materials and Methods

Plant Material, Growth Conditions, and Isolation of Male Sterile Lines. Sterile tobacco plants (*N. tabacum* cultivar Petit Havana) were grown on agar-solidified Murashige and Skoog's medium containing 30 g/liter sucrose (18). Transplastomic lines were rooted and propagated on the same medium. Male sterile tobacco lines were selected in tissue culture employing somaclonal variation [the frequency of which was enhanced by regeneration in the presence of sublethal concentrations of spectinomycin or streptomycin (5–10 μg/ml)]. Male sterility was confirmed by complete absence of self-fertilization and seed production in greenhouse-grown plants.

Construction of an *aadA-gfp* Plastid Transformation Vector. A GFP expression cassette was constructed by fusing the *gfp*-coding region to plastid expression signals. As a promoter, the strong constitutive plastid ribosomal RNA operon promoter Prrn was used in combination with a synthetic Shine–Dalgarno sequence (12). To ensure transcript stability, the 3′ end of the *gfp*-coding region was fused to the 3′ untranslated region of the plastid *rps16* gene (19). The expression cassette was cloned as a SacI/HindIII restriction fragment into plastid transformation vector pPRV111A (20), generating the final transformation plasmid pDK53. In vector pDK53, the *gfp* reporter gene is directly linked to the *aadA* selectable marker gene ensuring cointegration of the two markers into the plastid genome by homologous recombination via the flanking chloroplast sequences (Fig. 1). Unwanted recombination between the Prrn promoter of the *aadA* and the endogenous Prrn promoter (driving the ribosomal RNA operon beginning with the 16S rRNA gene; Fig. 1*A*) could, in theory, result in loss of the *aadA* gene. However, because such events would also delete the essential *trnV* gene (Fig. 1*A*), they would be homoplasmic lethal and thus cannot interfere with the spectinomycin selection for paternal leakage.

Plastid Transformation and Selection of Homoplasmic Transformed Tobacco Lines. Young leaves from sterile tobacco plants were bombarded with plasmid-coated, 0.6-μm gold particles by using a biolistic gun (PDS1000He; Bio-Rad, Hercules, CA). Primary spectinomycin-resistant lines were selected on RMOP regeneration medium containing 500 mg/liter spectinomycin (12, 21). Spontaneous spectinomycin-resistant plants were eliminated by double selection on medium containing spectinomycin and streptomycin (500 mg/liter each) (1, 12). Several independent transplastomic lines were subjected to three additional rounds of regeneration on RMOP/spectinomycin to eliminate residual wild-type genome copies and isolate homoplasmic tissue. Homoplasmy was assessed by DNA gel blot analysis and ultimately confirmed by seed assays.

Crosses and Selection for Paternal Chloroplast Transmission. Large-scale crosses were conducted by pollinating male sterile plants with pollen from homoplasmic transplastomic plants. The progeny were assayed for paternal chloroplast transmission by germinating seeds on 0.5× Murashige and Skoog's medium in the presence of spectinomycin (500 mg/liter). Seedling phenotypes were analyzed by visual inspection under a stereo microscope (Zeiss, Oberkochen, Germany). Green sectors were excised from cotyledons or primary leaves and regenerated on RMOP medium containing 500 mg/liter spectinomycin. To eliminate spontaneous spectinomycin-resistant mutants, tissue samples were exposed to double selection on medium containing spectinomycin and streptomycin (500 mg/liter each). Whereas spontaneous resistance mutants bleach out on this medium, cells with transgenic chloroplasts remain green and continue to grow (1).

Isolation of Nucleic Acids and Hybridization Procedures. Total plant DNA was isolated by a rapid cetyltrimethylammoniumbromide-based miniprep procedure (22). DNA samples were digested with the restriction enzymes EcoRV and XhoI, separated on 1.0% agarose gels, and blotted onto Hybond N nylon membranes (Amersham, Piscataway, NJ). For hybridization, α[^{32}P]dCTP-labeled probes were generated by random priming (Multiprime DNA labeling kit; Amersham). A PCR product covering part of the 16S rRNA gene (prepared by amplification with primers P16Srrn-F 5′-CAAGCGGTGGAGCATGTGG-3′ and P16Srrn-R 5′-GGCGGTGTGTACAAGGCCC-3′) was used as probe for the RFLP analyses. Hybridizations were carried out at 65–68°C in Rapid Hybridization Buffer (Amersham) according to the manufacturer's instructions.

Microscopy. Green sectors in germinating seedlings were identified by visual inspection and light microscopy using a stereomicroscope (Stemmi 2000-C; Zeiss). GFP fluorescence in green sectors of germinating seedlings was detected with the MZ FLIII fluorescence stereomicroscope (Leica, Wetzlar, Germany) by using filters GFP2 (excitation filter, BP 480/40 nm; barrier filter, LP 510 nm) and GFP3 (excitation filter, BP 470/40 nm; barrier filter, BP 525/50 nm). Subcellular localization of GFP fluorescence was determined by confocal laser scanning microscopy (TCS SP2; Leica) with an argon laser for excitation (at 488 nm) and a 500–510 nm filter for detection of GFP fluorescence and a 610–700 nm filter for detection of chlorophyll autofluorescence.

We thank Maryam Mashagi, Beritt Bleicher, Stefanie Seeger, Annett Kassner, Sandra Stegemann, Claudia Hasse, and Daniela Ahlert for technical assistance and the Max-Planck-Institut für Molekulare Pflanzenphysiologie Green Team for plant care and cultivation. This work was supported by a grant from the European Union (Sixth Framework Program, Integrated Project "Co-Extra," Contract 007158) and by the Max Planck Society.

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