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Exchange of Genetic Material Between Cells in Plant Tissue Grafts

Sandra Stegemann and Ralph Bock*

Tissue grafting includes applications ranging from plant breeding to animal organ transplantation. Donor and recipient are generally believed to maintain their genetic integrity, in that the grafted tissues are joined but their genetic materials do not mix. We grafted tobacco plants from two transgenic lines carrying different marker and reporter genes in different cellular compartments, the nucleus and the plastid. Analysis of the graft sites revealed the frequent occurrence of cells harboring both antibiotic resistances and both fluorescent reporters. Our data demonstrate that plant grafting can result in the exchange of genetic information via either large DNA pieces or entire plastid genomes. This observation of novel combinations of genetic material has implications for grafting techniques and also provides a possible path for horizontal gene transfer.

Grafting is widely used in plant breeding programs in order to modify plant architecture, improve vigor, or increase disease resistance. Grafting also occurs naturally; for example, when the stems or roots of trees contact each other (*1*). Although the grafted tissues fuse and establish vascular connections, the stock (the lower part of the graft) and scion (the upper part, usually supplying solely aerial parts to the graft) are thought not to exchange their genetic materials (*2*).

To test this assumption, we generated two transgenic tobacco lines carrying different selection markers and reporters. One line, Nuc-kan:yfp, harbors a kanamycin resistance gene (*nptII*) and the yellow fluorescent protein gene (*yfp*) in its nuclear genome, whereas the other, Pt-spec:gfp, possesses a spectinomycin resistance gene (*aadA*) and the green fluorescent protein gene (*gfp*) in its plastid (chloroplast) genome (Fig. 1A and fig. S1).

We performed grafting experiments in which Pt-spec:gfp scions were grafted onto Nuc-kan:yfp

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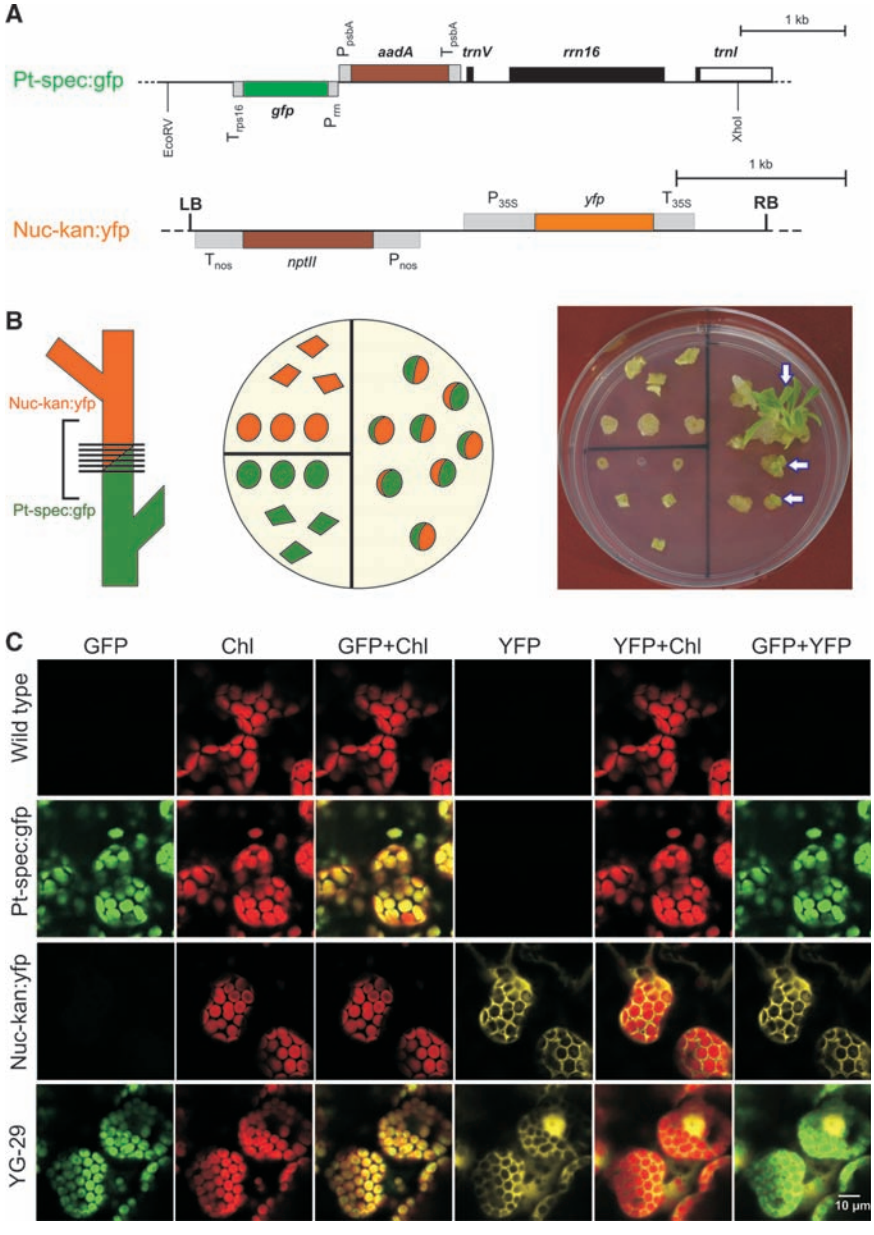

Fig. 1. Genetic screen for intercellular gene transfer. (**A**) Maps of the plastid genome in Pt-spec:gfp plants and the transgenic locus in Nuc-kan:yfp plants. P_{psbA} and T_{psbA}, promoter and terminator from the plastid *psbA* gene; P_{rrn}, promoter from the plastid rRNA operon; T_{rps16}, terminator from the plastid *rps16* gene; P_{nos} and T_{nos}, promoter and terminator from the nopaline synthase gene from *Agrobacterium tumefaciens*; P_{35S} and T_{35S}, promoter and terminator from the cauliflower mosaic virus 35*S* transcript; LB and RB, left and right borders of the T-DNA region; Eco RV and Xho I, restriction sites used for restriction fragment length polymorphism analysis (fig. S3). (**B**) Selection experiments. The grafted stem region was either sectioned (horizontal lines) or directly exposed to selection (bracket). The middle panel shows the arrangement of tissue explants, the right panel a selection plate (right half, stem sections from the graft site; upper left quarter, three stem sections and three leaf explants from Nuc-kan:yfp; lower left quarter, the corresponding explants from Pt-spec:gfp). After 4 weeks on medium with spectinomycin and kanamycin, some explants from the graft site developed growing callus tissue or regenerating shoots (arrows). (**C**) Expression and subcellular localization of the fluorescent reporters. The wild type, the two grafting partners, and a YG line were assayed for GFP, chlorophyll (Chl), and YFP fluorescence.

stocks and vice versa. After the establishment of a physical connection, the graft site was excised and analyzed for gene flow between scion and stock by testing for the presence of cells that harbor both the kanamycin resistance gene (from Nuc-kan:yfp) and the spectinomycin resistance gene (from Pt-spec:gfp). Exposure of stem sections to double selection for kanamycin and spectinomycin resistance frequently yielded resistant calli (that is, mounds of undifferentiated cells) and regenerating shoots (Fig. 1B). Cell lines isolated from grafts with Pt-spec:gfp as scion and Nuc-kan:yfp as stock are referred to as GY; lines from the reciprocal grafting are referred to as YG.

We next assayed GY and YG lines for expression of the fluorescent reporters. All cells in the regenerated plants showed GFP fluorescence in chloroplasts and YFP fluorescence in the cytosol, indicating that the two reporter proteins were present in the same cell (Fig. 1C and fig. S2). It is known that some proteins and RNAs can travel between cells and across graft junctions (*3*). We therefore performed Northern blot analyses to confirm transcription of all four transgenes in GY and YG lines (Fig. 2A) and also demonstrated the presence of all transgenes at the DNA level (Fig. 2B and fig. S3).

We recovered doubly resistant lines at high frequency (94 events from 74 grafted plantlets, table S1). The frequency of intercellular gene transfer was independent of the orientation of the graft (table S1). When we subjected leaf explants and distant stem sections to selection, no resistant lines were obtained (table S2), suggesting that gene transfer is confined to the graft site and no long-distance transfer may occur. Whether the gene transfer is strictly dependent on direct cell-to-cell contact remains to be investigated.

Experiments in which whole graft sites were subjected to selection (Fig. 1B) also produced resistant cell lines, suggesting that tissue injury (by cross-sectioning) was not involved in the gene transfer process. In contrast, when stock and scion were separated before fusion, no resistant lines were obtained (table S1). Karyotype analyses excluded the possibility that YG and GY plants arose through fusion of Nuc-kan:yfp cells with Pt-spec:gfp cells (fig. S4). Finally, genetic crosses demonstrated stable inheritance of the transferred genes and additionally confirmed the absence of polyploidy (fig. S5).

Two directions of gene transfer are possible: movement of plastid genes from Pt-spec:gfp cells into Nuc-kan:yfp cells or transfer of nuclear genes from Nuc-kan:yfp to Pt-spec:gfp cells. To distinguish between these possibilities by tracking molecular markers in the plastid and nuclear genomes, we grafted two different tobacco varieties, Petit Havana (PH; Pt-spec:gfp) and Samsun NN (SNN; Nuc-kan:yfp). Analysis of a polymorphism in the plastid genomes of the two cultivars [>15 kb away from the transgene insertion site (*4*)] revealed that all GY and YG plants analyzed carried the PH marker (Fig. 2C), indicating that large DNA pieces or even entire plastid genomes are transferred. Polymorphisms in pathogen resistance genes were used as nuclear markers (*5*). All GY and YG plants tested positive for the SNN-specific marker and negative for the PH-specific marker (Fig. 2C), suggesting that the plastid transgenes moved from PH cells into SNN cells. Plant cells are connected via plasmatic bridges called plasmodesmata, but the passage of large macromolecules requires the action of specific plasmodesmata-widening proteins (*3*). Whether large DNA pieces or even

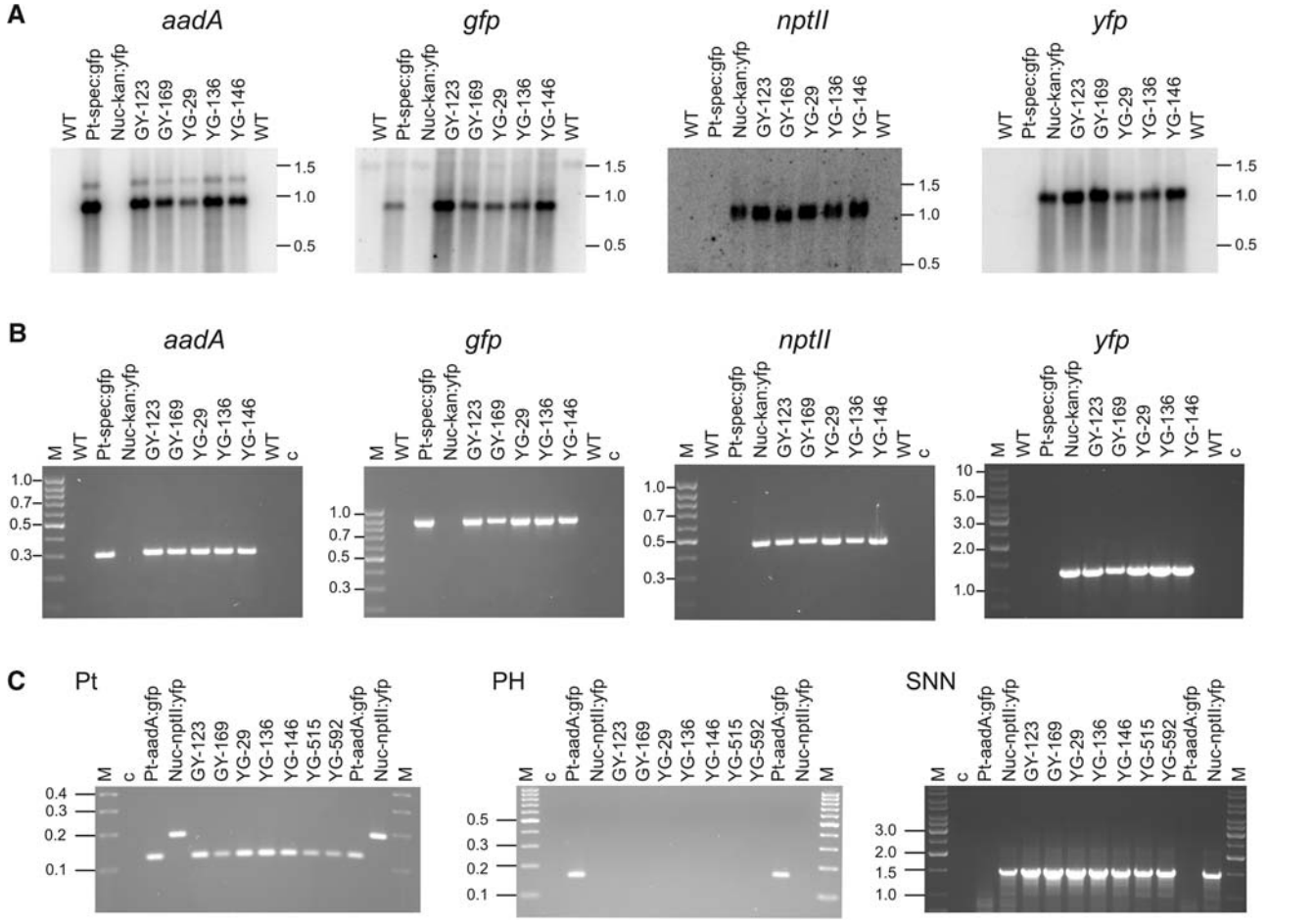

Fig. 2. Analysis of transgenes and molecular markers in gene transfer lines. (**A**) Northern blot analyses of transgene expression. (**B**) Transgene detection at the DNA level by polymerase chain reaction (PCR). (**C**) PCR analysis of plastid and nuclear markers to determine the direction of intercellular gene transfer. Pt, assay of a length polymorphism in the plastid genomes of the two grafted varieties PH [Pt-spec:gfp, 125–base pair (bp) product] and SNN (Nuc-kan:yfp, 191-bp product); PH, a PH-specific nuclear marker (189-bp product); SNN, an SNN-specific nuclear marker (1569-bp product). WT, wild type; M, marker (sizes in kilobase pairs); c, buffer control.

entire organelles can travel through plasmodesmata requires further investigation.

Our discovery of grafting-mediated gene transfer further blurs the boundary between natural gene transfer and genetic engineering and suggests that grafting provides an avenue for genes to cross species barriers. Phylogenetic evidence suggests that DNA can be transferred horizontally between reproductively isolated species (*6*). We propose that grafting (whether natural or assisted) provides a path for horizontal gene transfer.

Finally, although our data demonstrate the exchange of genetic material between grafted plants, they do not lend support to the tenet of Lysenkoism that "graft hybridization" would be analogous to sexual hybridization. Instead, our finding that gene transfer is restricted to the contact zone between scion and stock indicates that the changes can become heritable only via lateral shoot formation from the graft site. However, there is some reported evidence for heritable alterations induced by grafting (*7*) and, in light of our findings, these cases certainly warrant detailed molecular investigation.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/324/5927/649/DC1
Materials and Methods
Figs. S1 to S5
Tables S1 and S2
References

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Circadian Clock Feedback Cycle Through NAMPT-Mediated NAD^+ Biosynthesis

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The circadian clock is encoded by a transcription-translation feedback loop that synchronizes behavior and metabolism with the light-dark cycle. Here we report that both the rate-limiting enzyme in mammalian nicotinamide adenine dinucleotide (NAD^+) biosynthesis, nicotinamide phosphoribosyltransferase (NAMPT), and levels of NAD^+ display circadian oscillations that are regulated by the core clock machinery in mice. Inhibition of NAMPT promotes oscillation of the clock gene *Per2* by releasing CLOCK:BMAL1 from suppression by SIRT1. In turn, the circadian transcription factor CLOCK binds to and up-regulates *Nampt*, thus completing a feedback loop involving NAMPT/NAD^+ and SIRT1/CLOCK:BMAL1.

Many aspects of mammalian behavior and physiology are coordinated through interconnected networks of 24-hour central and peripheral oscillators that synchronize cycles of fuel storage and utilization to maintain organismal homeostasis. In mice, circadian disruption has been tied to metabolic disturbance (*1*, *2*), whereas a high-fat diet alters both behavioral and molecular rhythms (*3*, *4*). The underlying mechanism of the mammalian clock consists of a transcription-translation feedback loop in which CLOCK and BMAL1 activate transcription of *Cryptochrome* (*Cry1* and *2*) and *Period* (*Per1*, *2*, and *3*), leading to subsequent repression of CLOCK:BMAL1 by CRY and PER proteins (*5*). An additional feedback loop involves the transcriptional regulation of *Bmal1* by RORα and REV-ERBα (*6*, *7*). Previous studies have also implicated a role for cellular nicotinamide adenine dinucleotide (NAD^+) in the regulation of CLOCK and NPAS2 activity (*8*), an observation consistent with the recent finding that the NAD^+-dependent protein deacetylase SIRT1 modulates activity of the clock complex (*9*, *10*).

NAD^+ is a classic coenzyme that is synthesized from three major precursors: tryptophan, nicotinic acid, and nicotinamide (fig. S1). In yeast, it has been reported that enzymes involved in the NAD^+ salvage pathway play an important role in the regulation of Sir2 activity (*11*, *12*). On the other hand, in mammals, the predominant NAD^+ biosynthetic pathway involves the conversion of nicotinamide and 5′-phosphoribosylpyrophosphate to nicotinamide mononucleotide (NMN) by the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT) (fig. S1) (*13*–*15*). NAMPT-mediated NAD^+ biosynthesis has been demonstrated to play a critical role in a number of biological processes through the NAD^+-dependent deacetylase SIRT1 (fig. S1) (*13*, *16*–*18*).

These findings led us to hypothesize that circadian regulation of NAD^+ may provide a mechanism for regulation of the core clock. Expression levels of *Nampt* RNA displayed a robust diurnal pattern in both wild-type (WT) mouse liver and white adipose tissue (WAT), peaking at the beginning of the dark period [*zeitgeber* time (ZT) 14] (Fig. 1A) (*19*). Expression levels of NAMPT protein also showed a diurnal pattern of oscillation across the 24-hour light-dark cycle in liver (Fig. 1B), with a reduction in NAMPT protein levels before the onset of the dark period [Fig. 1B and supporting description 1 in the supporting online material (SOM)]. *Nampt* RNA oscillation is circadian in nature, as we found a robust oscillation of *Nampt* RNA for 48 hours in liver isolated from WT mice that had been maintained in constant darkness [$P < 0.01$, one-way analysis of variance (ANOVA)] (Fig. 1C). Furthermore, the levels of *Nampt* RNA and protein were lower across the entire light-dark cycle in liver and WAT of $Clock^{\Delta 19}$ mutant mice, and the diurnal oscillation of *Nampt* RNA and protein was abolished in $Clock^{\Delta 19}$ mutant mice (Fig. 1, A and B). The robust *Nampt* RNA oscillation during constant darkness was also abolished completely in the $Clock^{\Delta 19}$ mutant mouse liver (Fig. 1C), indicating that the core clock machinery is required for the circadian control of NAMPT expression.

Because NAMPT is the rate-limiting enzyme in the predominant NAD^+ biosynthetic pathway in mammals, we tested whether tissue NAD^+ levels also display a circadian oscillation pattern in WT liver across 48 hours from mice maintained in constant darkness. Liver NAD^+ levels showed a very similar bimodal circadian oscillation pattern to that of NAMPT protein, and the lowest levels of NAD^+ occurred with a rhythmicity of 24 hours (Fig. 1D). We also observed a decrease in NAD^+ levels around CT10-14 and again 24 hours later (CT34), generating the observed bimodal oscillation pattern (Fig. 1D and supporting description 1). The degree to which NAD^+ levels increased from baseline (40 to 53%) during these daily cycles, as well as the concentrations of NAD^+ that we measured, fall within the physiological range of reported alterations in NAD^+ levels (supporting description 2) (*20*, *21*).

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