Supporting Online Material for

Exchange of Genetic Material Between Cells in Plant Tissue Grafts

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Materials and Methods

Plant material

Sterile tobacco (*Nicotiana tabacum* cv. Petit Havana and cv. Samsun NN) plants were grown on agar-solidified synthetic medium containing 30 g/l sucrose (*1*). Homoplasmic plastid-transformed (=transplastomic) Pt-spec:gfp plants carry a previously described GFP expression cassette (*2*) which had been cloned into plastid transformation vector pPRV111A (*3*). Nuclear transgenic Nuc-kan:yfp lines harbor a 35S YFP expression cassette and were obtained by *Agrobacterium*-mediated transformation using a pGreen vector. The nuclear-transgenic lines (Nuc-kan:yfp) were kanamycin resistant, showed YFP accumulation in the cytosol and the nucleus and transmitted both transgenes to progeny in a Mendelian fashion. The transplastomic lines (Pt-spec:gfp) were resistant to spectinomycin, showed GFP accumulation in chloroplasts and inherited the transgenes maternally (fig. S1).

Grafting and selection for intercellular gene transfer

To exclude possible influences of pathogens or endophytic microbes, all grafting experiments were performed with sterile plants under aseptic conditions. Nuclear transgenic and homoplasmic plastid-transformed plants were raised from surface-sterilized seeds on synthetic medium under aseptic conditions (*1*). Grafted plants were produced by cutting the stems of 5-10 cm tall plantlets in an approximately 45° angle and using the upper part as scion and the lower part as stock in reciprocal grafting experiments. After stock and scion had been allowed to fuse for 9-14 days, the graft site was sliced in 1-2 mm thin cross sections with a scalpel. The explants were then exposed to antibiotic selection on a plant regeneration medium (*4*) containing 500 mg/l spectinomycin and 250 mg/l kanamycin. In an alternative experimental setup, the entire graft site was excised and exposed to antibiotic selection (Fig. 1B). As selection controls, stem cuttings and leaf explants from Nuc-kan:yfp and Pt-spec:gfp plants raised under identical conditions were exposed to the same medium (Fig. 1B). In additional control experiments, stock and scion were separated prior to fusion (2 days after grafting) and exposed to double selection. Selected doubly resistant calli and shoots were transferred to fresh plates and regenerated again under antibiotic selection, to eliminate possible cross-protected cells. Regenerated shoots were rooted on phytohormone-free synthetic medium (*1*) and subsequently transferred to soil and grown to maturity under standard greenhouse conditions.

Isolation of nucleic acids and gel blot analyses

Total plant DNAs were isolated from fresh leaf tissue by a cetyltrimethylammoniumbromide (CTAB)-based method (*5*). Total cellular RNA was extracted using the peqGOLD TriFast reagent (Peqlab GmbH, Erlangen, Germany). For

Southern blot analysis, DNA samples were digested with restriction enzymes, separated by gel electrophoresis in 0.8% agarose gels, and transferred onto Hybond nylon membranes (GE Healthcare, Buckinghamshire, UK) by capillary blotting using standard protocols. Total cellular RNA samples were electrophoresed in formaldehyde-containing 1 % agarose gels and blotted onto Hybond nylon membranes. Hybridization probes were purified by agarose gel electrophoresis following extraction of the DNA fragments of interest from excised gel slices using the Nucleospin Extract II kit (Macherey-Nagel, Düren, Germany) and then radiolabeled with ^{32}P-dCTP using the MegaPrime kit (GE Healthcare). Hybridizations were performed at 65 °C in Church buffer (*6*) or Rapid-Hyb buffer (GE Healthcare).

Hybridization probes specific for the four transgenes used in this study (*aadA, nptII, gfp* and *yfp*) were produced by excising the entire coding regions from plasmid clones. A PCR product covering part of the 16S rRNA gene *(rrn16*; prepared by amplification with primers P16Srrn-F 5' CAAGCGGTGGAGCATGTGG 3' and P16Srrn-R 5' GGCGGTGTGTACAAGGCCC 3') was used as probe for the restriction fragment length polymorphism (RFLP) analysis of the plastid genome.

Polymerase chain reactions (PCR)

Total cellular DNA was amplified in an Eppendorf thermal cycler using GoTaq® Flexi DNA Polymerase (Promega) and gene-specific primer pairs. The standard PCR program was 30 to 40 cycles of 1 min at 94 °C, 40 s at 49-58 °C, and 1 to 2.5 min at 72°C with a 5 min extension of the first cycle at 94°C and a 5 min final extension at 72°C. To detect the transferred reporter and marker genes, the following synthetic oligonucleotides were used as primers:

aadA: PaadAfor 5’ CGCCGAAGTATCGACTCA 3’; PaadArev 5’ TCGCGCTTAGCTGGATAAC 3’

nptII: Pnptfor 5’ GAGGCAGCGCGGCTATC 3’; Pnptrev 5’GCGGTCCGCCACACCCA 3’

gfp: Pgfpfor 5’ AAAGAGCTCGCTCCCCCGCCGTCG 3’; Pgfprev 5’ TTTTCTAGATTAGTTCATCCATGCCAT 3’

yfp: P35Sfor 5’ GACCAAAGGGCTATTGAGAC 3’; P35Srev 5’ CGGGGGATCTGGATTTTAGTAC 3’.

A length polymorphism in the plastid genomes of the tobacco cultivars Petit Havana and Samsun NN was detected by PCR amplifications with primers P5’JLA (5’ GTAGTAAATAGGAGAGAAAATCG 3’) and P3’JLA (5’ AAGGCAGTGGATTGTGAATC 3’). A primer pair for amplification of a Petit Havana-specific nuclear genetic marker was derived from the gene sequence for Avr9/Cf-9 rapidly elicited protein 4 (ACRE4; GenBank accession number AF211528): P5’PH4 (5’ GGTTTCATTGAGTATTTGTC 3’) and P3’PH4 (5’ GTTATCGTACCTTTGTTTCC 3’). Published primer sequences derived from exon 4 of the TMV resistance gene N were employed to amplify a Samsun NN-specific nuclear marker (*7*).

Confocal laser scanning microscopy and karyotyping

Subcellular localization of GFP and YFP fluorescence was determined by confocal laser-scanning microscopy (TCS SP2; Leica, Wetzlar, Germany) using an argon laser for excitation (at 488 nm) and a 500-510 nm filter for detection of GFP fluorescence, a

514-527 nm filter for detection of YFP fluorescence and a 610-700 nm filter for detection of chlorophyll fluorescence.

Chromosome numbers were determined by staining of root tips harvested from aseptically grown plants following standard procedures. Briefly, root tips were washed, incubated in water overnight at 4°C followed by fixation in a 3:1 mix of glacial acetic acid and ethanol overnight. After 5 min incubation in 45% acetic acid, the root tips were stained in 1% acid orcein solution for 1-2 days. Stained root tips were then squashed on a glass slide in a drop of 45% acetic acid and metaphase plates were analyzed by light microscopy.

Inheritance assays

To determine the inheritance patterns of the transferred genes, seeds were produced by selfing GY and YG plants and by crossing them reciprocally to wild-type plants. Surface-sterilized seed samples were then germinated on synthetic medium (*1*) in the presence or absence of antibiotics. Maternal inheritance of the plastid transgenes was assayed by germination in the presence of spectinomycin, Mendelian inheritance of the nuclear transgenes was determined by germination on kanamycin-containing medium.

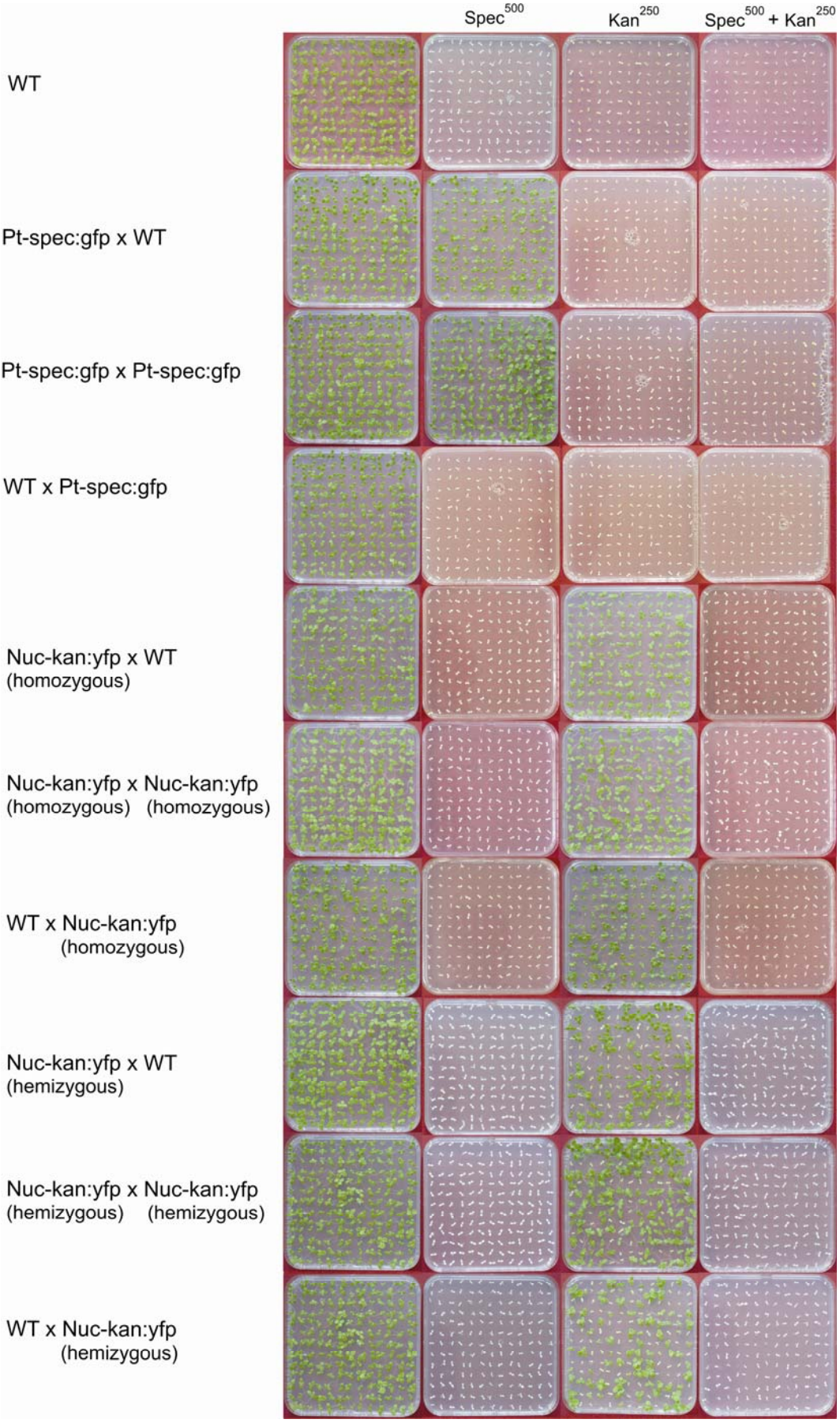
Spec500
Kan250
Spec500 + Kan250
WT
Pt-spec:gfp x WT
Pt-spec:gfp x Pt-spec:gfp
WT x Pt-spec:gfp
Nuc-kan:yfp x WT
(homozygous)
Nuc-kan:yfp x Nuc-kan:yfp
(homozygous) (homozygous)
WT x Nuc-kan:yfp
(homozygous)
Nuc-kan:yfp x WT
(hemizygous)
Nuc-kan:yfp x Nuc-kan:yfp
(hemizygous) (hemizygous)
WT x Nuc-kan:yfp
(hemizygous)

Fig. S1. Genetic analysis of transgenic lines used in grafting experiments. Transgenic plants were either selfed or reciprocally crossed to wild-type plants (WT) and the resulting progenies were subjected to resistance tests on media without antibiotics, with spectinomycin (500 mg/l; $Spec^{500}$), kanamycin (250 mg/l; Kan^{250}) or both spectinomycin and kanamycin ($Spec^{500}$ + Kan^{250}). As expected, the plastid-encoded spectinomycin resistance is maternally inherited and does not show segregation in the progeny. In contrast, the nuclear encoded kanamycin resistance displays Mendelian inheritance and crosses with hemizygous plants result in segregating progeny. Segregation ratios are as expected: 3:1 (127:42) if the hemizygous Nuc-kan:yfp line is selfed and 1:1 (84:85 and 80:88, respectively) if it is crossed to the wild type (Nuc-kan:yfp x WT and WT x Nuc-kan:yfp, respectively).

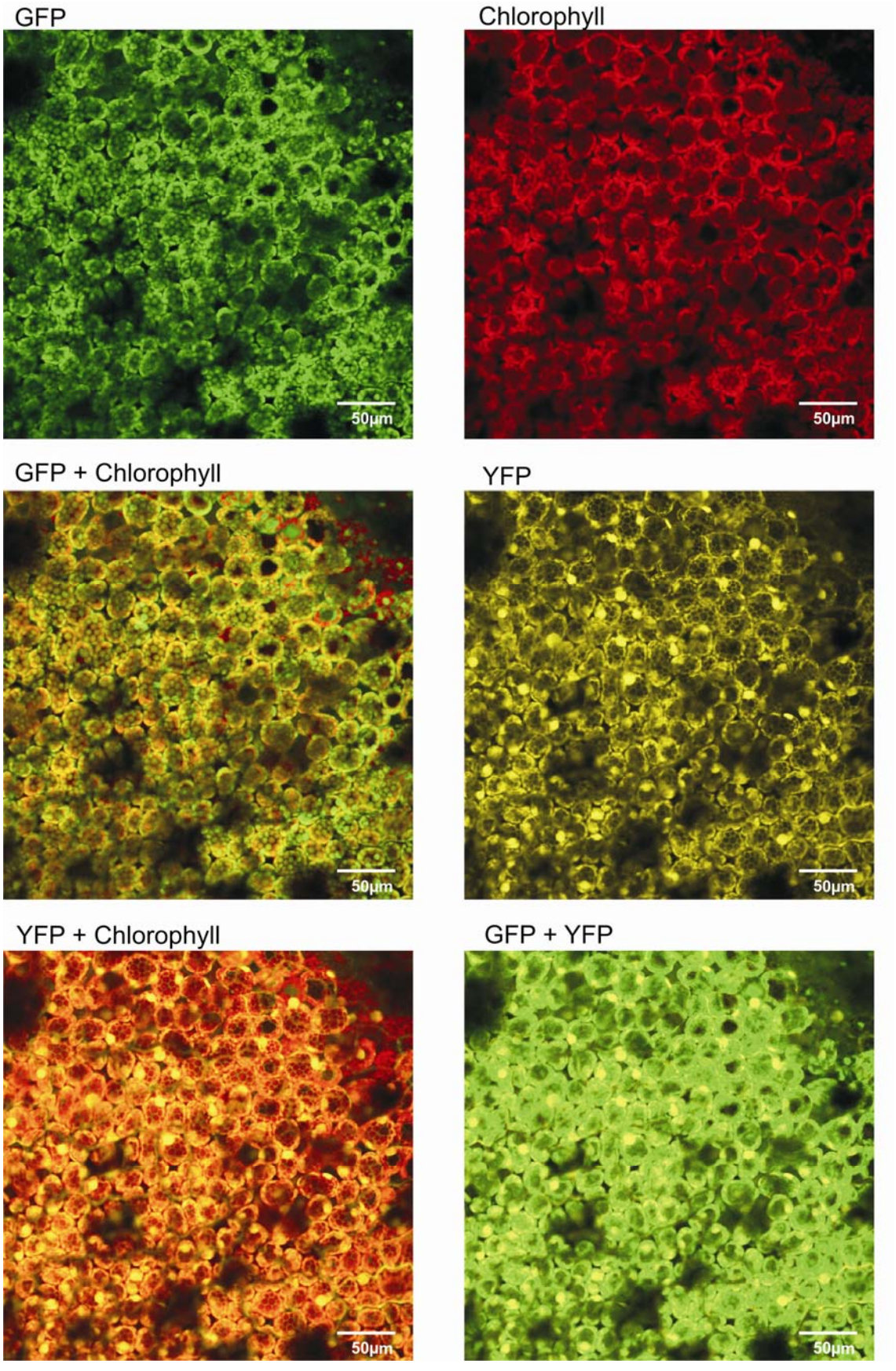
GFP
50µm
Chlorophyll
50µm
GFP + Chlorophyll
50µm
YFP
50µm
YFP + Chlorophyll
50µm
GFP + YFP
50µm

Fig. S2. Expression of both fluorescent reporter proteins in all cells of a regenerated doubly antibiotic-resistant YG line. Filters used to detect the fluorescences of chlorophyll, GFP and YFP are indicated above each panel. All cells of the YG line show both green GFP fluorescence in the chloroplast and yellow YFP fluorescence in the nucleocytosolic compartment. The three pairwise overlays (GFP + Chlorophyll, YFP + Chlorophyll and GFP + YFP) are also shown.

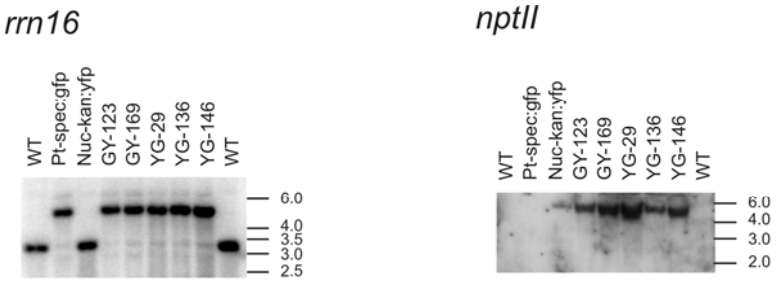

Fig. S3. Southern blot analyses. To assay for the presence of the two plastid transgenes, DNA samples were digested with EcoRV and XhoI and hybridized to a radiolabeled probe detecting the region of the plastid genome that flanks the transgene insertion site (*rrn16*; Fig. 1A). The size difference between the fragment detected in wild-type and Nuc-kan:yfp plants and the fragment detected in Pt-spec:gfp plants, GY plants and YG plants corresponds to the sum of the sizes of the *aadA* and *gfp* cassettes. To test for the presence of the nuclear kanamycin resistance gene, DNA was cut with HindIII and hybridized to an *nptII*-specific probe. The chloroplast RFLP analysis also established that (i) all chloroplast genome copies in GY and YG plants harbor the two transgenes, a status referred to as homoplasmy, and (ii) the plastid transgenes in GY and YG lines still reside in the same genomic location. This was expected, because DNA integration into plastid genomes is known to occur exclusively via homologous recombination (*8*).

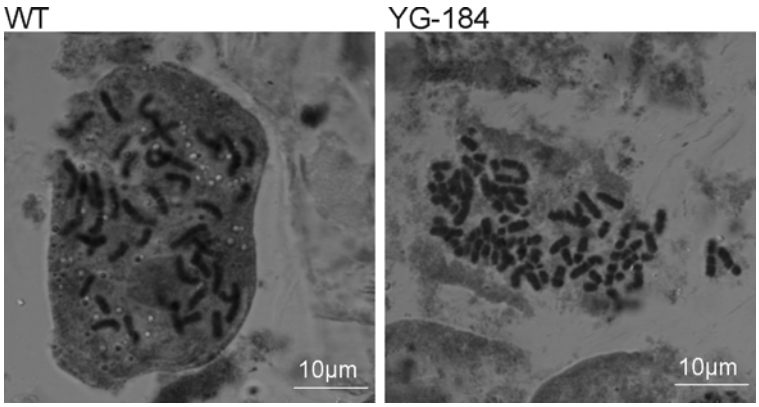

Fig. S4. Metaphase plate of a wild-type tobacco cell (left) and a cell from gene transfer line YG-184 (right). Chromosome numbers are identical in both cells (48 chromosomes) suggesting that the YG line does not originate from fusion of a Pt-spec:gfp cell and a Nuc-kan:yfp cell.

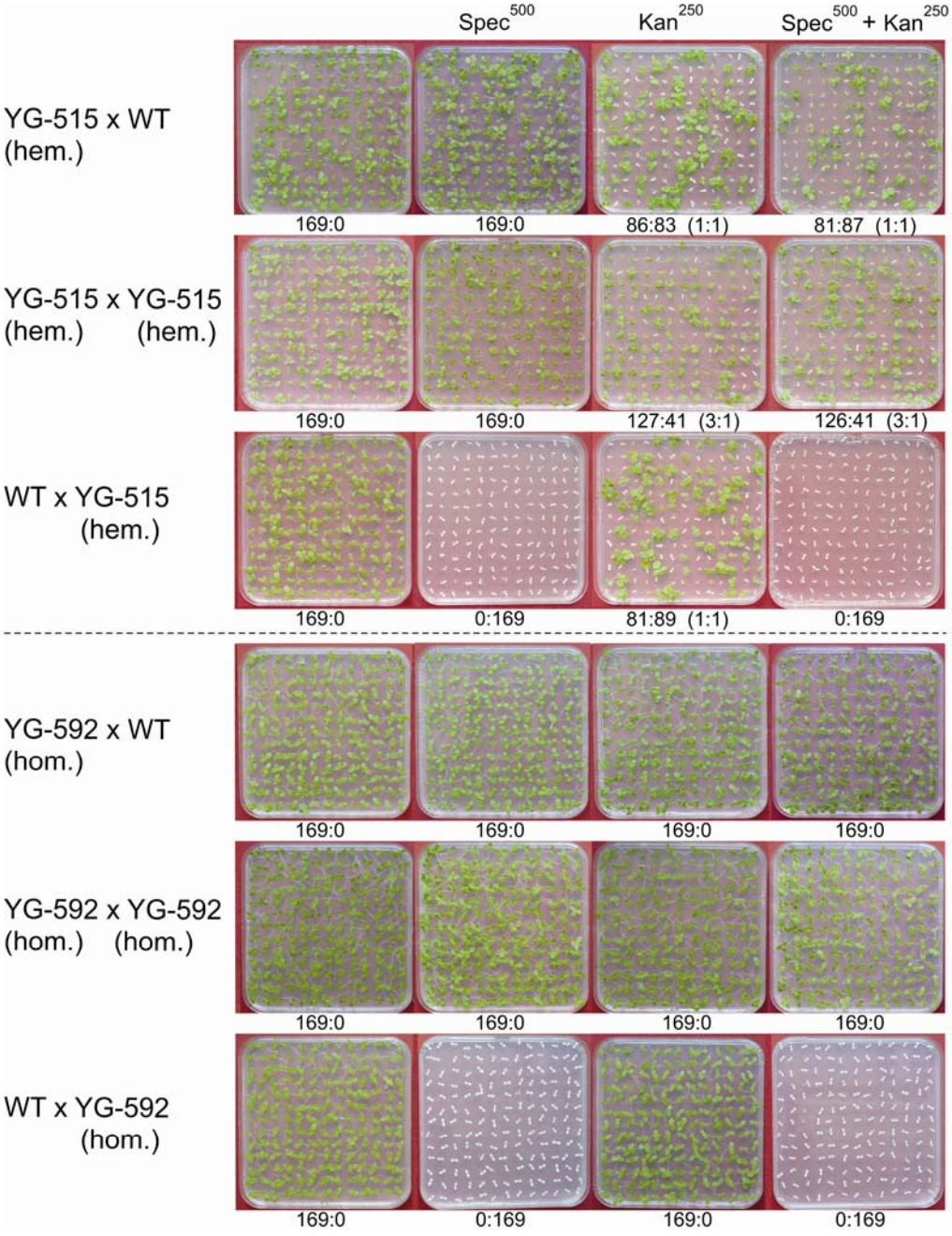

Fig. S5. Seed assays to confirm stable inheritance of the transferred antibiotic resistance genes. Selected gene transfer plants were either selfed or reciprocally crossed to wild-type plants (WT) and the resulting progenies were subjected to resistance tests on media without antibiotics, with spectinomycin (500 mg/l; $Spec^{500}$), kanamycin (250 mg/l; Kan^{250}) or both spectinomycin and kanamycin ($Spec^{500}$ + Kan^{250}). The upper three

crosses represent a gene transfer line selected from a graft of a hemizygous Nuc-kan:yfp plant (hem.) with a Pt-spec:gfp plant, whereas the lower three panels show the analogous crosses for a gene transfer line selected from a graft of a homozygous Nuc-kan:yfp plant (hom.; see fig. S1). Note that the spectinomycin resistance is maternally inherited, as expected of a plastid-encoded trait, whereas the kanamycin resistance shows Mendelian inheritance. Segregation ratios (resistant: sensitive) are indicated for all crosses. Lack of segregation of the kanamycin resistance in the progeny of the YG-592 (hom.) line, which was further confirmed in large-scale assays with more than a thousand seeds, excludes cell fusion as a source of doubly resistant cell lines. This is because fusion of a homozygous Nuc-kan:yfp cell with a Pt-spec:gfp cell would result in tetraploid plants of the constitution RRrr (with R denoting the dominant kanamycin resistance gene and r the recessive kanamycin sensitivity), which, if selfed, would segregate in the next generation 35:1 (resistant:sensitive). (Note that, as *Nicotiana tabacum* is an allotetraploid species, strictly speaking, such plants would be autoallooctoploid.) The inheritance data also provide important information about the mechanism of intercellular gene transfer. If the nuclear transgenes are transferred from a Nuc-kan:yfp cell to a Pt-spec:gfp cell, movement of a DNA fragment carrying the *ntpII* and *yfp* transgenes out of a homozygous Nuc-kan:yfp cell into a Pt-spec:gfp cell, most plausibly, would produce cells that are hemizygous for both *nptII* and *yfp*. In contrast, if the plastid transgenes moved out of a Pt-spec:gfp cell into a neighboring homozygous Nuc-kan:yfp cell, the resulting cell lines should be homozygous for the kanamycin resistance gene and not segregate in the next generation. Lack of genetic segregation in in the progeny of the YG-592 (hom.) line indicates that the intercellular

genetic exchange is more likely to be explained by movement of plastid genes than transfer of nuclear genes.

Table S1. Overview of the genetic screens for intercellular gene transfer.

Tissue exposed to selection	**No. of grafts**	**No. of candidate lines (Kan^{250} + $Spec^{500}$)**	**No. of GFP and YFP-positive lines**
Fused grafts (sliced)	GY: 33 YG: 41 Total: 74	GY: 38 YG: 56 Total: 94[(1)]	GY: 38 YG: 56 Total: 94
Fused grafts (whole graft site)	GY: 5 YG: 5 Total: 10	GY: 3 YG: 3 Total: 6	GY: 3 YG: 3 Total: 6
Unfused grafts	GY: 3 YG: 3 Total: 6	GY: 0 YG: 0 Total: 0	GY: 0 YG: 0 Total: 0

[(1)] The majority of grafts (45 out of 74) gave rise to at least one positive line and the maximum obtained was 5 lines from a single graft. In addition to a stochastic component, differences in the size of the fusion area between scion and stock may account for the different numbers of events per graft.

Table S2. Selection for long-distance gene transfer across graft junctions.

Graft / grafting partner analyzed	**Number of leaf pieces[(1)] subjected to double selection**	**Number of selected gene transfer lines from leaves**	**Number of stem slices subjected to double selection**	**Number of selected gene transfer lines from stems**
GY / Pt-spec:gfp	135	0	80	0
YG / Pt-spec:gfp	114	0	51	0
GY / Nuc-kan:yfp	136	0	43	0
YG / Nuc-kan:yfp	114	0	51	0

[(1)] Average size of leaf pieces: 5 x 5 mm

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