Transgene containment by maternal inheritance: Effective or elusive?

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Transgene containment is a central concern in genetically modified (GM) crops, especially for those with out-crossing wild relatives. However, plant cells contain genomes in the nucleus, mitochondria, and chloroplasts. Whereas nuclear genes are biparentally inherited, organelle genes are in general maternally inherited (1, 2). Therefore, engineering foreign genes in the chloroplast genome may provide containment from pollen transmission. This topic was hotly debated when the first herbicide-resistant (Round-Up-ready) transplastomic plants (transformed with chloroplast genomes) were reported (3). In this issue of PNAS, two articles highlight different aspects of this important question. The article by Svab and Maliga (4) examines whether alien cytoplasm contributes to rare paternal plastid transmission. They report low frequencies (10^{-4} to 10^{-5}) of paternal plastids in both normal and alien cytoplasm and caution that this observation is biased by tissue culture selection, and therefore transgenes are less likely to get into the germ line under field conditions. Most importantly, these investigators show that the entire plastid genome is transmitted by pollen instead of plastid DNA fragments and that the mitochondria are also cotransmitted. In a parallel study, Ruf *et al.* (5) set up a stringent selection system for paternal transmission by using male-sterile maternal parents and transplastomic pollen donors, conferring plastid-specific antibiotic resistance and green fluorescence for visual screening. This selection system identified 6 among 2.1 million seedlings screened (frequency of 2.86×10^{-6}) that showed paternal transmission of transgenes, and the authors concluded that plastid transformation provides an effective tool to increase biosafety of GM crops.

Mechanism of Maternal Inheritance

Most flowering plants show uniparental maternal inheritance of plastids (2). This observation was first reported a century ago in *Milabilis jalapa* and is achieved through a variety of mechanisms (2). During pollen development, at the microspore mitosis, all plastids are distributed into vegetative cells, and, therefore, the generative cells that form sperm cells are free of plastids (the *Lycopersicon* type). In some species young generative cells may have a few plastids, but maternal generative cells are free of plastids because they degenerate (the *Solanum* type). In monocots, sperm cells contain plastids, but the plastids are not transmitted into the egg cell because they are degraded before fertilization in the synergid cell. In *Chlamydomonas*, the paternal plastid DNA (ptDNA) is degraded within 10 min after zygote formation by specific nucleases; the maternal plastid DNA is protected by methylation. Thus, plastid DNA is degraded at different stages from the very first step of pollen mitosis, before fertilization, or even after zygote formation. However, there are rare exceptions to uinparental maternal inheritance. For example, in *Medicago* or *Oenothera*, there is an equal distribution of plastids during the first pollen mitosis into the generative and vegetative cells; therefore, sperm cells transmit plastids into egg cells.

Evaluation of Transgene Containment

Ruf *et al.* (5) devised an interesting experimental system consisting of male-sterile plants as maternal parents and transplastomic lines as pollen donors, which carry two transgenes: the *aadA* gene to confer spectinomycin/streptomycin resistance and *gfp* to provide green fluorescence for visible screening. More than 2.5 million seedlings were screened for green tissue sectors among bleached-out seedlings. To eliminate specific point mutations in the 16S rRNA gene that also result in green sectors, the authors also screened seedlings under a fluorescent stereomicroscope. Homoplasmic transgenic lines were regenerated from all seedlings. Among 2.1 million seedlings screened, 118 seedlings with green sectors were identified. Among these 79 were spontaneous 16S rRNA mutants. In the remaining 39 lines, a significant number contained transgenic chloroplasts only in their cotyledons, and these chloroplasts were lost quickly. Only 6 seedlings contained stable paternal plastids in apical meristems. The authors conclude that the frequency of occasional paternal transmission in the field will be in the range of 10^{-8}, and this makes plastid transformation an excellent tool for the prevention of transgene dispersal by pollen. This frequency will be even less because of the absence of selection pressure under field conditions.

Svab and Maliga (4) tested ptDNA transmission by using a very different approach. An alloplastic tobacco line (CMS92) was used as the maternal parent. The paternal line contained the *aadA* gene. Transformed seedlings were therefore antibiotic resistant and male sterile. Ten events in 47,859 seedlings were identified to transfer ptDNA paternally. Testing of paternal ptDNA transmission between parents with normal cytoplasm was carried out with a transplastomic spectinomycin-resistant father and a mother containing nuclear gentamycin resistance. Three ptDNA transfer events were recovered in 34,115 seedlings (9×10^{-5} frequency). On the basis of restriction fragment length polymorphism markers, the authors conclude that the entire ptDNA is transmitted by pollen as intact organelles and not as small ptDNA fragments. There is no evidence for a transformation-like process that was reported for transgenes from plastid to nuclear genome (6). Using polymorphic restriction sites within the *atpB* gene, the authors conclude that mitochondria were cotransmitted with plastids by pollen, a novel observation.

Impact of Transgene Containment on Biotechnology Applications

Both of these articles are timely because the chloroplast genetic engineering field is now entering the phase of field testing and commercial development. Table 1 summarizes biotechnology advances through chloroplast genetic engineering. The first field test of transplastomic tobacco expressing human IFN was conducted recently (7). Chloroplast-derived IFN-α2b had *in vitro* biological activity similar to commercially produced PEG–Intron (Schering, Kenilworth, NJ) when tested for its ability to protect cells against cytopathic viral replication in the standard vesicular stomatitis virus cytopathic effect

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See companion articles on pages 6998 and 7003.

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Table 1. Biotechnology advances by means of chloroplast genetic engineering

Trait or product	Gene	% total soluble protein, functionality	Ref.
Agronomic traits			
Insect resistance	*cry1A(c)*	2–3%, killed Bt-susceptible insects	22
Insect resistance	*cry2Aa2*	46.1%, killed Bt-resistant insects	23
Disease resistance	*msi-99*	21%, killed bacterial and fungal pathogens	24
Drought tolerance	*tps1*	21 days drought, 10% PEG survival	25
Phytoremediation	*merA,B*	Up to 400 μM phenylmercuric acetate	26
Herbicide	*aroA*	Lethal dose, 5 mM glyphosate	3
Herbicide, soybean	*hppd*	5%, lethal dose isoxaflutole (1–2 kg/ha)	27
Salt tolerance, carrot	*badh*	Up to 400 mM sodium chloride	19
Male sterility	*phaA*	Lack of pollen, reversible to fertility	18
Biopharmaceuticals			
Somatotropin	*hst*	7.0%, NB2 cell growth	14
Interferon α2b	*IFNα2b*	19%, antiviral VSV, HIV, antitumor	7
Serum albumin	*hsa*	11.1%, inclusion bodies	16
Interferon γ	*IFN-γ*	6%, antiviral, EMC	15
Proinsulin	*ctxB-pris*	16%, protection against insulitis	17
Vaccines			
Cholera vaccine	*ctxB*	4.1%, GM1 binding	9
Canine parvovirus	*ctxB-2L21*	31.1%, neutralize canine parvovirus	12
Anthrax vaccine	*pag*	18.1%, lethal toxin challenge	8
Tetanus toxin	*tetC*	25%, pathogen challenge	10
Amebiasis	*LecA*	6.3%, systemic immune response	13

First reports of agronomic traits, biopharmaceuticals, or vaccine antigens engineered by using the chloroplast genome are tabulated. The highest reported total soluble protein or evaluated functions are listed. Bt, *Bacillus thuringiensis* toxin; VSV, vesicular stomatitis virus; EMC, encephalomyocarditis virus.

assay and to inhibit early stage HIV infection. The antitumor and immunomodulating properties of IFN-α2b were also seen *in vivo*. CpIFN-α2b increased the expression of MHC I on splenocytes and the total number of NK cells and protected mice from a highly metastatic tumor line (7).

Tobacco is ideally suited for production of biopharmaceuticals or vaccine antigens because up to 40 metric tons of leaves can be harvested from 1 acre (0.40 hectare). Therefore, up to 360 million doses of fully functional anthrax protective antigen expressed in tobacco chloroplasts could be harvested from a single acre of this nonfood/nonfeed crop (8). Several vaccine antigens against bacterial (8–10), viral (11, 12), or protozoan (13) pathogens and human therapeutic proteins (14–17) have already been developed in transgenic tobacco chloroplasts (Table 1). Such high levels of expression, in addition to transgene containment, make this concept commercially attractive. However, when absolute containment for such transplastomic lines is required, additional failsafe methods should be used. One such failsafe method is reversible cytoplasmic male sterility engineered by means of the chloroplast genome by using β-ketothiolase (18). In this system seeds were produced in the greenhouse under continuous light, but under normal day/light conditions plants were sterile.

Oral delivery of biopharmaceutical proteins expressed in plant cells should reduce costs associated with purification, processing, cold storage, transportation, and delivery. Oral administration of transplastomic leaves expressing cholera toxin subunit B (CTB)-proinsulin conferred protection against development of insulitis (diabetes) in nonobese diabetic mice (17). Insulin-producing β-cells in the pancreatic islets of the CTB-proinsulin-treated mice were well preserved, resulting in lower blood and urine glucose levels. Toxicology studies to conduct human clinical trials are in progress. Carrot (19), tomato (20), and lettuce (21) plastid transformation systems have been developed for oral delivery, and human proinsulin has been produced in lettuce chloroplasts (17).

Several valuable agronomic traits have already been introduced by means of the chloroplast genome, including insect resistance (22, 23), herbicide resistance (2), disease resistance (24), drought tolerance (25), salt tolerance (19), and phytoremediation (26). These are examples of the highest levels of expression (up to 46% total soluble protein) or resistance or tolerance reported in the literature. Multigene engineering is also feasible in a single transformation step (23, 26). Chloroplast genomes of several major crops have been transformed, including soybean (27) and cotton (28), tree species such as poplar (29), and vegetables or fruits, including tomato (20), potato (30), and lettuce (21). Further research is required to achieve similar success in monocots. Thus, the field of chloroplast genetic engineering is poised for major advances in the near future.

Both PNAS articles (4, 5) report pollen transmission in the range of 0.0087–0.00024%. Currently, in Europe, non-GM products with 0.9% contamination of GM products are marketed without labeling. Chloroplast genetic engineering should therefore offer containment levels far superior to currently available methods or much lower levels than current contamination guidelines. However, maternal inheritance will contain only pollen transmission. An ideal system, especially for production of vaccines or biopharmaceuticals, should contain transgenes in pollen and seeds. Because the chloroplast transformation system is ideally suited for transgene expression in leaves, it would be necessary to harvest them before the development of any reproductive structures.

1. Daniell H (2002) *Nat Biotechnol* 20:581–586.
2. Hagemann R (2004) in *Molecular Biology and Biotechnology of Plant Organelles*, eds Daniell H, Chase C (Springer, Dordrecht, The Netherlands), pp 93–113.
3. Daniell H, Datta R, Varma S, Gray S, Lee SB (1998) *Nat Biotechnol* 16:345–348.
4. Svab Z, Maliga P (2007) *Proc Natl Acad Sci USA* 104:7003–7008.
5. Ruf S, Karcher D, Bock R (2007) *Proc Natl Acad Sci USA* 104:6998–7002.
6. Stegeman S, Hartmann S, Ruf S, Bock R (2003) *Proc Natl Acad Sci USA* 100:8828–8833.
7. Arlen PA, Falconer R, Cherukumilli S, Cole A, Cole AM, Oishi K, Daniell H (2007) *Plant Biotechnol J*, in press.
8. Koya V, Moayeri M, Leppla SH, Daniell H (2005) *Infect Immun* 73:8266–8274.
9. Daniell H, Lee SB, Pahchal T, Wiebe P (2001) *J Mol Biol* 311:1001–1009.
10. Tregoning JS, Nixon P, Kuroda H, Svab Z, Clare S, Bowe F, Fairweather N, Ytterberg J, van Wijk KJ, Dougan G, Maliga P (2003) *Nucleic Acids Res* 31:1174–1179.
11. Birch-Machin I, Newell CA, Hibberd JM, Gray JC (2004) *Plant Biotechnol J* 2:261–270
12. Molina A, Herva-Stubbs S, Daniell H, Mingo-Castel AM, Veramendi J (2004) *Plant Biotechnol J* 2:141–153.
13. Chebolu S, Daniell H (2007) *Plant Biotechnol J* 2:230–239.
14. Staub JM, Garcia B, Graves J, Hajdukiewicz PT, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L, *et al.* (2000) *Nat Biotechnol* 18:333–338.
15. Leelavathi S, Reddy V (2003) *Mol Breed* 11:49–58.
16. Fernandez-San MA, Mingeo-Castel AM, Miller M, Daniell H (2003) *Plant Biotechnol J* 1:71–79.
17. Ruhlman T, Ahangari R, Devine A, Samsam M, Daniell H (2007) *Plant Biotechnol J*, in press.
18. Ruiz ON, Daniell H (2005) *Plant Physiol* 138:1232–1246.
19. Kumar S, Dhingra A, Daniell H (2004) *Plant Physiol* 136:2843–2854.
20. Ruf S, Hermann M, Berger I, Carrer H, Bock R (2001) *Nat Biotechnol* 19:870–875.
21. Kanamoto H, Yamashita A, Asao H, Okumura S, Takase H, Hattori M, Yokota A, Tomizawa K (2006) *Transgenic Res* 15:205–217.
22. McBride K, Svab Z, Schaaf D, Hogan P, Stalker D, Maliga P (1995) *Biotechnology* 13:362–365.
23. De Cosa B, Moar W, Lee SB, Miller M, Daniell H (2001) *Nat Biotechnol* 19:71–74.
24. DeGray G, Rajasekaran K, Smith F, Saford J, Daniell H (2001) *Plant Physiol* 127:852–862.
25. Lee, S-B, Kwon H, Kwon S, Park S, Jeong M, Han S, Daniell H (2003) *Mol Breed* 11:1–13.
26. Ruiz O, Hussein S, Terry N, Daniell H (2003) *Plant Physiol* 132:1344–1352.
27. Dufourmantel N, Dubald M, Matringe M, Canard H, Garcon F, Job C, Kay E, Wisniewski JP, Ferullo JM, Pelissier B, *et al.* (2007) *Plant Biotechnol J* 5:118–133.
28. Kumar S, Dhingra A, Daniell H (2004) *Plant Mol Biol* 56:203–216.
29. Okumura S, Sawada M, Park YW, Hayashi T, Shimamura M, Takase H, Tomizawa K (2006) *Transgenic Res* 15:637–646.
30. Sidorov VA, Kasten D, Pang SZ, Hajdukiewicz PT, Staub JM, Nehra NS (1999) *Plant J* 19:209–216.