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Jumping genes and containment

A recent study on the transfer of foreign genes from chloroplasts to the nucleus raises technical issues that require resolution.

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Because chloroplast DNA is usually maternally inherited during seed formation, the engineering of transplastomic crops has been put forward as a promising means of containing transgenes[1–3]. For this strategy to progress, greater understanding of the process of gene transfer from chloroplasts to the nuclear genome is required. A recent report in *Nature* by Timmis and colleagues[4] describes the transfer of foreign genes from the chloroplast to the nuclear genome of tobacco at unprecedented rates. Among 250,000 progeny obtained by fertilization of wild-type females with pollen from two chloroplast transgenic lines, sixteen independent lines were identified that contained transgenes (0.006%). Although Timmis and colleagues claim DNA transfer from plastid to nucleus at a rate of one in ~16,000 pollen grains, they note that none of the transgenes functional within transgenic chloroplasts is functional in the nuclear genome. Contrary to media interpretations of the data, their results do not call into question the use of chloroplast engineering as a transgene containment strategy.

Evidence from genome projects suggests that the cytoplasmic organelles (e.g., chloroplasts or mitochondria) have lost many genes during evolution[5–7]. In their new paper, Timmis and colleagues attempt to measure directly the transfer rate of chloroplast DNA into the nucleus of tobacco plants using transgenic constructs. Their paper is an ambitious attempt to reproduce in an experiment the process of DNA depletion/transfer from organelle to nucleus that takes place over evolutionary timescales.

To demonstrate transposition of genes from the chloroplast to nuclear genome, Timmis and colleagues inserted a nuclear specific *neo STL2* gene that contained a constitutive plant viral promoter (CaMV 35S). This cassette was designed to be functional only when transposed to the nucleus, and to confer kanamycin resistance. An intron from the potato nuclear *ST-LS1* gene and eukaryotic regulatory signals were inserted within, upstream, or downstream of the *neo* coding sequence to prevent synthesis of functional neomycin phosphotransferase inside transgenic chloroplasts. To select for chloroplast transgenic lines, the authors also inserted into the transgene cassette the *aadA* gene encoding aminoglycoside 3′ adenyltransferase under the control of the chloroplast *psbA* gene sequences (5′- and 3′-untranslated regions). Both genes, along with regulatory sequences, were inserted into the spacer region between chloroplast *trnV* and *rps* 12/17 genes to facilitate homologous recombination and site-specific integration of transgenes.

The authors obtained only two independent chloroplast transgenic lines (tp7 and tp17) and confirmed homoplasmy (integration of transgenes into all chloroplast genomes) by Southern blots probed with the same chloroplast DNA flanking sequences used to facilitate homologous recombination. In Southern blots of the transgenic lines (tp7, tp17) probed with *neo* or *aadA* coding sequences, several hybridizing fragments of lower intensity are quite evident (see original paper[4], Fig. 3B,D). Such low-intensity hybridizing fragments would be expected if one or two copies of transgenes integrated into the nuclear genome as opposed to thousands of copies of transgenes integrated into chloroplast genomes. These additional fragments are not explained by partial digestion of plant DNA because all other expected size hybridizing DNA fragments are observed in the same blot (5.9 kb and 3.4 kb for *neo* probe and 11.4 kb for *aadA* probe). These hybridizing fragments are more prominent in subsequent generations because of an increase in transgene copy numbers after sexual crosses resulting in homozygous nuclear transgenes. Additional hybridizing fragments observed in primary chloroplast transgenic lines should not result from nonspecific chloroplast integration because there are no illegitimate recombination events in chloroplast genomes.

On the basis of the data presented, it is not clear that Timmis and colleagues can exclude the possibility that their transgenic lines had transgene integration only into the chloroplast genome. Instead, it is possible they obtained two transgenic lines with simultaneous integration into the nuclear and chloroplast genomes. If this were indeed the case, an alternative interpretation of the results is clearly warranted.

It would have been helpful if the authors had tested primary chloroplast transgenic lines (tp7 and tp 17) by inverse polymerase chain reaction and analyze junction sequences to look for simultaneous nuclear and chloroplast integration of transgenes. In previous published reports[1,2], numerous chloroplast transgenic lines have been screened by probing with transgene coding sequences and prolonged exposure on the Southern blots (to compensate for the few copies of nuclear integration that arise). Using this strategy, transgenic lines with simultaneous integration into nuclear or mitochondrial genomes can be eliminated. Simultaneous integration into the nuclear genome would also explain segregation ratios that deviated from the 3:1 ratio of mendelian inheritance, complex transgene integration patterns, and the loss of neo^R genes in subsequent generation reported by the authors.

The argument that the transgenic material detected in the nucleus by Timmis and colleagues resulted from integration during transformation is made even more compelling by the authors' observation that nonplastid DNA (nuclear DNA or genes) adjoins the 'transposed' chloroplast DNA. If indeed chloroplast DNA were transposed to the nucleus, transgenes should be flanked by regions longer or shorter than those present in chloroplast vectors used for transformation. Lack of chloroplast DNA flanking sequences longer or shorter than those present in chloroplast vectors and the presence of only nuclear DNA at junctions of transgene integration in all transgenic lines confirm direct integration of transgenes into the nuclear genome during bombardment and argues against any transposition from chloroplast genomes.

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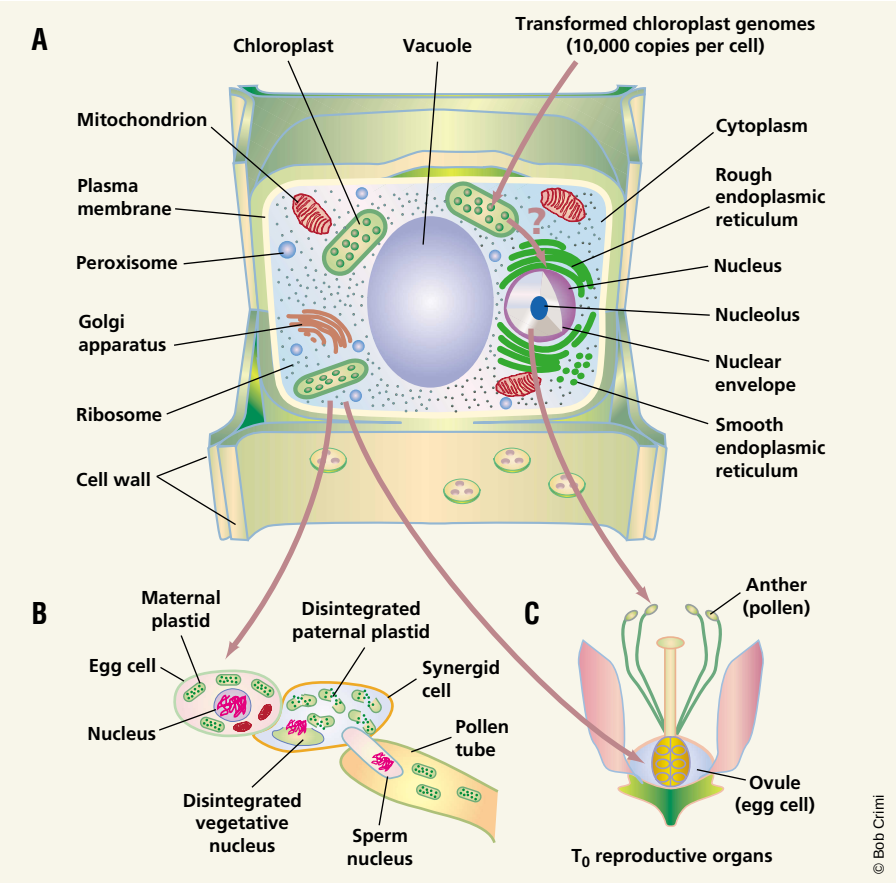

Figure 1. Maternal inheritance and gene containment. (A) The plant cell shows three compartments that contain DNA: nuclear, chloroplast, and mitochondrial genomes. The question mark raises the possibility that transgenes can jump from the chloroplast to nuclear genome. (B) During meiosis, haploid egg and sperm cells are formed. The synergid cell attracts the pollen tube by secretion of calcium, carbohydrates, and proteins. Fertilization begins when the pollen tube enters the synergid cell. Once inside the cytoplasm of the synergid cell, the pollen tube ruptures releasing its contents. The paternal chloroplasts are disintegrated and only the sperm nucleus enters the egg cell and fuses with the egg to form zygote. The zygote contains only maternal plastids because the paternal plastids disintegrate in the synergid cell. Thus, maternal inheritance of transgenes offers containment because of lack of gene flow through pollen. (C) Reproductive floral organs. Anthers produce pollen. Ovules contain egg cells. If transgenes were ever transposed from chloroplast to nuclear genomes, the data from Timmis and colleagues[4] indicates they would be passed on via pollen at a very low frequency of 0.006%. However, even if transgenes are passed on to the nuclear genome, they are not functional.

Timmis and colleagues also observe multiple copies of transgenes integrated into several transgenic lines, which is typically observed in nuclear transgenic lines obtained via particle bombardment. If this were to happen by transposition, it would have required several independent transposition events from the chloroplast to nuclear genome with varied lengths of chloroplast flanking sequences and one or both transgenes. Again, presence of only the same length of chloroplast DNA sequences as used in chloroplast vectors and both transgenes in all transgenic lines confirm direct integration in the nuclear genome during bombardment and argue against multiple independent transposition events.

Notwithstanding these technical issues, one wonders whether the findings present any concerns for gene containment strategies based on maternal inheritance (see Fig. 1). In their study, Timmis and colleagues screen 250,000 progeny and identify 16 plants of independent origin that show stable integration of the *neo STLS2* and *aadA* genes into the nuclear genome. While the *neo* gene with eukaryotic regulatory sequences was functional, the *aadA* gene (encoding/conferring resistance to spectinomycin) with chloroplast regulatory sequences was nonfunctional. This is a significant observation with regard to genetically modified crops engineered via chloroplast genomes. Timmis and colleagues conclude that even if foreign genes are transposed from chloroplast to nuclear genomes, they will not be functional, practically eliminating concerns about such transfer[8]. For proper expression in the nucleus, chloroplast genes transposed to the nucleus would have to acquire appropriate nuclear regulatory sequences for transcription and translation. This would be a major challenge for chloroplast genes transposed into the nuclear genome. Lack of functionality of the *aadA* gene with prokaryotic regulatory sequences in any of the 250,000 seedlings screened by Timmis and colleagues confirms the rarity of such an event (if it ever, in fact, did occur). As an aside, it is also highly unlikely that anyone would ever insert a transgene, for genetic engineering purposes, into the chloroplast genome with eukaryotic regulatory sequences that are nonfunctional in chloroplasts.

Finally, it is worth thinking about the authors' findings in the context of evolution. The chloroplast genome has undergone extreme reduction in size and gene content post endosymbiosis from a cyanobacterial ancestor[7]. Many of these losses occurred in parallel during diversification of photosynthetic eukaryotes, while a certain number of these losses were functionally transferred to the nucleus before land plant diversification[7,9] (*ca*. 470 million years ago). However, after the evolution of land plants, two cases of transfer of functional chloroplast genes to the nucleus have been documented[10]. Intact gene transfer from the chloroplast to nuclear genome and expression requires the acquisition of regulatory elements for transcription, translation, and protein transport, the rate of which must be minimal, as documented above (two cases detected so far in *ca*. 470 million years). The incredibly high rate of chloroplast DNA transfer into the nucleus reported by the authors (eighteen transposition events in a single generation from two transgenic lines) thus appears to be an overestimate. Otherwise, as nuclear genomes do not seem to be continuously expanding in size, one must assume a mechanism exists that maintains genome size by selectively deleting such nonfunctional transferred genes. To our knowledge, no such mechanisms have been identified that could accomplish such selective gene removal from nuclear genomes of plants. Current data refute this: although large tracts of plastid DNA are found in the nucleus of many plants[5,6], their size is several orders of magnitude smaller than that implied by the rates proposed by these authors.

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