LETTER

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Horizontal genome transfer as an asexual path to the formation of new species

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Allopolyploidization, the combination of the genomes from two different species, has been a major source of evolutionary innovation and a driver of speciation and environmental adaptation[1–4]. In plants, it has also contributed greatly to crop domestication, as the superior properties of many modern crop plants were conferred by ancient allopolyploidization events[5,6]. It is generally thought that allopolyploidization occurred through hybridization events between species, accompanied or followed by genome duplication[6,7]. Although many allopolyploids arose from closely related species (congeners), there are also allopolyploid species that were formed from more distantly related progenitor species belonging to different genera or even different tribes[8]. Here we have examined the possibility that allopolyploidization can also occur by asexual mechanisms. We show that upon grafting—a mechanism of plant–plant interaction that is widespread in nature—entire nuclear genomes can be transferred between plant cells. We provide direct evidence for this process resulting in speciation by creating a new allopolyploid plant species from a herbaceous species and a woody species in the nightshade family. The new species is fertile and produces fertile progeny. Our data highlight natural grafting as a potential asexual mechanism of speciation and also provide a method for the generation of novel allopolyploid crop species.

Grafting commonly occurs in nature[9–13] and the observation of natural grafting may have inspired the application of grafting in agriculture and horticulture hundreds of years BCE[14]. Natural stem grafts result from the mechanical pressure of interlocking stems or branches, leading to tissue fusion and establishment of new vascular connections. Natural root grafts[11,12] may influence population ecology by allowing a community of plants to share water, minerals and metabolites[14]. In nature, interspecific grafts have been observed between very distantly related species[9]. Recent work has demonstrated that entire chloroplast genomes can be horizontally transferred across the graft junction, potentially explaining so-called organelle capture events that have occurred in evolution[15–17]. If nuclear genomes are also exchanged between neighbouring cells in natural grafts, this would provide a straightforward asexual mechanism by which new allopolyploid species could form. Indeed, microscopic evidence supports the idea of nuclear material occasionally moving from cell to cell in intact plant tissues, a phenomenon known as cytomixis[18–20].

To investigate the possibility that grafting enables the transfer of nuclear DNA, we generated two transgenic tobacco (*Nicotiana tabacum*) plants carrying different selectable marker genes. Line Nt-kan:yfp carries the kanamycin resistance gene *nptII* (and the gene for the yellow fluorescent reporter YFP) in its nuclear genome, whereas line Nt-hyg contains

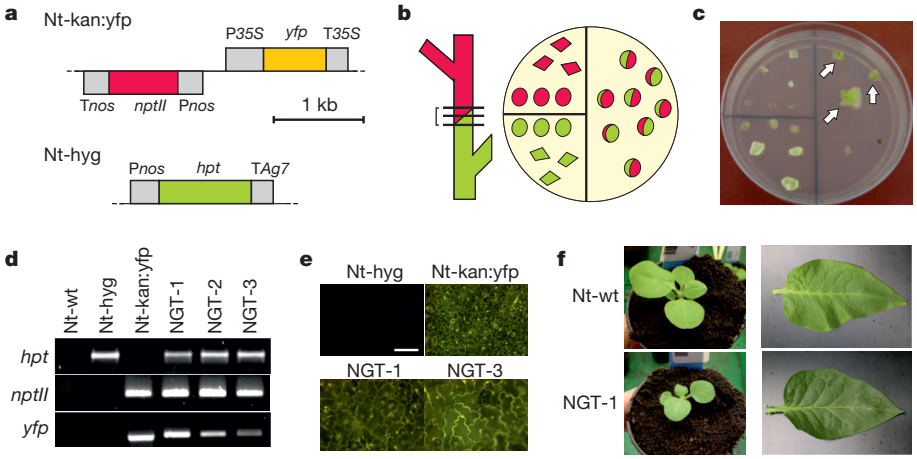

Figure 1 | Grafting and experimental selection for horizontal transfer of nuclear DNA between tobacco plants. **a**, Map of the transgenic loci in the two tobacco lines used for grafting and selection. Genes above the line are transcribed from left to right, genes below the line in the opposite direction. Expression elements (promoters, terminators) are shown in grey. P*nos*, nopaline synthase promoter; P*35S*, CaMV 35S promoter; T*nos*, nopaline synthase terminator; T*35S*, CaMV 35S terminator; T*Ag7*, *Ag7* terminator from *Agrobacterium tumefaciens*; *nptII*, kanamycin resistance gene; *hpt*, hygromycin resistance gene; *yfp*, yellow fluorescent protein gene. **b**, Schematic drawing of the grafted stem regions, the sectioning of the graft site (horizontal lines) and the selection for gene transfer. The two transgenic lines are coloured according to the antibiotic resistance gene they carry (see **a**). The Petri dish is split in three parts, with the left two quarters containing leaf and stem sections from the two graft partners and the right half containing sectioned graft sites. **c**, Selection of callus resistant to kanamycin and hygromycin on medium containing both antibiotics. White arrows indicate green resistant tissue. **d**, Presence of all three transgenes in doubly resistant lines (nuclear gene transfer, NGT lines). PCR assays were performed with wild-type tobacco (Nt-wt), the two graft partners (Nt-kan:yfp, Nt-hyg) and three independently generated NGT lines (see Methods). **e**, Analysis of YFP fluorescence by ultraviolet microscopy. Note the differences in cell size between Nt-kan:yfp plants and NGT plants. Scale bar, 100 μm. **f**, Phenotype of NGT plants. Growth of NGT seedlings is delayed compared to wild-type seedlings (left panel, picture taken 23 days after sowing). Comparison between a wild-type leaf and a leaf from an NGT plant reveals enhanced pigmentation of NGT leaves (right panel).

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the hygromycin resistance gene *hpt* (Fig. 1a). The two lines were grafted onto each other and, after fusion of scion and stock had occurred, the graft site was excised and subjected to double selection for kanamycin and hygromycin resistance (Fig. 1b). Although tissue explants from the two graft partners cannot grow on this medium and bleach out (Fig. 1c), surprisingly, the formation of resistant calli from the graft sites was observed frequently (29 independent events selected from 13 grafts). Doubly resistant tissue readily regenerated into shoots, which were tested for the presence of the two resistance genes and the *yfp* reporter gene (Fig. 1d). PCR assays detected all three transgenes in the selected lines (referred to as NGT lines, for nuclear gene transfer), tentatively suggesting that nuclear gene transfer across the graft junction had occurred (Fig. 1d). As a preliminary investigation to distinguish between transfer of individual genes and transfer of entire nuclear genomes, the macroscopic and microscopic phenotypes of the regenerated plants were examined. Cell size is correlated to nuclear DNA content[21] and, therefore, polyploid plants often have larger cells than their diploid ancestors. Indeed, NGT plants had larger cells than *N. tabacum* (Fig. 1e), indicating that they contain significantly more DNA. When NGT plants were grown in the greenhouse, they were smaller than wild-type plants and exhibited more intense leaf pigmentation (Fig. 1f). NGT plants were fertile and produced seeds from which the next generation was raised to determine chromosome number and DNA content in individual seedlings. The chromosome set of *N. tabacum* comprises 48 chromosomes. Although 48 chromosomes were present in Nt-kan:yfp and Nt-hyg plants, all T_1 progeny of the NGT lines displayed increased chromosome numbers (Extended Data Fig. 1). The numbers were variable, but a large fraction of the plants contained 96 chromosomes. Hence, the NGT lines are autopolyploid (tetraploid or, considering the allopolyploid origin of *Nicotiana tabacum*, autoallooctoploid). Newly formed autopolyploids may be meiotically unstable and experience the loss of individual chromosomes due to missegregation[4], potentially explaining why some NGT progeny had fewer than 96 chromosomes. Indeed, when meiotic chromosome segregation was analysed in pollen mother cells, aberrant segregation patterns were observed (Extended Data Fig. 2). Consistent with occasional chromosome loss, the F_1 progeny displayed considerable phenotypic variation (Extended Data Fig. 3). Measurements of nuclear DNA content by flow cytometry confirmed the tetraploid status of NGT plants (and the triploid status of progeny from a cross with the wild type; Extended Data Fig. 1c). Tetraploidy was further confirmed by inheritance tests analysing the segregation of the two antibiotic resistances (Extended Data Table 1).

Grafting and tissue culture procedures can occasionally induce spontaneous polyploidization or formation of chimaeras. Although our inheritance analyses excluded the possibility of NGT plants being chimaeras composed of Nt-kan:yfp cells and Nt-hyg cells, the data do not entirely rule out the remote possibility that the resistance gene was horizontally transferred and the genome duplication occurred independently through spontaneous autopolyploidization in all NGT lines. To definitively prove that entire nuclear genomes are horizontally transferred and to examine whether this transfer provides an asexual mechanism by which new allopolyploid species can form, grafting experiments with two different plant species were performed using the tree tobacco, *Nicotiana glauca* (a woody species), and the cigarette tobacco, *Nicotiana tabacum* (a herbaceous species). *N. glauca* was equipped with the *nptII* gene, whereas *N. tabacum* harboured the *hpt* gene (Fig. 1a). Grafting experiments were performed both *in vitro*[15] and in the greenhouse, mimicking natural stem grafting as occurring in 'kissing trees' (Fig. 2a). From a total of twelve grafted plants, 45 doubly resistant lines were obtained (Fig. 2b) and regenerated into plantlets (Fig. 2c). DNA content measurements revealed that the plants have genome sizes equalling the sum of the *N. glauca* and *N. tabacum* genomes (Fig. 2d). Analysis of molecular markers confirmed the presence of genetic material from both species (Fig. 3), indicating that allopolyploid plants had indeed been obtained through grafting. As plants combining the genomes of *N. tabacum* and *N. glauca* potentially represent a new species, we tentatively named them *Nicotiana tabauca*.

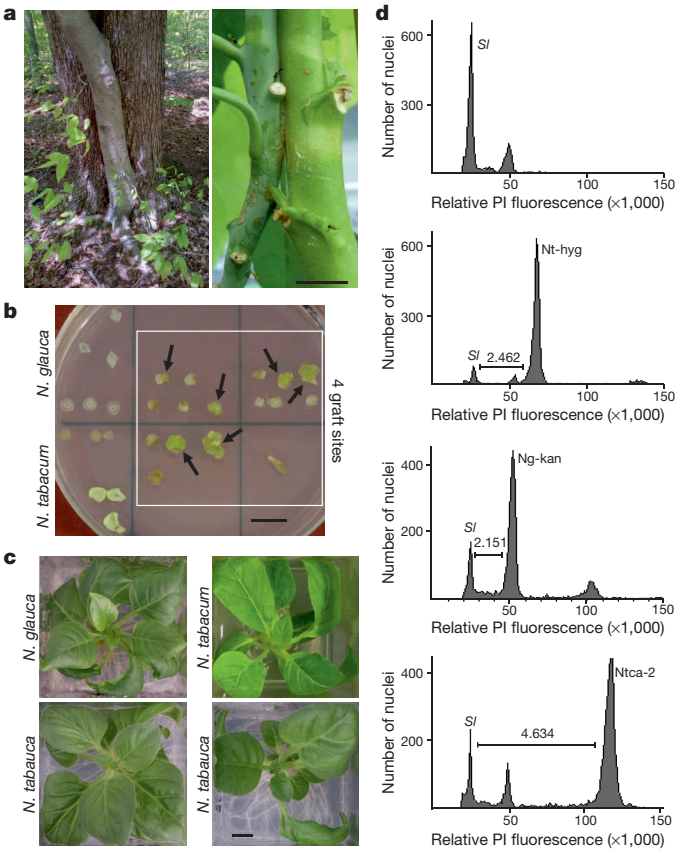

Figure 2 | Grafting and horizontal transfer of nuclear DNA between different species. **a**, A natural stem graft between a beech (front) and a maple (back) in a forest near Monroe, New Jersey (left picture), and an similar stem graft between the tree tobacco, *Nicotiana glauca* (left), and the cigarette tobacco *Nicotiana tabacum* (right) in the greenhouse. **b**, Selection for horizontal transfer of nuclear DNA between plants by exposing graft sites to selection for double resistance to hygromycin and kanamycin. Control explants from the two graft partners (left) and explants from four graft sites (right; framed in white) are shown (compare with Figure 1a–c). **c**, Growth of plantlets regenerated from grafts between *N. tabacum* and *N. glauca* (here termed *Nicotiana tabauca*) on synthetic medium. **d**, Genome size determination in *N. tabauca* by flow cytometry (compare with Extended Data Fig. 1c). *Sl*: *Solanum lycopersicum* (tomato) standard; Ntca: *Nicotiana tabauca*. Note that the calculated genome size for *N. tabauca* equals the sum of the genome sizes of *N. tabacum* (Nt-hyg) and *N. glauca* (Ng-kan).

N. tabauca plants combine many traits from their two progenitor species, for example, perennial growth and axillary anthocyanin accumulation from *N. glauca* and the lighter green leaf pigmentation from *N. tabacum*. Other traits are intermediate, such as leaf shape and flower morphology (Fig. 4a, b). Under greenhouse conditions, *N. tabauca* overgrows its two progenitor species (Fig. 4a), possibly indicating that it could outcompete them. The fitness advantage of many natural allopolyploids and the superior yield of allopolyploid crops have been linked to heterosis-like effects[1,5].

N. tabauca plants are fertile and produce fertile progeny (Fig. 3b), thus fulfilling all criteria of a new species. Inheritance assays indicate that *N. tabauca* behaves like a true amphipolyploid in that the genomes of the two parental species are sufficiently different to largely prevent pairing of *N. glauca* chromosomes with (homeologous) *N. tabacum* chromosomes. Consequently, nuclear marker genes show disomic inheritance and segregate like in a diploid (Fig. 3b).

Interestingly, the phenotypic variation in the progeny of *N. tabauca* plants was less pronounced than in the autotetraploid NGT lines (Extended Data Fig. 4). None of 24 analysed *N. tabauca* progeny showed a reduction in nuclear DNA content that would be detectable with the (limited)

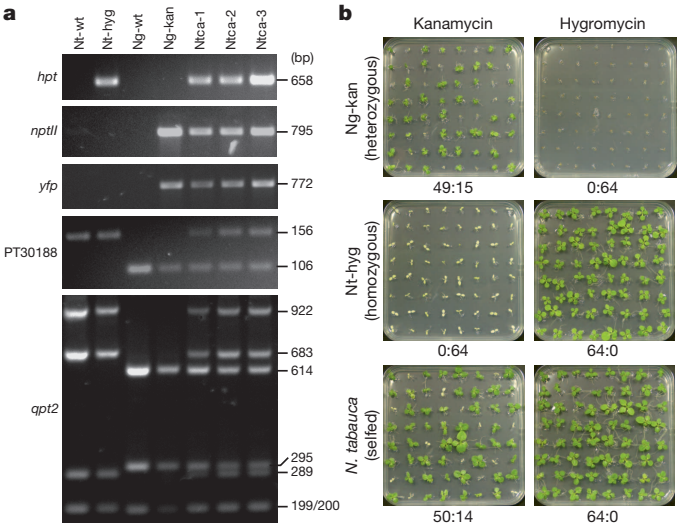

Figure 3 | Presence of nuclear genes from both *Nicotiana tabacum* and *Nicotiana glauca* in *Nicotiana tabauca* and their Mendelian inheritance. **a**, Wild-type *N. tabacum* (Nt-wt), wild-type *N. glauca* (Ng-wt), the two graft partners (Nt-hyg, Ng-kan) and three independently generated *N. tabauca* lines (Ntca-1, 2 and 3) were analysed by PCR using species-specific primers for endogenous genes and transgenes (see Extended Data Table 2 and Methods). PT30188 is a polymorphic microsatellite marker. Amplification products of the *qpt2* gene were digested with a restriction enzyme (for details and fragments sizes, see Methods). **b**, Segregation analysis of *N. tabauca* in comparison to its two parental species, *N. glauca* (Ng-kan) and *N. tabacum* (Nt-hyg). Segregation of the two antibiotic resistances is shown for the two graft partners (a heterozygous Ng-kan and a homozygous Nt-hyg) and a selfed *N. tabauca* line. Segregation ratios are given as numbers of antibiotic-resistant to antibiotic-sensitive seedlings. Note that the transgenes show disomic inheritance and segregate like in a diploid. Consequently, as the *N. tabacum* parent was homozygous for *hpt*, no hygromycin-sensitive progeny were obtained from selfed *N. tabauca*.

sensitivity of flow cytometry. However, karyotype analyses in root meristems revealed, in addition to plants with the full allopolyploid set of chromosomes (Fig. 4c), also cases of chromosome loss (Extended Data Fig. 5), suggesting that there is some somatic genome instability, at least in rapidly dividing root tip cells. Thus, full evolutionary stabilization of the new allopolyploid species will require further genomic adaptations, which occur rapidly after polyploidization events[22,23]. Cases of chromosome loss and genome instability have been documented in both recently evolved natural allopolyploids and synthetic allopolyploids[24,25], indicating that genomic imbalances are a common consequence of allopolyploidization.

The movement of entire nuclear genomes from cell to cell and across graft junctions raises questions about the underlying transfer mechanism. Two mechanisms appear plausible: (1) fusion of neighbouring cells at the graft site, or (2) migration of nuclei from cell to cell through plasmodesmata in a cytomixis-like process, as observed in microscopic studies[18–20]. To obtain insights into the transfer mechanism, we investigated the genomes of plastids and mitochondria. These organelles occur at high numbers per cell and, if cell fusion was involved, one might expect *N. tabauca* plants to harbour a mixed population of organelles from *N. glauca* and *N. tabacum*, a genetic heterogeneity known as heteroplasmy. Analysis of several independently generated *N. tabauca* plants revealed that some contained the *N. glauca* plastid genome, whereas others contained the *N. tabacum* plastid genome, but none was heteroplasmic (Extended Data Fig. 6), arguing against cell fusion as the mechanism underlying genome transfer. By contrast, some heteroplasmy was detected for the mitochondrial genome (Extended Data Figs 7 and 8). However, as mitochondria are often tightly associated with the nuclear membrane and sometimes even found within the nucleus[26], this finding does not necessarily provide evidence against cytomixis. Alternatively, segregation of plastids could occur faster than segregation of mitochondria.

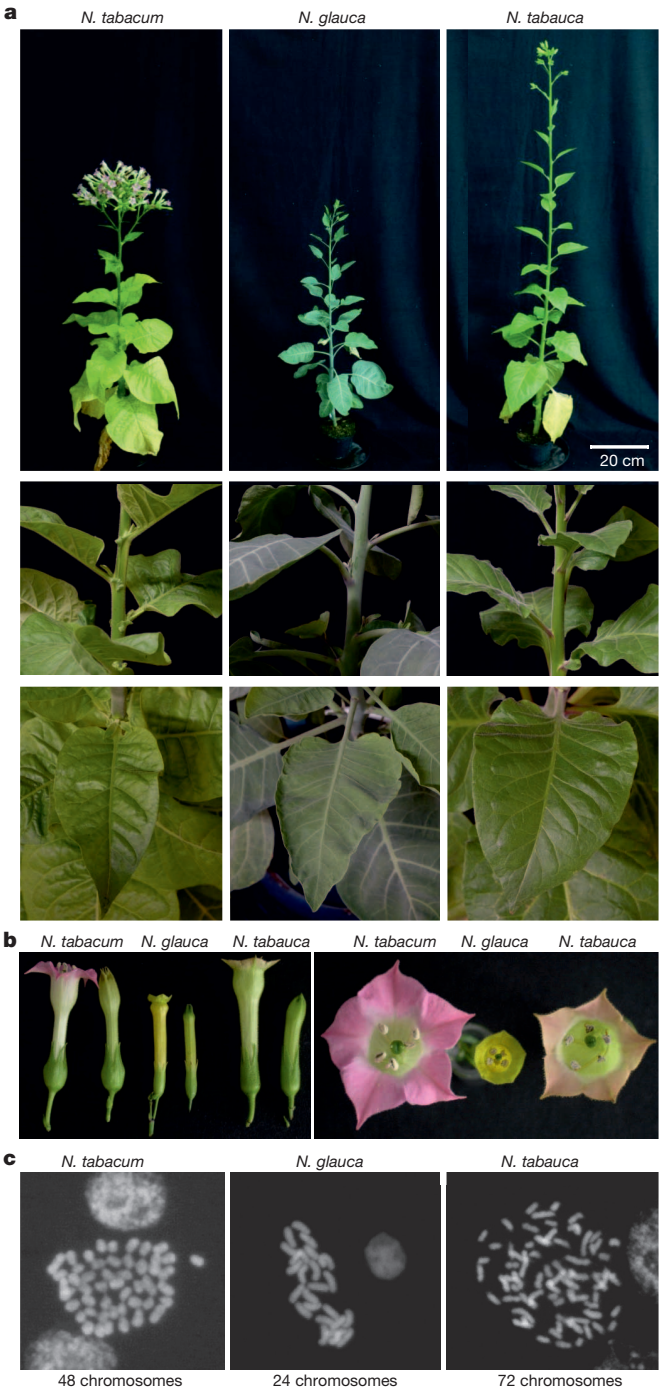

Figure 4 | Phenotype and karyotype of *Nicotiana tabauca* plants. **a**, Vegetative phenotypes of *N. tabacum*, *N. glauca* and *N. tabauca*. Plants in the upper panel were photographed 44 days after transfer to soil. Note the difference in leaf colour (blue-green in *N. glauca* and lighter green in *N. tabacum* and *N. tabauca*). The middle panel shows the difference in anthocyanin accumulation. *N. glauca* has accumulation visible in the whole leaf; *N. tabauca* has accumulation restricted to the petiole base; *N. tabacum* has no anthocyanin. The lower panel depicts the difference in leaf morphology. **b**, Comparison of flower morphology. **c**, Karyotype analysis by DAPI (4′,6-diamidino-2-phenylindole) staining reveals the allopolyploid status of *N. tabauca*.

The initial interspecies genome transfer in a natural graft represents a somatic event. However, it can become heritable by lateral shoot formation from the graft site, which is facilitated by the presence of a meristematic ring surrounding the stem, the cambium[12]. Lateral shoot formation

is commonly induced by wounding, for example, upon herbivory or grafting[10,14]. This would allow the allopolyploid cell to enter the germline by becoming part of a newly formed apical meristem.

As most modern crop plants arose through allopolyploidization, the possibility to generate new allopolyploid species by grafting-mediated genome transfer also provides a new tool for crop improvement. Although genome combination can also be achieved synthetically using protoplast fusion techniques[27,28], the procedures involved are technically demanding and available only for a limited number of species. In cases where hybridization is possible, the genomes of two species can also be combined sexually, but the resulting hybrids are often sterile unless their chromosome sets are doubled. This is also the case for most interspecific hybrids between *Nicotiana* species, including *N. tabacum* and *N. glauca*[29]. Finally, genome transfer by grafting creates new combinations of plastid and mitochondrial genomes (Extended Data Figs 6–8). This is important because the organellar genotypes influence important agronomic traits, including growth and stress tolerance[30].

We have demonstrated that grafting results in the transfer of entire nuclear genomes between species. The significance of this is twofold. First, natural grafting provides a potential asexual mechanism of speciation that is much less restricted by incompatibilities than sexual hybridization[9,14] (Fig. 2a). Thus, grafting should be considered as an alternative mechanism of polyploidization, especially in allopolyploids that formed from distantly related species. Second, interspecies grafting provides a method to produce new allopolyploid crop species. As polyploidy confers the superior properties of modern crops over their diploid progenitor species[6,7], this has significant potential in breeding and agricultural biotechnology.

METHODS SUMMARY

Nicotiana plants were grown under greenhouse conditions or under aseptic conditions on synthetic medium. Transgenic plants were produced by *Agrobacterium*-mediated transformation. Interspecies grafting experiments were performed reciprocally by transplanting *N. glauca* scions onto *N. tabacum* stocks and vice versa. Selection for gene transfer was done on regeneration medium containing kanamycin and hygromycin. Regenerated doubly resistant shoots were rooted on synthetic medium, transferred to soil and grown to maturity in the greenhouse. For molecular analyses, microscopy, karyotyping and flow cytometry, see the Methods section.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions I.F., S.S. and H.G. performed the experiments. All authors participated in data evaluation and experimental design. R.B. conceived the study, and wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.B. (rbock@mpimp-golm.mpg.de).

METHODS

Plant material and growth conditions. Sterile *Nicotiana tabacum* cv. SNN and *Nicotiana glauca* plants were grown under aseptic conditions by germinating surface-sterilized seeds in Petri dishes with MS medium[31] supplemented with 3% sucrose and transferring seedlings into magenta boxes containing the same medium. Plants were raised in controlled environment chambers under a diurnal cycle of 16 h light (50 μE m^{-2} s^{-1}) at 24 °C and 8 h dark at 22 °C. For cultivation in soil, plants were either germinated in soil or transferred from tissue culture into soil and grown under standard greenhouse conditions in a diurnal cycle of 16 h light at 25 °C and 8 h darkness at 20 °C.

Generation of transgenic plants. The vector containing the *nptII* and *yfp* expression cassettes and the generation of transgenic *N. tabacum* and *N. glauca* plants have been described previously[16]. A binary vector containing the hygromycin phosphotransferase (*hpt*) cassette (Fig. 1a) was provided by Dr. Karin Köhl (MPI-MP). Nuclear transformation was performed by *Agrobacterium tumefaciens* mediated leaf-disc transformation using standard protocols. Nuclear transgenic lines were selected on antibiotic-containing MS-based regeneration medium, rooted in sterile boxes with MS medium containing 2% sucrose and then transferred to the greenhouse.

Grafting and selection for horizontal gene transfer. Grafting experiments were performed with sterile plants under aseptic conditions or with plants in the greenhouse. For grafting under aseptic conditions, plants were raised on MS medium containing 3% sucrose. Plants with a similar stem diameter were selected as graft partners. Stems were cut at an angle of approximately 45°, the graft partners were joined and the scion was fixed with a sleeve produced from a silicon tube. Grafting experiments were performed reciprocally in that each plant was used as both stock and scion, giving rise to two grafts. Successful grafting was evidenced by establishment of a physical connection between scion and stock and continued growth of the scion. After 2–4 weeks, the graft site was excised, cut with a sharp scalpel in cross sections and exposed to regeneration medium containing 250 or 500 mg l^{-1} kanamycin and 50 or 150 mg l^{-1} hygromycin. Selection was performed at 50 μE m^{-2} s^{-1} under 16 h light at 24 °C and 8 h dark at 22 °C. Grafting in the greenhouse was performed by attaching surface-wounded stems to each other (by fixing them with tape), to mimic the natural grafting process (Fig. 3a). Following establishment of the graft, the graft site was surface-sterilized with Plant Preservative Mixture (Plant Cell Technology) and exposed to selection for DNA transfer as described above.

Preparation of nuclei from plants tissues. Young leaves from *N. tabacum*, *N. glauca*, *N. tabauca* and *S. lycopersicum* were collected and nuclei were prepared according to published protocols[32,33]. To determine the absolute genome size of the material, *S. lycopersicum* leaves were used as an internal standard. Immediately after removing the leaves from the plant, leaf pieces (sample plus internal standard in a 1:1 ratio) were chopped with a disposable blade in ice-cold Galbraith's buffer[32] with 10 mM DTT and supplemented with 50 μg ml^{-1} of RNase A solution and 50 μg ml^{-1} propidium iodide solution. The suspension of cell nuclei was then filtered through a 20 μm filter (CellTrics, Partec, Germany) and kept on ice in the dark for 10 min before determining the DNA content.

Measurement of nuclear DNA contents. DNA contents were determined by flow cytometry using the FACSAria II cell sorter (BD Bioscience). Propidium iodide fluorescence was measured using a blue laser (488 nm), a 616/23 nm band-pass filter and a 610 LP mirror. A minimum of 2,000 gated nuclei per sample were recorded. DNA content was calculated based on the ratio between the mean value for the 4C peak of the internal standard (*S. lycopersicum*, $2n = 2\times = 24$ chromosomes = 2C = 2.05 pg DNA) and the mean value of the 2C sample peak (*N. tabacum*, $2n = 4\times = 48$ chromosomes = 2C = 10.35 pg DNA; *N. glauca*, $2n = 2\times = 48$ chromosomes = 2C = 10.65 pg DNA). C-values were obtained from (http://data.kew.org/cvalues/). Changes in the ploidy level measured by flow cytometry were further verified by chromosome counting (see below).

Chromosome staining in pollen mother cells (PMCs). Aceto-orcein solution (2% w/v orcein in 45% v/v acetic acid) and Vectashield Mounting Medium with DAPI (Linaris B.P., Germany) were used to stain chromosomes from meiotic PMCs. Flower buds were fixed overnight in ethanol:acetic acid (3:1) solution at room temperature. Afterwards, the buds were washed with H_2O for 10 min, followed by incubation in 1 M HCl at 60 °C for 8 min. The material was then rinsed three times with H_2O and the anthers were placed onto a glass slide. One drop of aceto-orcein solution or Vectashield Mounting Medium was added and the anthers were covered by a coverslip. PMCs were released from the anthers by gently squashing the sample with the forefinger. PMCs with meiotic chromosomes were analysed by light microscopy or ultraviolet (UV) microscopy.

Preparation of mitotic chromosomes. For mitotic chromosome preparations, root tips from freshly germinated seedlings were collected, transferred to a saturated solution of 1-bromonaphthalene and incubated at 4 °C in the dark for 24 h. The samples were then washed several times with distilled water, transferred to freshly prepared fixative solution (3:1 v/v mix of ethanol and glacial acetic acid) and softened by enzymatic maceration (5% pectinase, 2% cellulase (Sigma-Aldrich) and 1% cellulase Onozuka RS (Serva), dissolved in citric acid/sodium citrate buffer, pH 4.6) for 15 min at 37 °C or, alternatively, by incubation in 1 N HCl at 60 °C for 10 min. Afterwards, the tissue was washed several times with distilled water and gently squashed under a coverslip in a drop of 45% acetic acid. The samples were shortly frozen in liquid nitrogen, the cover glass was removed and the tissue was air dried. Preparations from enzymatically digested material were mounted in DAPI (containing antifade solution (Vectashield, Vector Laboratories)). Those obtained by hot HCl hydrolysis were stained with a drop of 0.1% aqueous toluidine blue solution, washed in distilled water, air-dried and mounted in Entellan (Merck). Metaphase plates were subsequently analysed by light microscopy or epifluorescence microscopy.

Isolation of plant genomic DNA, polymerase chain reactions (PCR) and DNA sequencing. Genomic DNA was extracted from fresh leaf tissue using a CTAB-based method[34]. PCR amplification was performed with synthetic oligonucleotides as primers (Extended Data Table 2) and GoTaq Flexi DNA polymerase (Promega) in a Mastercycler EPgradient (Eppendorf, Germany). The standard reaction involved an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 50 to 60°C and 60 to 90 s at 72 °C, and a final elongation for 2 min at 72 °C.

PCR analysis of NGT lines was performed using the following oligonucleotide combinations: PHygFor + PHygRev for *hptII* (658 bp), PnptIIFor + PnptIIRev for *nptII* (795 bp) and P35SInternRev + PyfpRev for *yfp* (772 bp; Extended Data Table 2). PCR analysis of *Nicotiana tabauca* plants was performed using the following oligonucleotide combinations (Extended Data Table 2): PHygFor + PHygRev for *hptII* (658 bp), PnptIIFor + PnptIIRev for *nptII* (795 bp), P35SInternRev + PyfpRev for *yfp* (772 bp), PT30188F + PT30188R for a polymorphic microsatellite marker[35] (156 bp in *N. tabacum* and 106 bp in *N. glauca*) and Pqpt2F + PqptR for the quinolinate phosphoribosyltransferase 2 gene (*qpt2*). The *qpt2* gene occurs in two copies in *N. tabacum* (yielding PCR fragments of 1,121 bp and 1,172 bp, respectively) and one copy in *N. glauca* (giving rise to a 1,162 bp product). The amplification products were digested with the restriction enzyme DraI resulting in fragments of 199 bp and 922 bp (for the *N. tabacum* 1,121 bp PCR product); 200 bp, 289 bp and 683 bp (for the *N. tabacum* 1,172 bp PCR product); and 54 bp, 199 bp, 295 bp and 614 bp (for the *N. glauca* 1,162 bp PCR product; note that the 54 bp fragment is not visible in the gel in Fig. 3a because of its small size). The fragments were separated on 4% agarose gels.

For DNA sequencing, amplified PCR products were purified by agarose gel electrophoresis and recovered from excised gel slices with the NucleoSpin Extract II kit (Macherey-Nagel). Purified PCR products were sequenced with specific primers using the chain termination method (Eurofins MWG Operon).

Crosses and inheritance tests. Crosses were performed by hand-pollinating emasculated plants grown in the greenhouse. To accelerate flowering of the tree tobacco (*N. glauca*) and the tree-like new allopolyploid species *N. tabauca*, plants were kept in small pots. Inheritance assays were performed by sowing surface-sterilized seeds on MS medium containing the appropriate antibiotics. Kanamycin was used at a concentration of 250 μg ml^{-1}, hygromycin at 25 μg ml^{-1}.

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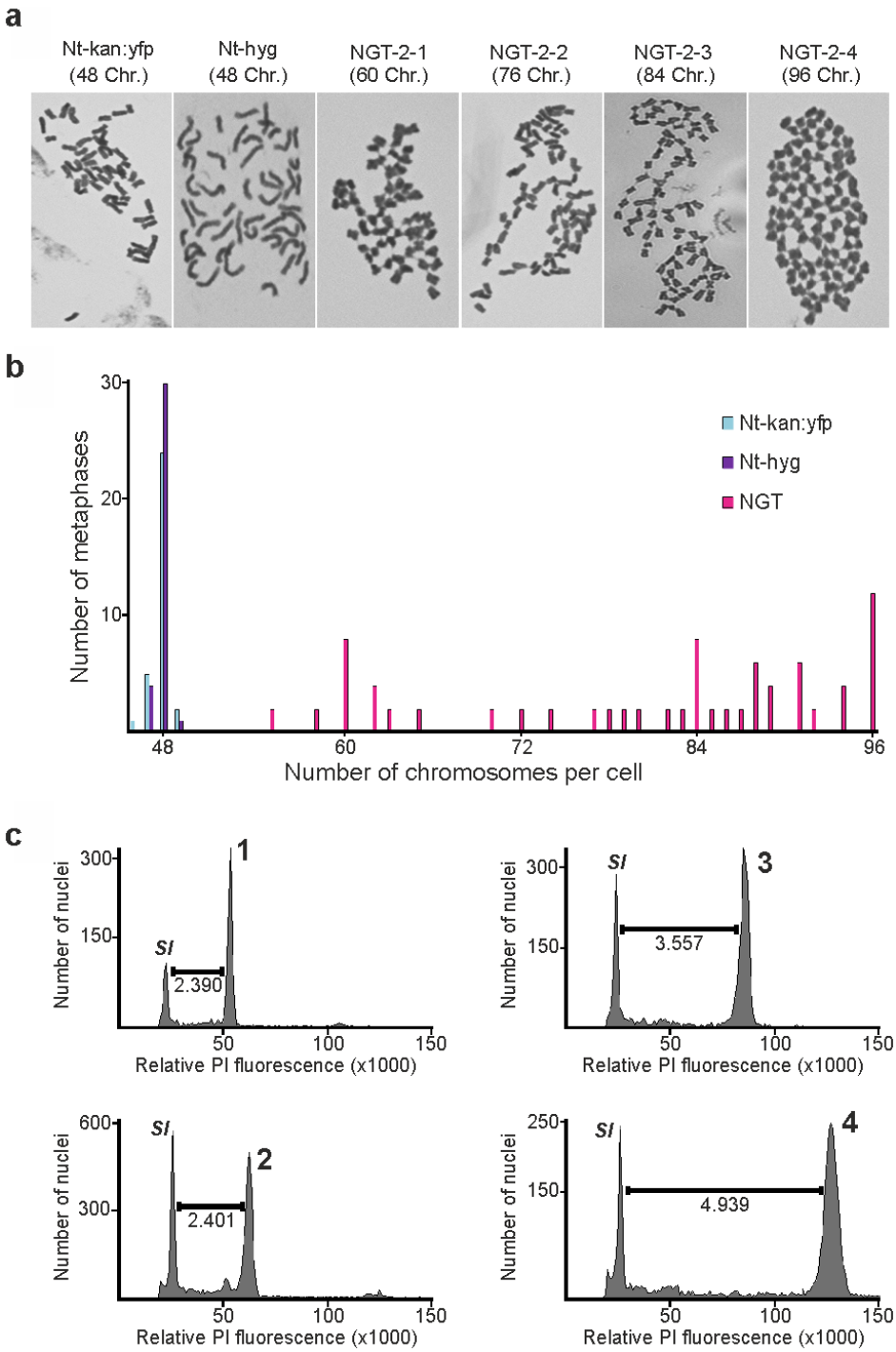
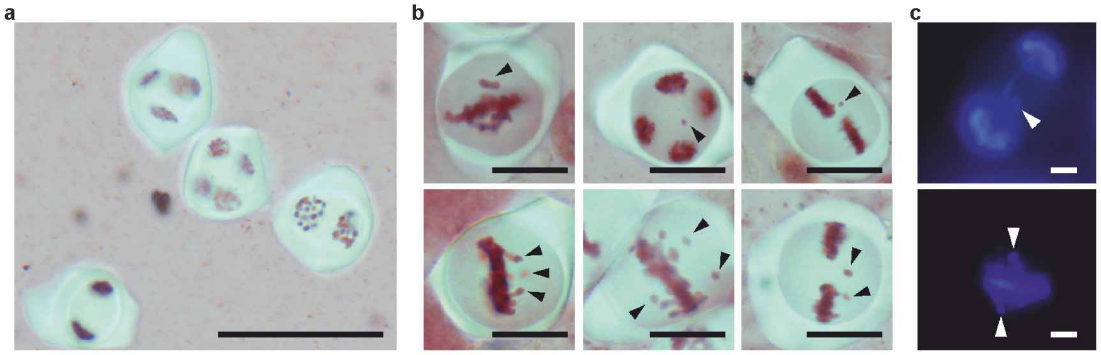
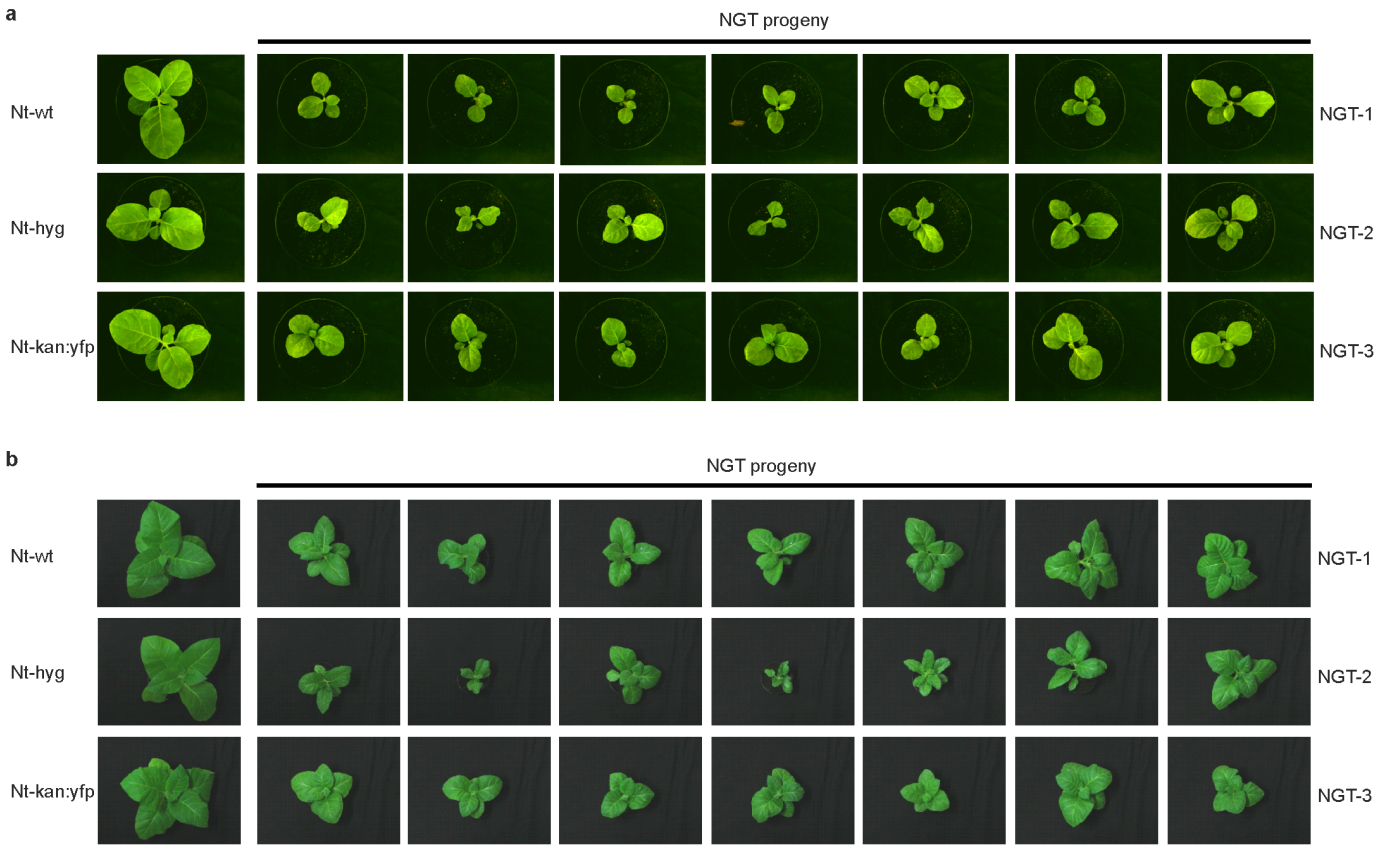


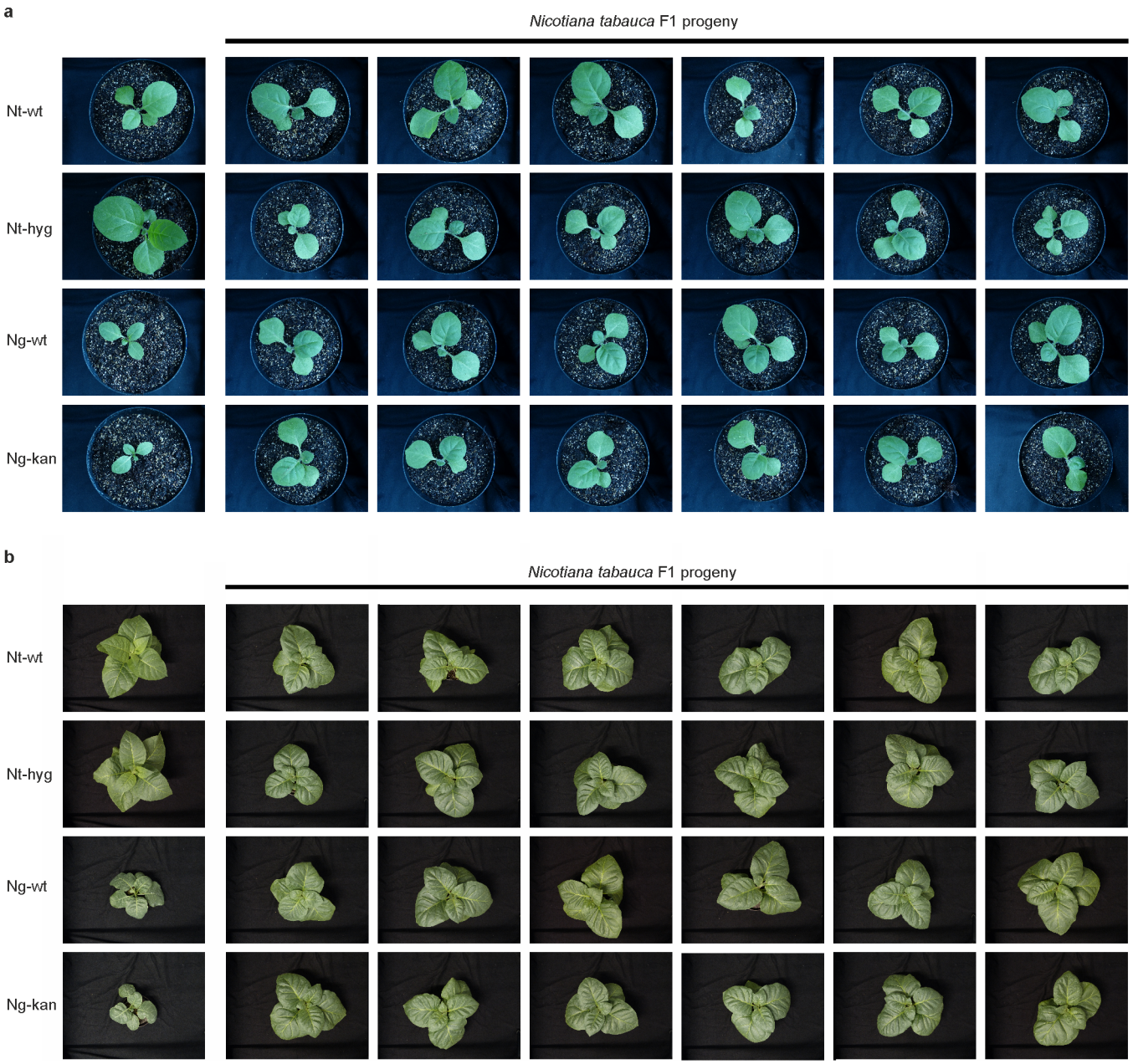
Extended Data Figure 1 | Autotetraploidy and chromosome loss in NGT plants. **a**, Chromosome preparations of mitotic cells. Along with preparations from each of the two graft partners (Nt-kan:yfp and Nt-hyg), four examples of mitotic cells from four individual progeny plants of the self-pollinated line NGT-2 are shown. Chromosomes are visualized by hot tissue hydrolysis in HCl and staining with toluidine blue. Chromosome numbers are given in parentheses. Chr, chromosomes. **b**, Chromosome counts for individual seedlings from the two graft partners and the selfed line NGT-2. Mitotic cells from the root tips were analysed. The total number of metaphases investigated was 32 for Nt-kan:yfp (blue), 35 for Nt-hyg (purple) and 86 for NGT-2 (pink). **c**, Absolute genome size determination by flow cytometry. Leaf samples were mixed with tomato leaves (*Solanum lycopersicum*, *Sl*) that served as internal standard, nuclei were isolated and the relative fluorescence intensity of propidium iodide (PI) was measured. Each peak corresponds to a population of nuclei. For each sample, the ratio between the peak of the analysed tobacco line and the peak of the internal standard (4C tomato nuclei) was calculated to determine the absolute genome size. Sample 1: Nt-hyg; sample 2: Nt-kan:yfp; sample 3: seedling from a cross between NGT-1 and wild type; sample 4: seedling from a self-pollinated NGT-2 plant.



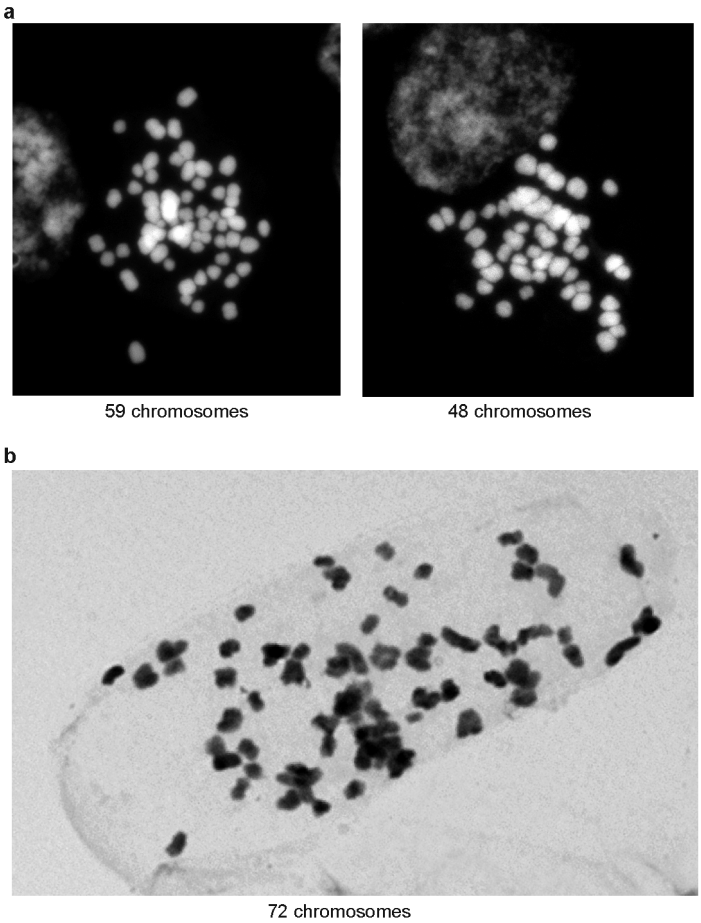
Extended Data Figure 2 | Meiotic chromosome missegregation in autopolyploid NGT tobacco lines. **a**–**c**,Young flower buds were fixed and stained, either with aceto-orceine (**a**, **b**) or with DAPI (**c**). The anthers were collected and the pollen mother cells (PMC) were examined under the microscope. Two plants from the F_1 generation of self-pollinated NGT plants were used. **a**, Four representative PMCs of a wild-type tobacco plant. Scale bar, 50 µm. **b**, Six PMC from NGT plants. The upper three cells are from line NGT-2, the lower three from line NGT-3. Scale bar, 20 µm. **c**, Two PMCs from line NGT-3 stained with DAPI. Scale bar, 5 µm. Mis-segregating chromosomes are indicated by arrowheads in **b** and **c**.



Extended Data Figure 3 | Phenotypes of NGT progeny plants. **a**, **b**, Wild-type tobacco (Nt-wt), the two transgenic graft partners (Nt-hyg and Nt-kan:yfp) and the second generation of three NGT lines (NGT-1, NGT-2 and NGT-3) were grown under greenhouse conditions. A total of 21 NGT progeny plants were investigated, 14 of them resulted from self-pollinated lines (NGT-2 and NGT-3) and the remaining 7 from the cross of the NGT-1 line with a wild-type plant (which was male sterile and could not be selfed). Pictures were taken 30 (**a**) and 45 (**b**) days after sowing.

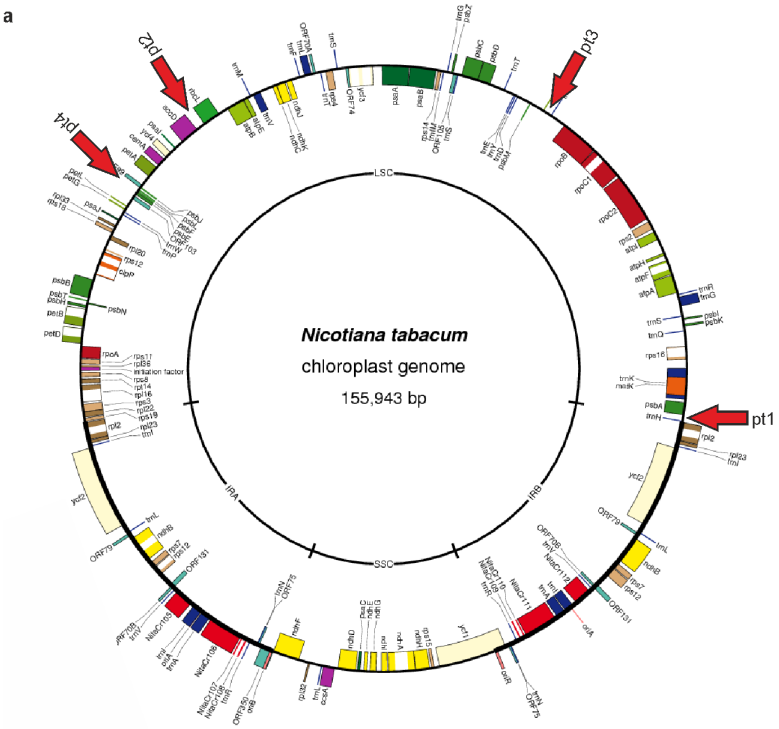


Extended Data Figure 4 | Phenotypes of *Nicotiana tabauca* progeny plants. **a**, **b**, Wild-type tobacco (Nt-wt), the two transgenic graft partners (Nt-hyg and Ng-kan) and the F_1 generation of an *N. tabauca* line were raised from seeds and grown under greenhouse conditions. Pictures were taken 28 (**a**) and 47 (**b**) days after sowing.



Extended Data Figure 5 | Detection of allopolyploid and aberrant karyotypes in *Nicotiana tabauca* F_1 progeny plants. **a**, Two examples of DAPI-stained metaphases with fewer than 72 chromosomes. **b**, As an alternative to the DAPI staining shown in Fig. 4c and in panel **a**, a method based on hot tissue hydrolysis in HCl and staining with toluidine blue was used (see Methods). Shown is a *Nicotiana tabauca* F_1 plant with the full allopolyploid chromosome set of 72 chromosomes.

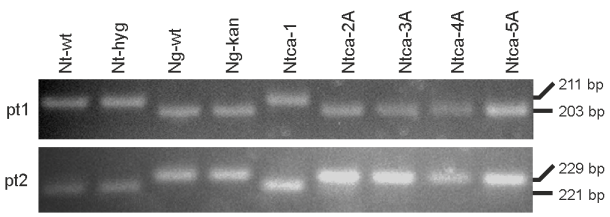
a



b

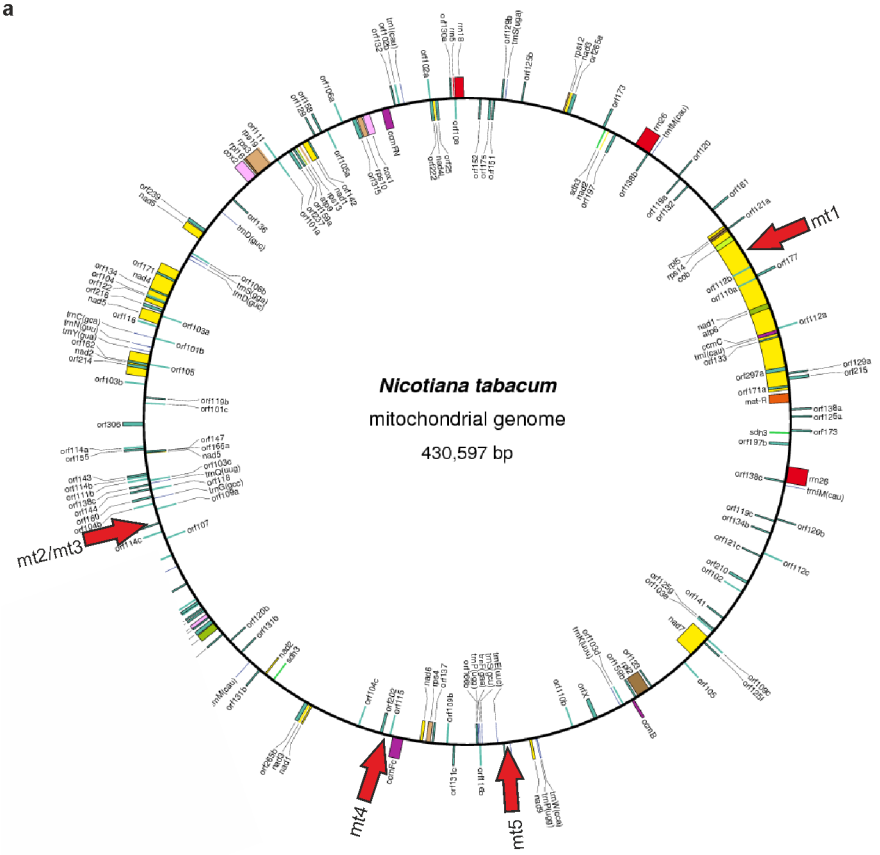
	Polymorphism			
Plant line	pt1	pt2	pt3	pt4
Nt-wt	Nt	Nt	Nt	Nt
Nt-hyg	Nt	Nt	Nt	Nt
Ng-wt	Ng	Ng	Ng	Ng
Ng-kan	Ng	Ng	Ng	Ng
Ntca-1	Nt	Nt	Nt	Nt
Ntca-2A	Ng	Ng	Ng	Ng
Ntca-2B	Ng	Ng	Ng	Ng
Ntca-3A	Ng	Ng	Ng	Ng
Ntca-3B	Ng	Ng	Ng	Ng
Ntca-4A	Ng	Ng	Ng	Ng
Ntca-4B	Ng	Ng	Ng	Ng
Ntca-5A	Ng	Ng	Ng	Ng
Ntca-5B	Ng	Ng	Ng	Ng

c



Extended Data Figure 6 | Molecular analysis of four polymorphic regions in the plastid genome of *Nicotiana tabauca* lines. **a**, Physical map of the *Nicotiana tabacum* plastid genome showing the four plastid polymorphic regions analysed (pt1, pt2, pt3 and pt4). pt1: polymorphism amplified with oligonucleotides Ppt1F and Ppt1R (Extended Data Table 2) resulting in a 211 bp fragment in *N. tabacum* cv. SNN and a 203 bp fragment in *N. glauca*; pt2: polymorphism amplified with oligonucleotides Ppt2F and Ppt2R resulting in a 221 bp fragment in *N. tabacum* cv. SNN and a 229 bp fragment in *N. glauca*; pt3: polymorphic region of ~3 kb (containing altogether 25 polymorphisms) amplified using the primer pairs Ppt3-1F + Ppt3-1R, Ppt3-2F + Ppt3-2R and Ppt3-3F + Ppt3-3R; pt4: polymorphic region of ~3 kb (containing altogether 32 polymorphisms) amplified with the primer pairs Ppt4-1F + Ppt4-1R, Ppt4-2F + Ppt4-2R and Ppt4-3F + Ppt4-3R. The polymorphisms were selected based on published sequence information of the plastid genomes of *N. tabacum* and *N. glauca*[16,36]. **b**, Overview of the plant lines and the polymorphic regions analysed. *Nicotiana tabacum* sequence is represented with Nt on yellow background, *Nicotiana glauca* sequence is represented with Ng on pink background. **c**, A 4% agarose gel showing the size difference of the PCR fragments for the pt1 and pt2 polymorphisms between *N. tabacum*, *N. glauca* and five *N. tabauca* lines.

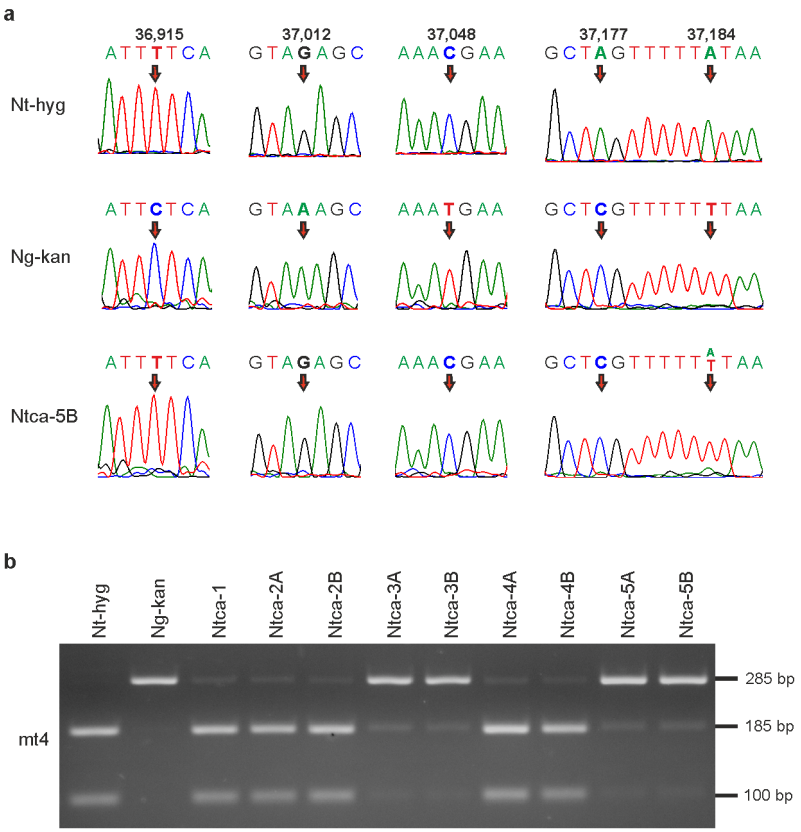
a



b

	Polymorphism				
Plant line	mt1	mt2	mt3	mt4	mt5
Nt-wt	Nt	Nt	Nt	Nt	Nt
Nt-hyg	Nt	Nt	Nt	Nt	Nt
Ng-wt	Ng	Ng	Ng	Ng	Ng
Ng-kan	Ng	Ng	Ng	Ng	Ng
Ntca-1	Nt	Nt	Nt	Nt	Nt
Ntca-2A	Nt	Nt	Nt	Nt	Nt
Ntca-2B	Nt	Nt	Nt	Nt	Nt
Ntca-3A	Nt	Ng	Ng	Ng	Ng>Nt
Ntca-3B	Nt/Ng	Nt	Ng	Ng	Ng>Nt
Ntca-4A	Nt	Nt	Nt	Nt	Ng>Nt
Ntca-4B	Nt	Nt	Nt	Nt	Ng>Nt
Ntca-5A	Nt/Ng	Nt	Ng	Ng	Ng>Nt
Ntca-5B	Nt/Ng	Nt	Ng	Ng	Ng>Nt

Extended Data Figure 7 | Molecular analysis of five mitochondrial genome polymorphisms in *Nicotiana tabauca* lines by sequencing of amplified PCR products. **a**, Physical map of the *Nicotiana tabacum* mitochondrial genome showing the five mitochondrial polymorphic regions analysed (mt1, mt2, mt3, mt4 and mt5). mt1: polymorphic region of 693 bp (containing 5 polymorphisms) amplified with oligonucleotides Pmt1F and Pmt1R (Extended Data Table 2); mt2: polymorphism (SNP) amplified with oligonucleotides Pmt2F and Pmt2R; mt3: polymorphism (SNP) amplified with oligonucleotides Pmt3F and Pmt3R; mt4: polymorphism (SNP) amplified with oligonucleotides Pmt4F and Pmt4R; mt5: polymorphic region amplified with oligonucleotides Pmt5F and Pmt5R and resulting in a 298 bp fragment in *N. tabacum* and a 288 bp fragment in *N. glauca*. The polymorphisms were selected based on published sequence information of the mitochondrial genomes of *N. tabacum* and *N. glauca*[16,37]. **b**, Overview of the plant lines and the polymorphic regions analysed. *Nicotiana tabacum* sequence is represented with Nt on yellow background, *Nicotiana glauca* sequence is represented with Ng on dark pink background. Heteroplasmy (that is, detectability of both the *N. tabacum* and the *N. glauca* sequence in an *N. tabauca* plant) is indicated by Nt/Ng on light pink background. A and B denote two different plants from the same *N. tabauca* line.



Extended Data Figure 8 | Detection of mitochondrial heteroplasmy in *N. tabauca* plants. **a**, Example of sequences amplified from the mt1 polymorphic region in Nt-hyg, Ng-kan and in an *N. tabauca* (Ntca) line. The mt1 polymorphic region was amplified with the oligonucleotide combination Pmt1F and Pmt1R (Extended Data Table 2) resulting in a 693 bp fragment (corresponding to nucleotide positions 36,746 to 37,438 in the *Nicotiana tabacum* mitochondrial genome, accession number: NC_006581.1). The fragment contains five single nucleotide polymorphisms (SNPs) that are denoted with an arrow in the sequence chromatograms and the nucleotide position in the *N. tabacum* mitochondrial genome. A mixed nucleotide position indicating heteroplasmy is denoted by two letters above each other. **b**, A 4% agarose gel analysing the mt4 polymorphism in *N. tabacum*, *N. glauca* and nine *N. tabauca* plants (representing five different lines). A 310 bp fragment was amplified by PCR and digested with the two restriction enzymes NcoI and BsaBI. Digestion of the PCR product amplified from the *N. tabacum* mitochondrial genome yields three restriction fragments (25 bp, 185 bp and 100 bp), whereas digestion of the PCR product amplified from the *N. glauca* mitochondrial genome results in two fragments (25 bp and 285 bp). This difference between the two species is due to a T to G substitution in *N. glauca* (relative to *N. tabacum*; position in the *N. tabacum* mitochondrial genome: 305,402), resulting in the loss of a BsaBI site. The 25 bp restriction fragment is not detectable in the gel because of its small size. Note that the low level of heteroplasmy detected by restriction-fragment length polymorphism (RFLP) analysis was not reliably detectable by DNA sequencing (compare with Extended Data Fig. 7b). A and B denote two different plants from the same *N. tabauca* line.

Extended Data Table 1 | Segregation ratios of three autopolyploid NGT lines

Plant line	Self-pollinated			Cross NGT x wt		
	Hr : Hs	Kr : Ks	HKr : HKs	Hr : Hs	Kr : Ks	HKr : HKs
NGT-1	-	-	-	326 : 45 (87.9%)	154 : 127 (54.8%)	195 : 223 (46.7%)*
NGT-2	396:15 (96.4%)	383 : 125 (75.4%)	317 : 130 (70.9%)	262 : 48 (84.5%)	223 : 121 (64.8%)	107 : 153 (41.2%)*
NGT-3	36 : 2 (94.7%)	55 : 20 (73.3%)	105 : 28 (78.9%)	165 : 32 (83.8%)	56 : 68 (45.2%)	85 : 109 (43.8%)

Hr, hygromycin resistant; Hs, hygromycin sensitive; Ks, kanamycin sensitive; Kr, kanamycin resistant; HKr, resistant to hygromycin + kanamycin; HKs, sensitive to hygromycin + kanamycin; −, not analysed (due to pollen sterility); *, segregation ratio significantly different from the expected segregation of a tetraploid plant (Pearson's chi squared, $P < 0.01$).

Extended Data Table 2 | List of oligonucleotides used in this study

Oligonucleotide	Sequence (5' to 3')
PHygFor	GACGTCTGTCGAGAAGTTTCTGATCG
PHygRev	GTATTGGGAATCCCCGAACATCGCCTC
PnptIIFor	ATGATTGAACAAGATGGATTGCAC
PnptIIRev	TCAGAAGAACTCGTCAAGAAGGCG
P35SIntern Rev	AAGGTGGCTCCTACAAATGCCAT
PyfpRev	GCCGTTCTTCTGCTTGTCGGCC
Pqpt2F	CTCTTTAAGAAGAAAAAAAGATTC
PqtpR	CGCGAATATCATCTCAGCAA
PT30188F	AACCATACGCCTTCAGATCG
PT30188R	TGGTTTGAGTAAAGAAATGTTGTGA
Ppt1F	CTTGATCCACTTGGCTACATCC
Ppt1R	GCTAATGTTACTATATCTTTTTGAT
Ppt2F	TTCTTTATAGGAGAGGACAAATC
Ppt2R	GCATAGAAATCCAATCACTAGG
Ppt3-1F	CAGGTATTGTAGATATTCCCTC
Ppt3-1R	AGGCGACTCCCGGATTTGAAC
Ppt3-2F	GAGTGGTAAGGCAGAGGAC
Ppt3-2R	CTAGAGTCCACTTCTTCCCC
Ppt3-3F	ATAGTAAGTCTTGCTTGGGC
Ppt3-3R	AAACAGTCAGTCAAAACGATTAA
Ppt4-1F	CAATTGGCCGAAATGAATTTCTA
Ppt4-1R	ATGGCCGATACTACTGGAAG
Ppt4-2F	TAGAGGGATGAACCCAATCC
Ppt4-2R	ATGTCTGGAAGCACAGGAGA
Ppt4-3F	GTAATGCTATGAATGACCCAGT
Ppt4-3R	GCCGCTAATAGAAAACCGAAATA
Pmt1F	TTCGACTGAACGACGGAATTC
Pmt1R	TCTTTGCTTGTTTCGTGGTATG
Pmt2F	GGTATTACACTATCGCGGAG
Pmt2R	ACTTTCGTCCCCAGTATTCTC
Pmt3F	AGGCGGTGATGAAGACAAAAG
Pmt3R	CTTTGGACTGCTTTTTTCAAAAG
Pmt4F	TCTTTTATGAACTCCACTGGTC
Pmt4R	ATGACTAAGGTAATTGCCAATAG
Pmt5F	TGTAGTTTAGGGGTGCGCAG
Pmt5R	TTAGGGGAAACAAAGCTTCAAC