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Removal of antibiotic resistance genes from transgenic tobacco plastids

Siriluck Iamtham and Anil Day*

*3.614 Stopford Building,School of Biological Sciences, Manchester University, Oxford Road, Manchester M13 9PT, UK. *Corresponding author (anil.day@man.ac.uk).*

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Removal of antibiotic resistance genes from genetically modified (GM) crops removes the risk of their transfer to the environment or gut microbes. Integration of foreign genes into plastid DNA enhances containment in crops that inherit their plastids maternally. Efficient plastid transformation requires the *aadA* marker gene, which confers resistance to the antibiotics spectinomycin and streptomycin. We have exploited plastid DNA recombination and cytoplasmic sorting to remove *aadA* from transplastomic tobacco plants. A 4.9 kbp insert, composed of *aadA* flanked by *bar* and *uidA* genes, was integrated into plastid DNA and selected to remove wild-type plastid genomes. The *bar* gene confers tolerance to the herbicide glufosinate despite being GC-rich. Excision of *aadA* and *uidA* mediated by two 174 bp direct repeats generated *aadA*-free T_0 transplastomic plants containing the *bar* gene. Removal of *aadA* and *bar* by three 418 bp direct repeats allowed the isolation of marker-free T_2 plants containing a plastid-located *uidA* reporter gene.

Keywords: glufosinate, GM crops, marker-free, plastid transformation, tobacco

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Clean gene transformation technologies allow the introduction of desirable genes into crops without antibiotic marker genes or vector sequences[1]. Such technologies remove the possibility of antibiotic gene transfer to weeds or microorganisms in the environment and gut. Integration of foreign genes into the plastid genome enhances gene containment because in many crop plants plastids are inherited from the maternal parent preventing the pollen-mediated spread of transgenes[2–4]. Homologous recombination in plastids allows precise gene targeting into a well-characterized genome[5] and elimination of bacterial vector sequences[6,7]. The promise of high levels of gene expression has resulted in an increasing number of foreign genes being located to plastids[4,8–10].

The production of transplastomic plants in tobacco[4,8–12], *Arabidopsis*[13], and potato[14] has relied almost exclusively on the efficiently selected *aadA* gene, which encodes aminoglycoside 3” adenyl transferase and confers resistance to the antibiotics spectinomycin and streptomycin. Around 10,000 copies of the *aadA* gene are present per cell in transplastomic plants, and their expression is driven by prokaryotic regulatory sequences that also function in bacteria. Strong expression in bacteria and high copy number increase the potential risk of *aadA* transfer from plants to bacteria. Methods to produce transplastomic plants that are free of the *aadA* gene are clearly desirable. Here we describe an efficient procedure for generating *aadA*-free plants that contain either a *uidA* reporter gene or herbicide resistance gene located in plastids. The procedure is applicable to numerous other passenger genes, which need not confer a selectable phenotype.

Results and discussion

Plastid transformation vector design. Tobacco plastid transformation vector pUM71 (Fig. 1) was made by cloning plastid expression cassettes containing the *uidA*[15], *aadA*[16], and *bar*[17] coding regions into the *Aoc*I site of pTB27[18]. The *AocI* site is located in the intergenic region between the *rbcL* and *accD* genes of tobacco plastid DNA[5]. The 5′-regulatory sequences used in the cassettes were composed of the plastid 16S rRNA promoter fused to the ribosome binding site of the *rbcL* gene and are similar to those described[11]. Two copies of rrnHv, denoted A and B in Figure 1, create a 174 bp direct repeat in pUM71. All three genes are terminated by a 3′-NtpsbA regulatory element creating three directly repeated sequences of 418 bp (NtpsbA 1, 2, and 3 in Fig. 1).

The combined size of the *uidA*, *aadA*, *bar* genes and regulatory sequences is 4.9 kbp. Flanking 5.7 and 1.5 kbp plastid sequences in pUM71 promote integration into the plastid genome by homologous recombination (Fig. 1). The insertion event introduces a new *Hin*dIII site at the site of integration and results in the replacement of an 11.5 kbp *Hin*dIII fragment by two *Hin*dIII fragments of 9.5 and 6.9 kbp. Nonintegrated plasmid or integration at alternative sites will not produce this pattern because the external *Hin*dIII sites lie outside the plastid DNA region cloned in pUM71. The predicted outcomes of excision events mediated by NtpsbA or rrnHv repeats are shown (Fig. 1). High rates of recombination between rrnHv and rrnBn were not expected given their limited sequence identity. The rrnHv and rrnBn promoters share 78% base identity and the largest duplicated stretch is 17 bp long.

Generation of transplastomic plants. Plastid transformants were identified as green shoots or green callus on media containing spectinomycin and streptomycin after particle bombardment with plasmid-coated microprojectiles[11]. Green clones were transferred to media containing the herbicide glufosinate. The majority of spectinomycin- plus streptomycin-resistant clones were also resistant to the herbicide (42/45 clones). Blot analyses confirmed integration of foreign genes into plastid DNA of all 42 herbicide-resistant T_0 clones (data not shown). Leaves from 39 of the 42 herbicide-resistant clones tested positive for β-glucuronidase (GUS), which is the product of the *uidA* gene (data not shown). DNA blot analysis on 10 of the 42 herbicide-resistant clones is presented in Figure 2.

Digests were hybridized with probes specific for the *uidA*, *aadA*, and *bar* genes (Fig. 2). Blots were also probed with a plastid DNA fragment (cpDNA probe) spanning the integration site and a nuclear ribosomal DNA probe (nrDNA) to verify similar DNA

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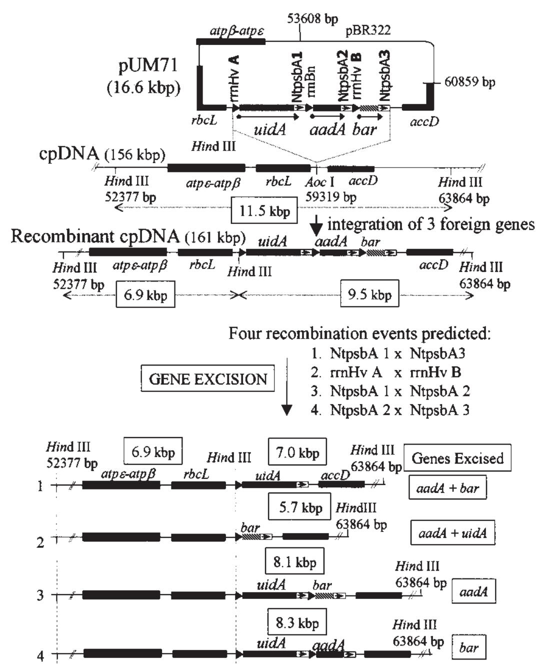

Figure 1. pUM71 integration into the tobacco plastid genome (cpDNA) and predicted gene excision events (not to scale). pUM71 contains a 4.9 kbp insert composed of the *uidA*, *aadA*, and *bar* genes cloned into the *Aoc*I site of pTB27. Common 5′ rrn and 3′ NtpsbA regulatory elements create two rrnHv direct repeats (▶) of 174 bp and three 418 bp NtpsbA direct repeats (⟶|). The integration event by homologous recombination replaces a wild-type 11.5 kbp *Hin*dIII plastid fragment with two fragments of 6.9 and 9.5 kbp. Gene excision events mediated by recombination between direct repeats result in a size reduction of the 9.5 kbp band. The map coordinates refer to locations in the 155,939 bp tobacco plastid genome.

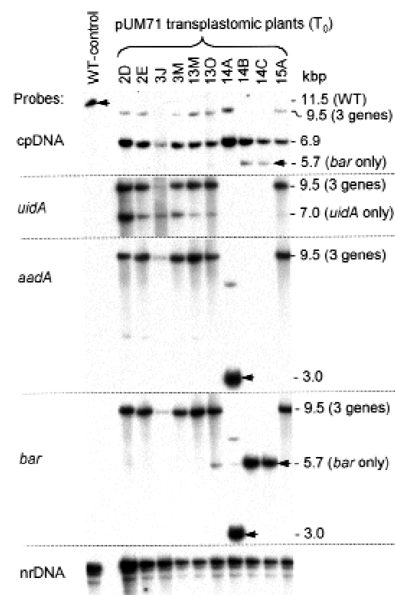

Figure 2. DNA blot analysis of pUM71 transformants. *Hin*dIII digests of DNA from 10 independent plastid transformants hybridized with the indicated probes. Band sizes were determined from the tobacco plastid DNA sequence and molecular weight markers. Bands located some distance from their indicated sizes (right) are arrowed. nrDNA, nuclear ribosomal DNA probe.

loadings per lane. Wild-type (WT) plants contain an 11.5 kbp *Hin*dIII fragment that hybridizes to the cpDNA probe. Three patterns of hybridization were observed in pUM71 transplastomic plants. In the majority of plants (39 clones) the 11.5 kbp WT plastid band is replaced by 9.5 kbp and 6.9 kbp bands, indicating integration of three foreign genes (see map of recombinant cpDNA in Fig. 1). The 9.5 kbp band hybridizes to the *uidA*, *aadA*, and *bar* gene probes. Clones 2D, 2E, 3J, 3M, 13M, 13O, and 15A illustrate this hybridization pattern (Fig. 2). Most of these clones also produce a 7.0 kbp band that hybridizes to the *uidA* gene probe but not the *aadA* or *bar* gene probes. The 7.0 kbp *uidA* band co-migrates with the 6.9 kbp plastid DNA band when probed with the cpDNA probe (Fig. 2). This 7.0 kbp *uidA* band is the result of excision of the *aadA* and *bar* genes (case 1 in Fig. 1) and is diagnostic of a marker-free plastid genome. The detection of 9.5 kbp and 7.0 kbp bands in DNA digests from a single plant is indicative of two recombinant plastid DNA types in a single plant (heteroplasmy). One plastid DNA type contains all three foreign genes, whereas the second type only contains the *uidA* gene.

Clones 14B and 14C illustrate the second pattern of hybridization obtained. In these plants, the 11.5 kbp WT band is replaced by 6.9 kbp and 5.7 kbp bands. The 5.7 kbp band results from excision of the *uidA* and *aadA* genes mediated by recombination between rrnHv A and B (Fig. 1, case 2), and this 5.7 kbp band hybridizes to the *bar* gene (arrowed in Fig. 2) but not the *uidA* or *aadA* gene probes. Leaves from 14B and 14C plants did not contain detectable GUS activities (data not shown). Polymerase chain reaction (PCR) analyses confirmed the absence of the *uidA* and *aadA* genes in these plants (see below and Fig. 6B). Clones 14B and 14C are homoplasmic for the *aadA*-free plastid genome containing the *bar* gene because they only contain one type of recombinant plastid genome. Plants 14B and 14C represent two of the original 42 clones resistant to both antibiotics and herbicide. The early switch from spectinomycin/streptomycin to glufosinate selection is compatible with the isolation of these herbicide-resistant *aad*-free plants in the T_0 generation.

Aberrant integration and/or recombination events after integration are responsible for the last pattern of hybridization. For example, clone 14A lacks the *uidA* gene and GUS activity but contains an aberrant 3 kbp band (Fig. 2, arrowed) that hybridizes to the *aadA* and *bar* gene probes but not the cpDNA probe.

A plastid-located GC-rich *bar* gene confers herbicide resistance and is inherited maternally. Nuclear transgenic plants expressing the *bar* gene exhibit high levels of resistance to glufosinate[19]. Most foreign genes that have been expressed successfully in plastids have GC contents of <55%(refs 4,8–11,20). The *bar* gene used here is 68% GC-rich[17]. Nevertheless, a plastid-located *bar* gene confers tolerance to the herbicide glufosinate. The glufosinate tolerance of T_1 transplastomic plants was compared with WT plants. Clone 13G (not shown in Fig. 2, but see Fig. 4) contains all three foreign genes integrated correctly and is representative of the major class of pUM71 transformants. Wild-type seeds germinated on medium containing 5 μg/ml glufosinate but produced white seedlings that died (Fig. 3A). In contrast, 13G seeds germinated to produce green, resistant seedlings on media containing 25 μg/ml glufosinate. When soil-grown plants were compared, WT plants were killed when sprayed with a 0.1% (vol/vol) solution of the herbicide Challenge (Fig. 3B), whereas 13G (T1) plants withstood a 2.5% (vol/vol) herbicide solution.

In crosses involving 13G as the maternal parent (emasculated female flowers) and nontransformed WT plants as the paternal parent (pollen donor), all progeny were herbicide resistant (Figs 3A–C). In contrast, all progeny from crosses involving 13G as paternal parent were herbicide sensitive (Fig. 3C). This maternal inheritance pattern is diagnostic of a resistance gene integrated into plastid DNA.

Recombination events in transplastomic plants. Foreign gene excision in pUM71 transplastomic plants is a continual process that accompanies vegetative growth and transmission through sexual crosses. Leaf samples from some of the T_1 progeny of parent plant

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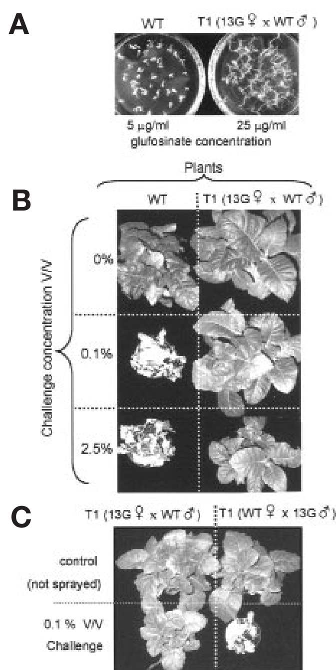

Figure 3. Herbicide tolerance of pUM71 transplastomic plants. (A) Control wild-type (WT) and pUM71 transplastomic seeds (T_1) germinated on MS medium containing glufosinate. (B) WT and pUM71 transplastomic plants (T_1) sprayed at 45, 49, and 53 days following sowing with the indicated dilutions of Challenge. Plants were photographed on day 71. Each pot contained four plants. (C) Five plants derived from reciprocal crosses of transplastomic clone 13G and wild-type (WT) tobacco were sprayed as indicated on days 36, 43, and 50 after sowing and photographed on day 57. Female recipient (♀) and pollen donor (♂) plants are labeled.

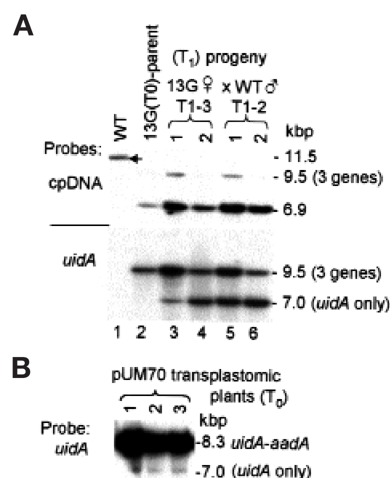

Figure 4. Marker gene excision in pUM71 and pUM70 transplastomic plants. (A) Blot analysis of *Hin*dIII digests of DNA from two progeny plants 13G(T1)-2 and 13G(T1)-3 and transplastomic parent 13G(T0). Two DNA extracts were prepared from separate leaves of a single plant. (B) Blot analysis of *Hin*dIII digests of DNA from three independent pUM70 transplastomic plants (T0). Hybridization probes and band sizes are indicated.

13G contain high levels of the marker-free plastid genome, revealed by a dark 7.0 kbp band (Fig. 4A, bottom panel, lanes 3–6), whereas parent 13G (Fig. 4A, lane 2) does not. The 11.5 kbp WT band was not detected in digests of DNA from 13G (T0) and 13G (T1) plants (Fig. 4A, top panel, lanes 2–6).

The products of single-gene excision (Fig. 1, cases 3 and 4) are *Hin*dIII bands of 8.1 (*aadA* loss) and 8.3 kbp (*bar* loss). These bands are not readily identified between the 9.5 kbp three-gene band and 7.0 kbp *uidA* band in digests probed with *uidA* (Figs 2 and 4A). The intermediates are not intrinsically unstable. This was deduced by using a two-gene construct (pUM70), which is identical to pUM71 except that it lacks the *bar* gene and associated 5′ and 3′ regulatory regions. The 8.3 kbp *Hin*dIII band, which contains *uidA* and *aadA*, is clearly stable and visible in digests of DNA from three independent pUM70 transformants (Fig. 4B). These results suggest that when plastid recombination is activated by the multiple direct repeats in pUM71 plants, recombination events do not stop until all local direct repeats have been removed. When two direct repeats are present, excision is not observed when repeat length is reduced to 137 bases in tobacco[21] and to 230 bases in *Chlamydomonas reinhardtii*[22]. A minor 7.0 kbp *uidA*-only band indicates a low frequency of excision mediated by the two 418-base NtpsbA repeats in pUM70 transformants (Fig. 4B).

Isolation of marker-free transplastomic plants containing *uidA*. Efficient recombination between NtpsbA 1 and NtpsbA 3 (Fig. 1, case 1) produces heteroplasmic plants, which contain the original 161 kbp recombinant genome selected with two antibiotics and glufosinate, and marker-free plastid genomes only containing *uidA*. Although persistence of heteroplasmy has been reported[23], cells with a mixed population of two plastid DNA types often convert to homoplasmy after repeated cell divisions[24]. This process of cytoplasmic sorting will generate cells, organs, and branches, which contain only marker-free recombinant plastid genomes. Partitioning of individual homoplasmic cells into eggs will, upon fertilization and germination of seeds, give rise to marker-free seedlings. Isolation of marker-free seedlings from a heteroplasmic parent is facilitated by maternal inheritance of tobacco plastids.

Seeds from selfed flowers of 13G (T1)-2 (Fig 4A, lanes 5 and 6) were collected and germinated on medium containing spectinomycin. Resistant seedlings containing the *aadA* gene germinate to produce green cotyledons, whereas the cotyledons of sensitive seedlings are white (Fig. 5A, left panel). Out of a total of three hundred and twenty-six 13G(T2) seedlings germinated on spectinomycin, 199 were green (61%), 79 were completely white (24%), and 48 variegated (15%). Green-white variegation is indicative of a heteroplasmic mixture of resistant and sensitive plastids, and these plants were not studied further. Green, resistant seedlings were GUS positive (data not shown). Spectinomycin-induced bleaching is reversible in tobacco[25]. Wholly white 13G(T2) seedlings were transferred to Murashige–Skoog (MS) medium lacking spectinomycin to allow recovery of plastid protein synthesis and *uidA* expression. Recovery of plastid protein synthesis allows greening of the first true leaves (Fig. 5A, middle panel). Thirty-three of the spectinomycin-sensitive 13G(T2) seedlings recovered to produce green true leaves. All 33 seedlings were GUS positive (data not shown). GUS activity in these seedlings was largely localized to the green true leaves (Fig. 5A, right panel), where restoration of plastid protein synthesis was complete. Six spectinomycin-sensitive seedlings, 13G(T2)-0.1, -1.1, -2.1, -3.1, -3.2, -7.1, and one resistant seedling, 13G(T2)-R, were grown up and studied in more detail.

Phenotype of marker-free 13G(T2) transplastomic plants. All six spectinomycin-sensitive 13G(T2) transplastomic plants exhibited identical responses, and only the results obtained with 13G(T2)-1.1 are shown in Figure 5B. Leaf pieces from 13G(T2)-1.1 resemble WT leaf pieces and bleached on medium containing either spectinomycin or glufosinate. Spectinomycin-resistant 13G(T2)-R leaf pieces regenerated extensively into green shoots on medium containing either spectinomycin or glufosinate (Fig. 5B, middle column) as a result of the presence of plastid-located *aadA* and *bar* genes. No GUS activity was detected in WT leaf pieces. In contrast, GUS staining was readily detected in leaf pieces from marker-free 13G(T2)-1.1 and resistant 13G(T2)-R transplastomic plants (Fig. 5B).

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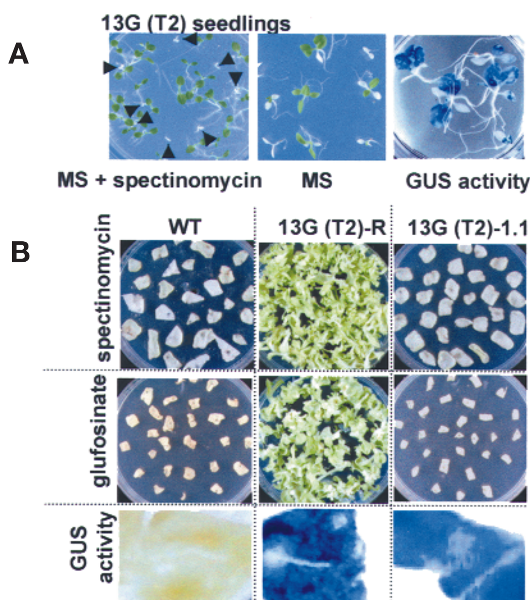

Figure 5. Isolation of marker-free transplastomic plants. (A) Selfed seeds (T_2) from transplastomic plant 13G(T1)-2 were surface-sterilized and plated on MS salts medium containing 500 µg/ml spectinomycin (left panel, bleached seedlings are arrowed). Re-greening of bleached 13G(T2) seedlings on MS salts medium lacking spectinomycin (middle panel), GUS activity in re-greened leaves of spectinomycin-sensitive 13G(T2) seedlings (right panel). GUS is the product of the *uidA* reporter gene and converts X-Gluc to a blue product. (B) Leaf pieces from aseptic WT, 13G(T2)-R, and 13G(T2)-1.1 placed on RMOP medium containing either 500 µg/ml spectinomycin (top row) or 5 µg/ml glufosinate (middle row). GUS assays on aseptic leaves (bottom row).

Molecular analyses of marker-free transplastomic plants. The plastid genomes in 13G(T2) marker-free transplastomic plants were analyzed using DNA blots and PCR (Fig. 6). The positions of restriction sites in plastid DNA and foreign genes are shown (Fig. 6C). The 11.5 kbp *Hin*dIII and 6.0 kbp *Pvu*II bands present in WT lanes were not visible in digests of DNA samples from marker-free plants or resistant plant 13G(T2)-R. In resistant plant 13G(T2)-R, 9.5 and 6.9 kbp *Hin*dIII bands and *Pvu*II bands of 8.6 and 2.4 kbp (not visible in Fig. 6A) replace these WT bands. A *Pvu*II site within the *bar* gene is responsible for the 2.4 kbp right junction fragment that hybridizes to the *bar* gene in digests of DNA from 13G(T2)-R (data not shown). *Pvu*II digests of DNA from marker-free transplastomic plants contain a single 8.4 kbp band that hybridizes with the cpDNA and *uidA* gene probes. This is consistent with retention of the 2.4 kbp *uidA* cassette in the 6.0 kbp WT *Pvu*II fragment. The cpDNA probe hybridizes to co-migrating 6.9 and 7.0 kbp *Hin*dIII bands in marker-free lanes as a result of a *Hin*dIII site located in front of the *uidA* gene. The 7.0 kbp *Hin*dIII band hybridizes to the *uidA* gene probe. No hybridization was detected between the *aadA* or *bar* probes and *Hin*dIII or *Pvu*II digests of DNA from marker-free plants. These results demonstrate that all six marker-free 13G(T2) plants contain a homoplasmic population of recombinant plastid genomes lacking the *aadA* and *bar* genes.

We used PCR to confirm the absence of marker genes and WT plastid genomes in marker-free 13G(T2) transplastomic plants. The locations of PCR primers are shown in Figure 6C. Amplification was carried out with primers cp-F, cp-R, and *uidA*-R (top panel, Fig. 6B). Any WT plastid DNA present in transplastomic plants would be revealed by the presence of a 0.82 kbp WT band amplified by primers cp-F and cp-R. A WT band was not detected in PCR products from transplastomic plants (Fig. 6B). A 1.14 kbp recombinant band amplified by the cp-F and *uidA*-R primers was visible in 13G(T2)-marker-free lanes and the

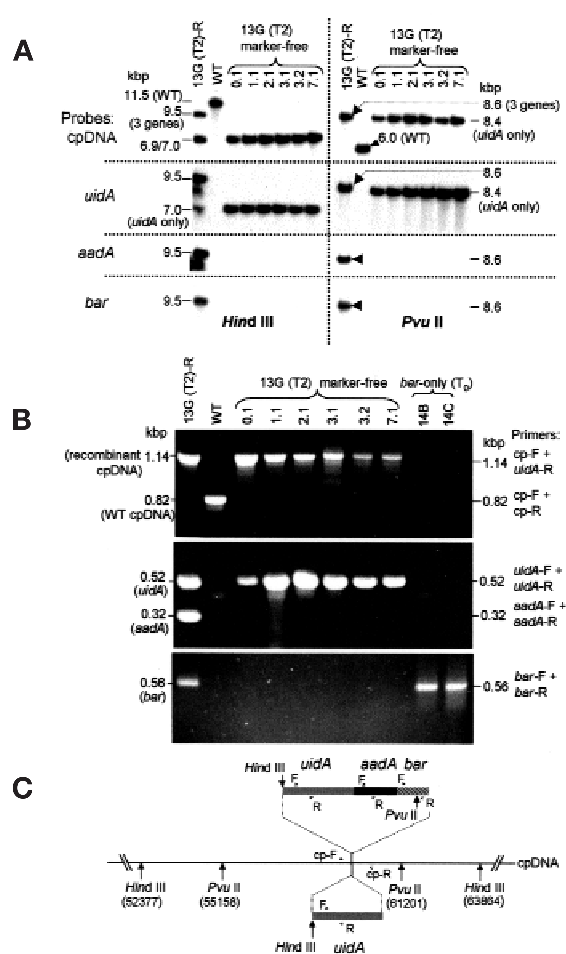

Figure 6. Molecular analyses of *aadA*-free transplastomic plants. (A) Blot analysis of *Hin*dIII digests (left) and *Pvu*II digests (right) of DNA samples from WT and transplastomic plants hybridized with the indicated probes. Band sizes are shown. (B) PCR analysis of WT and transplastomic plants. Top panel: amplification with primers cp-F, cp-R, and *uidA*-R. Middle panel: amplification with primers *aadA*-F, *aadA*-R, *uidA*-F, and *uidA*-R. Bottom panel: amplification with primers *bar*-F and *bar*-R. Band identities are indicated on the left and the primers responsible for amplifying the bands are shown on the right. (C) Locations of *Hin*dIII and *Pvu*II sites flanking the insertion site. Maps of the 4.9 kbp three-gene insert and 2.4 kbp *uidA* insert are shown above and below the map of plastid DNA, respectively. Locations of PCR primers are shown.

13G(T2)-R lane. This 1.14 kbp *uidA* left junction band was not visible in lanes 14B and 14C, which is consistent with complete replacement of *uidA* by *bar* at the left junction of the foreign insert in plants 14B and 14C (Fig. 1, case 2).

Amplification with primers *aad*-F, *aad*-R, *uidA*-F, and *uidA*-R produces 0.32 kbp *aadA* and 0.52 kbp *uidA* bands in the 13G(T2)-R lane (Fig. 6B, middle panel). No other lanes contained the *aadA* band, indicating the absence of this gene in WT plants, marker-free 13G(T2) plants, and herbicide-resistant plants 14B and 14C. The presence of a 0.52 kbp *uidA* band in 13G(T2)-marker-free lanes verifies successful amplification and rules out the absence of an *aadA* band due to a technical fault. Because of a high GC content, the primers *bar*-F and *bar*-R were used alone. A 0.56 kbp amplified *bar* band was detected only in DNA samples from 13G(T2)-R and the herbicide-resistant T_0 transplastomic plants 14B and 14C (bottom panel, Fig. 6B).

Our combined results based on phenotypic and molecular analyses demonstrate the isolation of homoplasmic *aadA*-free transplas-

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tomic plants containing either a plastid-located *uidA* or *bar* gene. We have used native recombination processes in plastids to excise undesirable genes. The sizes and number of direct repeats control the frequency of gene excision, whereas the organization of repeats directs the pattern of gene excision. Whereas the integration site chosen here has been previously used to isolate homoplasmic plastid transformants[4,11,14], some foreign DNA inserts targeted to this region may prevent homoplasmy[26]. The procedure we have outlined is not restricted to the insertion site used here. The facile removal of marker genes from transplastomic plants addresses some of the current concerns over GM crops and will facilitate the use of plastid transformation technology in agriculture.

Experimental protocol

Construction of plastid transformation vectors. Standard cloning methods were used[27]. The rrnHv and rrnBn sequences have been deposited in the EMBL/DDBJ/GenBank databases (EMBL accession numbers AJ276676, AJ276677). Primers for PCR as follow were used to amplify NtpsbA from tobacco DNA: 5´-CCCAAGCTTCTGCAGGCCTAGTCTATAGGAGG-3´ and 5´-GGGAAGCTTGGATCCTAAGGAATATAGCTCTTC-3´. A 0.80 kbp *Nco*I-*Pst*I aadA fragment16, 1.8 kbp *Nco*I-*Sma*I *uidA* fragment[15], and 0.57 kbp *Nco*I-*Pst*I *bar* fragment were used to construct pUM71. The ends of the *bar* coding region[17] were altered using PCR primers 5´-CCCCCCCATGGGC-CCAGAACGACGCCC-3´ (*bar*-F) and 5´-TTATTAGATCTCGGT-GACGGGCAG-3´ (*bar*-R).

Generation and analysis of transplastomic plants. Plastid transformants were generated by particle bombardment of *Nicotiana tabacum* cv. Wisconsin 38 essentially as described[11]. Shoots and green cell lines were selected on spectinomycin dihydrochloride pentahydrate (Duchefa, Haarlem, the Netherlands) plus streptomycin sulfate (Sigma, St. Louis, MO), each at 500 mg/ml. Clones were transferred to RMOP medium[7] containing 5 mg/ml glufosinate-ammonium (Dr. Ehrenstorfer GmbH, Augsburg, Germany) after 9–34 weeks for a second cycle of regeneration. Three of 45 clones were spectinomycin-plus-streptomycin resistant, GUS-positive, but sensitive to glufosinate. The 42 herbicide-resistant clones were rooted on MS medium containing 1 mg/ml glufosinate-ammonium and transferred to soil. T_0 plants were sprayed weekly with a 0.1% (vol/vol) solution of Challenge (AgrEvo, King's Lynn, UK) containing 150 g of glufosinate-ammonium per liter. Transformants originating from well-separated leaf pieces were presumed to result from independent transformation events. The clone number identifies the bombardment from which it was obtained. Blot analysis[27] was on DNA prepared using Promega (Madison, WI) extraction kits. The cpDNA probe used was a 3.4 kbp *Cla*I-*Eco*RV fragment of plastid DNA (map coordinates 57,176 to 60,604 bp; ref. 5). For PCR analysis (34 cycles) the following primers were used against 50–100 ng of total DNA from transplastomic plants: cp-F 5´-AGCAGTG-GACGTTTTGGATAAGTAA-3´, cp-R 5´-GCATGAAAATACAATAGAT-GAATAG-3´, *aadA*-F 5´-CGTCATCGAGCGCCATCTCGAA-3´, *aadA*-R 5´-TGGCTCGAAGATACCTGCAAGAAT-3´, *uid*A-F 5´-GCGTTACAA-GAAAGCCGGGCAAT-3´, *uid*A-R 5´-CACAGTTTTCGCGATCCAGACT-GAATG-3´, *bar*-F and *bar*-R (listed above). GUS assays were carried out according to Jefferson[15].

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