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ORIGINAL PAPER

Genetic transformation of plastids of different *Solanaceae* species using tobacco cells as organelle hosts

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Abstract The plastid genome of angiosperms represents an attractive target for genetic manipulations. However plastid transformation of higher plants, especially of agriculturally valuable crops is an extremely difficult problem. Transformation protocols developed for tobacco 15 years ago failed to produce similar results with more than a handful of other species so far. We have analyzed plastid transformability of remote cytoplasmic hybrids (cybrids) that combine nuclei of tobacco, an easily transformable species, and plastids of some other, recalcitrant *Solanaceae* species. Here, we demonstrate that the plastids of five species of *Solanaceae* family, representing two subfamilies and three tribes, can be easily transformed if the plastids of these species are transferred into a cell of a transformable species (tobacco). The results can be considered to be an alternative approach to the development of plastid transformation technologies for recalcitrant species using a transformable intermediary ("clipboard") host.

Abbreviations

*aad*A	Gene of aminoglycoside-3′-adenylyntransferase
ITS	Internal transcribed spacer
ndhD	Gene of NADH-dehydrogenase ND4 subunit
PEG	Polyethylene glycol
*psb*A	Gene of D1-protein (PS II)
*rbc*L	Rubisco large subunit gene
RBS	Ribosome binding site
*rpl*22	Gene of 50S ribosome protein CL22
*rpl*32	Gene of 50S ribosome protein CL32
*rps*19	Gene of 30S ribosome protein CS19
*trn*L	Gene of Leu-tRNA
*uid*A	Gene of β-glucuronidase
UTR	Untranslated region

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Introduction

Plastid transformation is a powerful tool to obtain plants with new properties and to study fundamental aspects of plastid function. Plastids are the site of some of the most important biosynthetic processes and pathways, such as photosynthesis, photorespiration, metabolism of amino acids, lipids, starch, carotenoids and other isoprenoids, etc. Since there are many (up to 10^5) copies of plastid DNA per plant cell, proteins encoded by plastid transgenes can be expressed at a very high level. Gene silencing, or the so-called position effects, were not described for plastid genes. Thus, the level of expression is much more predictable. Unlike integration into the nuclear genome, integration of heterologous DNA into a plastome occurs via a homologous recombination mechanism, thus allowing very precise genetic manipulations. The risk of uncontrolled transgene release into the environment is reduced since maternal inheritance of plastomes is typical for the majority of crop species (Birky 1995; Hagemann et al. 1998).

High reproducible plastid transformation methods have been reported mainly for tobacco. To our mind, limitations of plastome transformation technology may be caused by some technical problems (non-optimized protocols of foreign DNA delivery; selection specificity for every plant species, etc.). Tobacco is one of the most easy-to-handle species for tissue culture manipulations. One of the main reasons for its high transformability might be the availability of an effective regeneration system. Starting with pioneering work of Svab et al. (1990), only 13 plastomes have been transformed including *Arabidopsis thaliana* (Sidkar et al. 1998), *Solanum tuberosum* (Sidorov et al. 1999), *Lycopersicon esculentum* (Ruf et al. 2001; Nugent et al. 2005), *Brassica napus* (Hou et al. 2003), *Lesquerella fendleri* (Skarjinskaia et al. 2003), *Petunia hybrida* (Zubko et al. 2004), *Glycine max* (Dufourmantel et al. 2004; Dufourmantel et al. 2005), *Daucus carota* (Kumar et al. 2004a), *Gossypium hirsutum* (Kumar et al. 2004b) and *Lactuca sativa* (Lelivelt et al. 2005). In spite of the headily increasing number of plastome transformed species during the last years, this technology is still routine only for *Nicotiana tabacum*. In addition to marker and reporter genes, a number of pharmaceutical and agronomically important genes were introduced into the tobacco plastome. There are among them the genes encoding human growth hormone somatotropin (Staub et al. 2000), B subunit of the cholera toxin (Daniell et al. 2001b), *Bacillus thuringiensis* (Bt) toxin gene (McBride et al. 1995; DeCosa et al. 2001), and genes encoding synthesis of biodegradable polyester (Lossl et al. 2003; Arai et al. 2004). Some systems of marker gene elimination and antibiotic-free selection have also been reported for tobacco plants (Daniell et al. 2001a; Hajdukiewicz et al. 2001; Corneilly et al. 2001; Klaus et al. 2004).

We propose here an approach of plastid transformation using remote hybrids (cybrids) carrying tobacco nuclei and plastids of other *Solanaceae* species. This approach makes it possible to combine tobacco's high regeneration and transformation potential with the possibility to introduce transgenes into the plastomes of other (recalcitrant) species.

Materials and methods

Plant material

Four intertribal cybrids combining the nucleus of tobacco, *N. tabacum*, and plastids of *Atropa belladonna, Scopolia carniolica, Lycium barbarum*, or *Physochlaina officinalis* have been obtained earlier (Kushnir et al. 1987; Babiychuk et al. 1995) and were kept in the in vitro plant germplasm bank of the Institute of Cell Biology and Genetic Engineering (Kiev, Ukraine). Seeds of cybrids combining *N. tabacum* nucleus and chloroplasts of *S. sinuata* (Thanh and Medgyesy 1988) or recombinant tobacco/potato ("potacco") chloroplasts (Thanh et al. 1989) were kindly provided by Dr. Peter Medgyesy (Biological Research Centre of Hungarian Academy of Sciences, Szeged, Hungary). Aseptic plants were obtained from seeds and were kept on MS (Murashige and Skoog 1962) hormone-free-medium and subcultured every 3–4 weeks.

Vector DNA

Plastid transformation vectors pCB033 and pICF5822 (Fig. 3a) were obtained from Prof. H.-U. Koop (Institute of Botany, Munich University, Munich, Germany). The insertion cassette of the pCB033 plasmid contains selective gene of aminoglycoside-3′-adenylyntransferase (*aad*A) gene, tobacco *16S rDNA* promoter (Prrn) and 3′-*rbc*L UTR of *Chlamydomonas reinhardii* plastome. The cassette is flanked by *rpl*32 and *trn*L genes of the tobacco plastome for targeting *aad*A gene to this specific region of the chloroplast genome. The pICF5822 vector includes selective *aad*A and reporter gene of β-glucuronidase (*uid*A) genes. The transgenes are targeted downstream of *psb*A [gene of D1-protein (PS II)] and are transcribed as an operon. RBS is located upstream *aad*A gene and *rps*19/*rpl*22 spacer sequence is placed upstream of *uid*A gene (Fig. 3a).

Protoplast isolation, transformation and culture

Fully expanded leaves of 3–4-week-old plants were used for transformation experiments. Protoplasts were isolated and cultured according to standard recommendations (see, e.g., Sytnyk et al. 2005).

The protoplast transformation protocol was based on the method described by Koop et al. (1996) with some modifications. The density of protoplast suspension was adjusted to 10^6 cells/ml. The suspension (100 µl) was transferred to the lower edge of the tilted 6-cm Petri dish, and plasmid DNA (25 µl, 2 µg/µl) was added. Then, the suspension was mixed up with a pipette, and 125 µl of polyethylene glycol (PEG) solution was added dropwise. In 10–12 min incubation, 2–3 drops of transformation buffer were added (the suspension should not be mixed up) and after that 2–5 drops of W5 solution (Medgyesy et al. 1980) were added every 3–5 min until the dish was almost filled (approx. 3 ml). The dish was left in the hood for 1–3 h without mixing the suspension. After washing from PEG, protoplasts were

incubated in liquid medium at a cell density of 2–3×10^4 cells/ml, room temperature and low light intensity. Modified MS medium was used for cultivation of protoplasts and cell colonies (MSM: macro- and micronutrients of MS medium, 200 mg/l myoinositol, 1 mg/l thiamine-HCl, 1 mg/l pyridoxine-HCl, 1 mg/l nicotinic acid, 0.5 mg/l Ca-panthotenate, 0.02 mg/l biotin). Osmotic pressure was gradually reduced during the cultivation (Table 1). At the beginning of the culture, the protoplasts and cell colonies were subcultured in the liquid filter sterilized media (MSM1-MSM3, Table 1).

After that, colonies were transferred to the solidified medium (MSM4). Subsequent passages were performed every 3 weeks on the MSM5 medium until green calli or shoot formation. Selective antibiotics, 100 mg/l spectinomycin (Spe) and 100 mg/l streptomycin (Str), were added into MSM2 and MSM3 media. In the solid media, the concentration of selective agents was increased to 300 mg/l Spe and Str each.

Protoplast fusion and cell culture

Mesophyll protoplasts of transformed *N. tabacum* (+*S. sinuata*) cybrids and *S. sinuata* wild type plants were isolated as described above. Leaves of the plastome donor were γ-irradiated with the dose of 500–600 Gy. Protoplasts were fused as described earlier (Menczel et al. 1981) and cultured in KM8p medium (Kao and Michayluk 1975). After approximately 1 month of cultivation in the liquid medium, cell colonies were transferred to the agar-solidified MS medium supplemented with 0.2 M mannitol, 0.5 mg/l zeatin and 0.5 mg/l NAA. Subcultures were performed using the same medium, mannitol-free, containing 1 mg/l zeatin and 0.1 mg/l NAA. Regenerated shoots were rooted on hormone-free MS medium. Selection was started on the 5th–7th day of protoplast culture (when the first cell divisions were observed). The liquid media were supplemented with 100 mg/l Spe and 100 mg/l Str. In the solid media, the concentration of selective antibiotics was increased to 200 mg/l of Spe and Str each.

Chloroplast, mitochondria and nuclear DNA analyses

Plasmid DNA was isolated using the Qiagen tip 500 plasmid kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. Total plant DNA was isolated following Cheung et al. (1993) protocol. DNA was analyzed by PCR and PCR-RFLP.

The presence of foreign DNA sequence in the antibiotic resistant clones was analyzed using the pairs 1–3 of synthetic oligonucleotides (Table 2). PCR-RFLP analysis for the *trn*L–*ndh*D (genes of Leu-tRNA and NADH-dehydrogenase ND4 subunit) sequence was applied to distinguish the plastomes investigated here using primer pair 4 and restriction endonucleases *Eco* 47I, *Hae*III, *Sty*I, *Pvu*, *Hpa*II, *Eco*RI and *Dra*I. To analyze mitochondria origin in transplastomic *S. sinuata* plants, the mitochondrial *ndh*1, *ndh*4, *ndh*5 and *ndh*7 gene sequences were compared. Fragments were obtained by PCR amplification (primer pairs 5–8) and digested with *Eco*RI, *Bam*H1, *Hind* III (*ndh*1 fragment), *Msp*I, *Rsa*I, *Dra*I, *Ssp*I (*ndh*4 fragment), *Eco*RI, *Dra*I, *Ssp*I (*ndh*5 fragment) and *Mae*III, *Mso*I, *Bam*HI, *Hind*III, *Dra*I (*ndh*7 fragment). RAPD-PCR analysis of nuclear DNA of transplastomic *S. sinuata* plants was performed using primer pairs 9–12. Fragment of internal transcribed spacer (ITS) amplified with primers of pair 10 was treated with *Dra*I restriction endonuclease. Primer sequences are represented in Table 2.

Southern Blot analysis was performed with DNA extracted from *S. sinuata* transplastomic and wild type plants. Total DNA was digested with *Nco*I (Fig. 3a), fractionated by electrophoresis and transferred to nylon membranes (Gene Screen, NEN Life Science Products) under alkaline conditions using a VacuGene XL apparatus (Pharmacia LKB). The fragments were detected by hybridization with Biotin ULS labeled probe (Biotin ULS Labaling Kit, Fermentas). To synthesize the DNA probe, the *trn*L–*ndh*D fragment was amplified by PCR with primer pair 4 (Table 2). Then, the *trn*L–*ndh*D PCR fragment was digested with *Hae*III and the 473 bp fragment was isolated and

Table 1 MSM1–MSM5 media composition

Medium	Components	Cultivation period
MSM1	MSM + 0.5 M glucose, 0.5 g/l casein hydrolysate, 0.5 g/l xylose, 0.1 g/l glycine, 0.5 g/l NAA and 0.5 g/l BAP	1 week
MSM2	MSM1 + 0.4 M glucose instead of 0.5 M glucose	2 weeks
MSM3	MSM + 0.3 M mannitol, 60 mM glucose, 30 mM sucrose, 0.5 g/l hydrolysate casein, 0.1 g/l NAA and 1 g/l BAP	2 weeks
MSM4	MSM + 0.15 M mannitol, 90 mM sucrose, 0.1 g/l NAA, 1 g/l BAP and 7% agar	3 weeks
MSM5	MSM + 60 mM sucrose, 0.1 g/l NAA, 1 g/l BAP and 7% agar	Subculture every 3–4 weeks

Table 2 Primer sequences

Sequence	Targeted site
Pair 1: CACTACATTTCGCTCATCGCC and TGCTGGCCGTACATTTGTACG	*aad*A
Pair 2: CCACCAACGCTGATCAATTCC and GTTTCAGGGTCTCTACTTTACGG	*uid*A–*psb*A
Pair 3: GCCAGCAACGTCGGTTCG and CGATCGAAAAGGAAATGTGAG	*aad*A–ycf5
Pair 4: GTAGACACGCTGCTCTTAGG and CGCCAGATGTTCTATGGATAC	*trn*L–*ndh*D
Pair 5: GCATTACGATCTGCAGCTCA and GGAGCTCGATTAGTTTCTGC	*ndh*1
Pair 6: CAGTGGGTTGGTCTGGTATG and TCATATGGGCTACTGAGGAG	*ndh*4
Pair 7: TTTTTTCGGACGTTTTCTAG and TTTGGCCAAGTATCCTACAA	*ndh*5
Pair 8: GCTTTACCTTATTCTGATCG and TTCTTGGGCCATCATAGA	*ndh*7
Random primer 9: (TCC) × 5	Satellite DNA
Pair 10: TCCTCCGCTTATTGATATGC and GGAAGTAAAAGTCGTAACAAGG	ITS rDNA
Pair 11: GGATGGGTGACCTCCCGGGAAGTCC and CGCTTAACTGCGGAGTTCTGATGGG	*5S rDNA*
Random primer 12: (TTTAGGG) × 3	Telomer sequences

labeled using the Biotin ULS Labeling Kit. The signal was detected immunologically using chromogenic substrates NBT and BCIP (Roche Diagnostics, Mannheim, Germany).

Results

Analysis of hybrid plants

Cybrid plants of *N. tabacum* (+*Ph. officinalis*), *N. tabacum* (+*S. carniolica*), *N. tabacum* (+*L. barbarum*) and *N. tabacum* (+*A. belladonna*) were produced by transferring the corresponding plastids into the chlorophyll deficient tobacco nuclear background using the somatic hybridization method (Fig. 1). Resulting plants have revealed both partial chlorophyll deficiency (Fig. 1d) and natural photosynthetic capacity. In this study the fully photoautotrophic plants, without any visible bleaching, were used for transformation experiments (Fig. 1e).

To confirm the plastid genome origin of cybrid plants as well as to visualize the difference between the plastid genomes of plastid donor species and those of the nuclear host, restriction enzyme analysis of the *trn*L–*ndh*D fragment of the plastid DNA was performed. The tested *N. tabacum* (+*S. sinuata*), *N. tabacum* (+*Ph. officinalis*), *N. tabacum* (+*S. carniolica*) and *N. tabacum* (+*L. barbarum*) cybrids had their own digestion patterns each of which was different from each other as well as from the tobacco plastome (Fig. 2).

Transformation procedure

The method of PEG-mediated protoplast treatment was chosen to introduce foreign genes into cybrid cells.

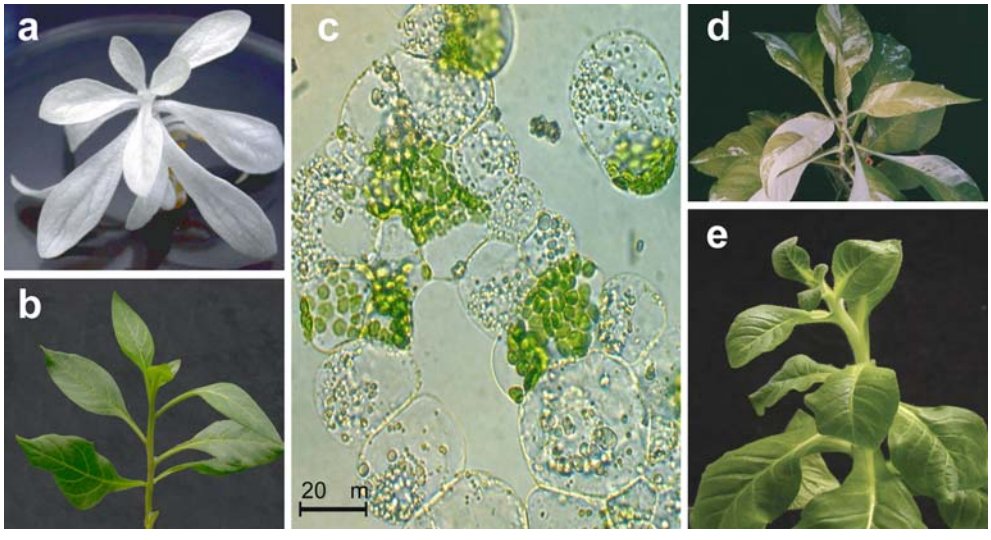

Fig. 1 **a** Chlorophyll deficient tobacco plastid mutant used as parent material for production of cytoplasmic hybrids. **b** One of the plastome donors, *A.belladonna*. **c** Fusion of green and albino mesophyll protoplasts. **d**, **e** Cytoplasmic hybrids produced by somatic cell fusion and following genetic segregation process

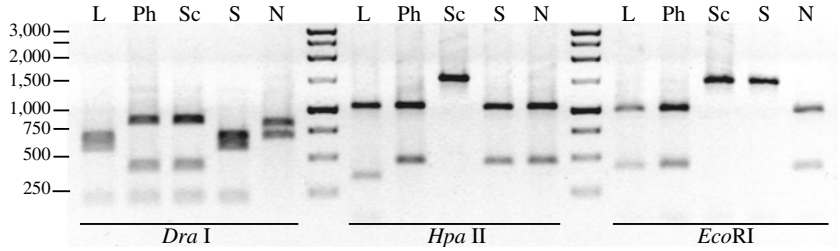

Fig. 2 Chloroplast genome RFLP analysis of cybrids carrying *Lycium* (*L*), *Physochlaina* (*Ph*), *Scopolia* (*Sc*) and *Salpiglossis* (*S*) plastids as well as *Nicotiana* wild type (*N*). Digestion patterns of *trn*L–*ndh*D sequence (1,536 bp) are shown for restriction analyses performed with *Hae* III, *Eco* RI and *Dra* I enzymes

We used the pCB033 plastome transformation vector (Fig. 3a) in experiments with *N. tabacum* (+*S. sinuata*), *N. tabacum* (+*S. carniolica*), *N. tabacum* (+*Ph. officinalis*), *N. tabacum* (+"potacco"), *N. tabacum* (+*A. belladonna*), *N. tabacum* (+*L.barbarum*) plants; and we also applied the pICF5822 construct for plastome transformation of *N. tabacum* (+*L.barbarum*) cybrid. Selection of resistant lines was performed using the mixture of two antibiotics (spectinomycin and streptomycin). During protoplast cultivation, the selective pressure was gradually increased from 100 to 300 mg/l of each antibiotic. The putative transformed colonies were recovered after approximately 4 months of selection procedure. In general, the number of resistant lines amounted to 1–3 cell lines per 10^6 treated protoplasts. The results of transformation experiments are shown in Table 3.

N. tabacum (+*S. sinuata*), *N. tabacum* (+"potacco"), *N. tabacum* (+*A. belladonna*) and *N. tabacum* (+*L. barbarum*) Spe-resistant colonies formed normally developed plants which were rooted on the medium supplemented with 200 mg/l Spe and 200 mg/l Str (Fig. 3b). *N. tabacum* (+*S. carniolica*) regenerated plants have revealed bleached parts and have been grown and rooted in the presence of the lower concentration of selective antibiotic (200 mg/l Spe). Higher selective pressure led to limited growth. The spe-resistant cell line of *N. tabacum* (+*Ph. officinalis*) did not regenerate on the selective medium but retained active growth of green callus tissue (Fig. 3c).

Molecular analysis of putative transplastomic cybrids

The plant material was analyzed to test both the presence of the transgene in the plant genome and its integration into specific region of the chloroplast chromosome. The presence of the internal part of *aad*A gene in the total DNA of selected plants has been shown for *S. sinuata, Ph. officinalis, S. carniolica, L. barbarum* and "potacco" plastomes (Fig. 3d, e). Unfortunately, transgenic state of *N. tabacum* (+*A. belladonna*) plastome has not been confirmed for any of three resistant lines (data not shown). Site specificity of transgene insertion has been tested by PCR with the simultaneous use of the transgene-specific primer and the primer specific to the adjacent sequence (see Fig. 3a, primer locations are shown with arrows). Thus, plastomes of *S. sinuata*, *Ph. officinalis*, *S. carniolica*, *L. barbarum* and "potacco" were proved to be transformed with the pCB033 vector (Fig. 3d, f) and pICF5822 vector (Fig. 3e, g).

Production of transplastomic *S. sinuata* plants

To illustrate the complete transformation cycle, we have transferred the transformed plastids of *S. sinuata* from the corresponding tobacco "clipboard" cybrid back to the original nuclear background of *S. sinuata*. As a result of the somatic hybridization experiments, a number of Spe/Str-resistant colonies were recovered and phenotypically normal *S. sinuata* plants were regenerated and rooted (Fig. 4a–c). Greenhouse-grown plants flowered and formed seeds. Plants of T1 generation retained the transplastomic nature.

To study the plastome status of three selected *S. sinuata* plants, Southern blot hybridization analysis was used (Fig. 4d). The whole of analyzed lines were defined as transgene positive homoplastomic plants. PCR-RFLP analysis of nuclear DNA did not reveal any fragments peculiar to *N. tabacum* samples. Only the sequences of *S. sinuata* DNA were amplified. Since initial *N. tabacum* (+*S. sinuata*) cybrid plants used in our transformation and somatic hybridization experiments contained recombinant mtDNA (Thanh et al. 1988), it seems reasonable to analyze mtDNA in resulting *S. sinuata* plants. Sequences of *ndh*1, *ndh*4, *ndh*5 and *ndh*7 mtDNA genes of transplastomic *S. sinuata* plants were tested. The comparison of the gene fragments showed the presence of both *N. tabacum* and *S. sinuata* *ndh*1 gene patterns (Fig. 4e).

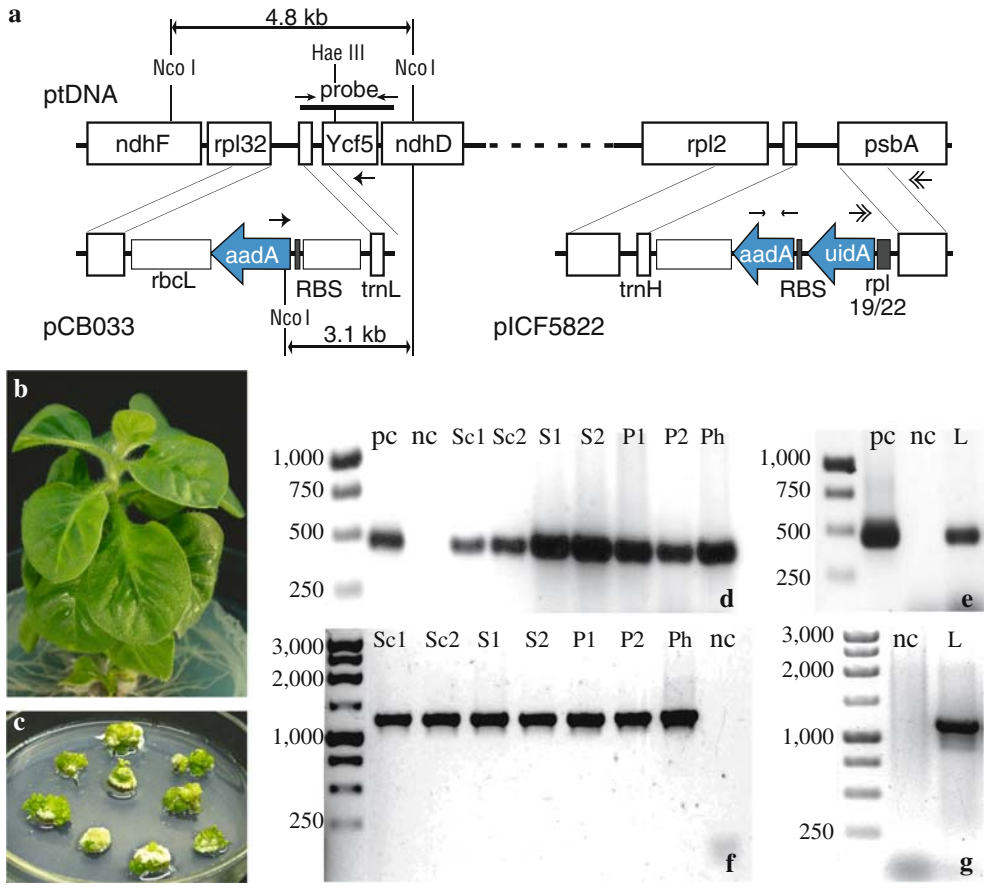

Fig. 3 **a** Scheme of pCB033 and pICF5822 vector integration into plastome, *black arrows* show the position of the primers used for analyses; **b** Recovered *N. tabacum* (+ *S. sinuata*) transplastomic plant. **c** Spe-resistant *N. tabacum* (+*Ph. officinalis*) cell line; **d**, **e** PCR analysis of transformed cybrids using internal *aad*A-specific primer pair 1 (fragment size is 479 bp); **f**, **g** PCR amplification for transgenic cybrid lines with primer pair 3 (fragment size is 1,189 bp); *pc* positive control, transformed tobacco ptDNA; *nc* negative control, non-transformed tobacco DNA; *L Lycium*; *P* "potacco"; *Ph Physochlaine*; *S Salpiglossis*; *Sc Scopolia*

Table 3 Frequencies of chloroplast transformation

Cybrid plants	Number of experiments	Number of spe-resistant clones	Number of transformed clones
N. tabacum (+*Ph. officinalis*)	4	1	1
N. tabacum (+*S. sinuata*)	2	2	2
N. tabacum (+*L. barbarum*)	2	1	1
N. tabacum (+*S. carniolica*)	2	2	2
N. tabacum (+*A. belladonna*)	1	1	0
N. tabacum (+"*potacco*")	2	3	3

Discussion

In the present work, we describe successful genetic transformation of plastids of *S. carniolica, Ph. officinalis, S. sinuata, L. barbarum* and recombinant plastids of *N. tabacum/S.tuberosum* through use of a transformable intermediary "clipboard" host (Fig. 5). Phylogenetically transformed plastids belong to the species representing two subfamilies, *Cestroideae* and *Solanoideae*, and two different tribes, *Salpiglossidae* and *Hyoscyameae*, of the *Solanaceae* family (for review, see D'Arcy 1991).

The majority of Spe-resistant lines contains the expected insertions and has most probably been generated through homologous recombination-based integration. Although complete DNA sequences of the majority of investigated plastomes are not known, the studied insertion regions are fairly conservative, especially within the same plant family, thus the results are in line with theoretical expectations. The negative result with *N. tabacum* (+*A. balladonna*) Spe-resistant plants could be explained by a mutation process. Spontaneous Spe resistance could be caused by point mutations in the plastid 16S rRNA gene (Fromm et al. 1987; Svab and Maliga 1991; Sidkar et al. 1998; Sidorov et al. 1999; Skarjinskaia

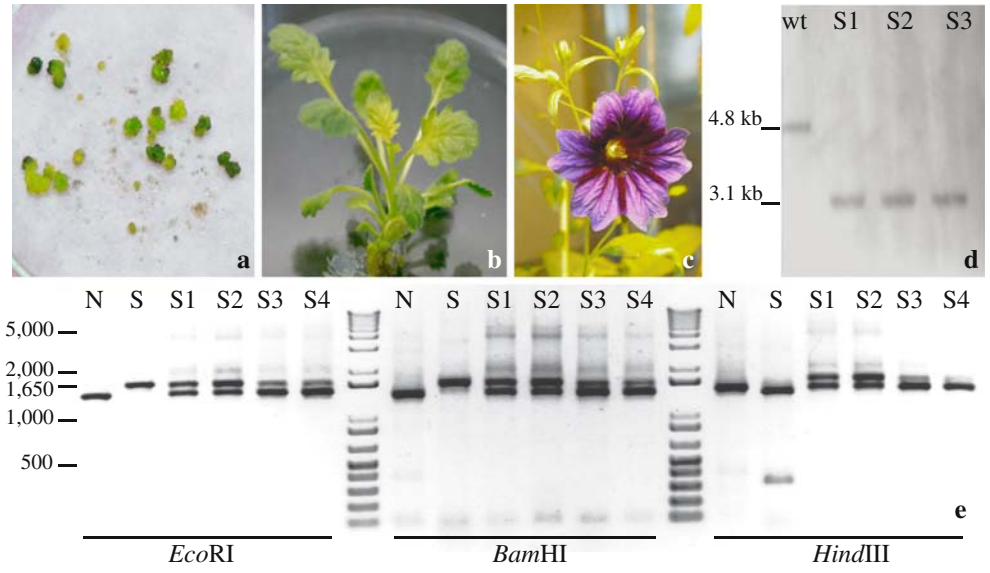

Fig. 4 **a–c** Recovering of transplastomic *S. sinuata* plant after somatic hybridization with *N. tabacum* (+*S. sinuata*) cybrid. **d** Southern-RFLP analysis of *S. sinuata* transplastomic plant performed with trnL–ycf5 probe (wt, non-transformed control plant; S1–S3, transformed lines). **e** RFLP analysis of PCR-amplified mitochondrial *ndh*1 gene of four transplastomic *S. sinuata* plants using *Eco*RI, *Bam*HI and *Hind*III restriction endonucleases; N, *N. tabacum* samples; S, wild type *S. sinuata*; S1–S4, *S. sinuata* transplastomic lines 1–4. 1 kb-Plus DNA Ladder (Gibco) was used

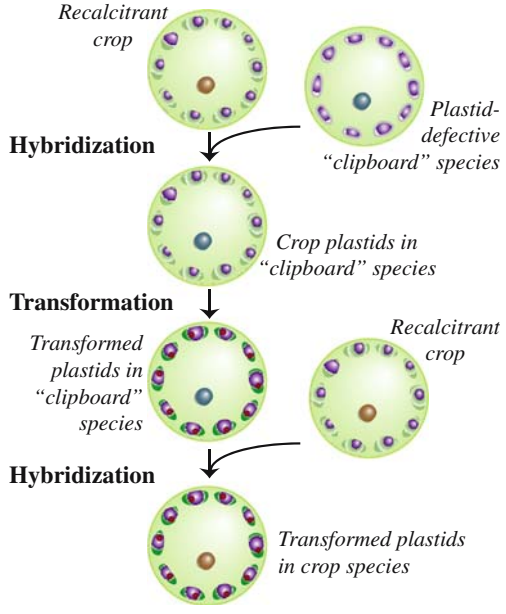

Fig. 5 Plastid transformation using "clipboard" host

et al. 2003). Finally, transplastomic *S. sinuata* plants have been produced by transferring transgenic plastids from "clipboard" *N. tabacum* (+*S. sinuata*) cybrids back to the nuclear genome background of *S. sinuata.* However, the mitochondrial genome differs from mtDNA of initial *S. sinuata* and *N. tabacum* species.

The outlined approach has certain limitations. The number of steps and increased duration of genetic manipulations may result in additional genetic variability; cell fusion technology may lead to the recombination of mitochondrial DNA. In addition, reliable protoplast regeneration protocols are required for both parental species, including the crop varieties. Probably, the most serious limitation is the need of at least one easily transformable plastid "clipboard" host within each plant family of interest that is compatible with plastomes of economically important crops. Experiments in our laboratory and some other ones have demonstrated that even within the *Solanaceae* family, tobacco is not a universal plastid host. Most notably, plastids of two the most important crop species of the family, potato and tomato, are incompatible with the tobacco nuclear genome. Thanh et al. (1988) attempted to circumvent such an incompatibility by selecting the recombinant plastids that combine genetic material of both species. It is unclear, however, whether such recombinant plastids are compatible with the potato genome and how plastome alterations will affect the practically relevant traits.

In addition, our experiments identified another *Solanaceae* species, *Lycopersicon peruvianum*, as a suitable "clipboard" species which nuclear genome is compatible with both potato and tomato cytoplasm genomes. In search for a suitable clipboard for other economically important plant families, we have recently generated cybrids in the *Cruciferae* family, by using *Brassica oleracea, Lesquerella fendleri* or *Orychophragmus violaceus* as plastid hosts.

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