GENETIC TRANSFORMATION AND HYBRIDIZATION

**G. D. Nugent · M. ten Have · A. van der Gulik ·
P. J. Dix · B. A. Uijtewaal · A. P. Mordhorst**

Plastid transformants of tomato selected using mutations affecting ribosome structure

Abstract Tomato plastid transformants were obtained using two vectors containing cloned plastid DNA of either *Nicotiana tabacum* or *Solanum nigrum* and including point mutations conferring resistance to spectinomycin and streptomycin. Transformants were recovered after PEG-mediated direct DNA uptake into protoplasts, followed by selection on spectinomycin-containing medium. Sixteen lines contained the point mutation, as confirmed by mapping restriction enzyme sites. One line obtained with each vector was analysed in more detail, in comparison with a spontaneous spectinomycin-resistant mutant. Integration of the cloned *Solanum* or *Nicotiana* plastid DNA, by multiple recombination events, into the tomato plastome was confirmed by sequence analysis of the targeted region of plastid DNA in the inverted repeat region. Maternal inheritance of spectinomycin and streptomycin resistances or sensitivity in seedlings also confirmed the transplastomic status of the two transformants. The results demonstrate the efficacy in tomato of a selection strategy which avoids the integration of a dominant bacterial antibiotic resistance gene.

G. D. Nugent · P. J. Dix
Biology Department, National University of Ireland,
Maynooth, County Kildare Ireland

M. ten Have · A. van der Gulik · B. A. Uijtewaal ·
A. P. Mordhorst (✉)
Nunhems Zaden BV,
P.O. Box 4005 6080 AA Haelen, The Netherlands
e-mail: andreas.mordhorst@basf-ag.de
Tel.: +49-621-6028854
Fax: +49-621-6027789

Present address:
G. D. Nugent
Department of Primary Industries, Primary Industries Research
Victoria, Plant Biotechnology Centre, La Trobe University,
3086 Bundoora, Victoria Australia

A. P. Mordhorst
BASF Plant Science GmbH,
Li444, Carl-Bosch-Str., 64,
67117 Limburgerhof, Germany

Abbreviations

BAP	Benzylaminopurine
HEPES	*N*-2-Hydroxyehtylpiperazine-*N′*-2-ethanesulfonic acid
MES buffer	2-(4-Morpholino)-ethane sulfonic acid
NAA	α-Naphthaleneacetic acid
PEG	Polyethylene glycol
ptDNA	Plastid DNA
Spec	Spectinomycin
Strep	Streptomycin
2,4-D	2,4-Dichlorophenoxyacetic acid

Introduction

Plastid transformation is a recently developed technique suitable for both fundamental studies on plastid gene function (Sugita et al. 1997) and biotechnological applications (Bock 2001; Maliga 2003, 2004). The advantages of plastid transformation over nuclear transformation, including the potential for a high level of foreign protein production from plastomically inserted foreign transgenes, have been reviewed elsewhere (Heifetz 2000). Plastid transformation of tobacco (*Nicotiana tabacum*) is routine in a number of laboratories, but there are only a limited number of isolated reports relating to genera outside *Nicotiana*. These include tomato (Ruf et al. 2001), rice (Khan and Maliga 1999), potato (Sidorov et al. 1999), carrot (Kumar et al. 2004) and several Brassicaceae, including *Arabidopsis* (Sikdar et al. 1998), *Brassica napus* (Hou et al. 2002) and *Lesquerella fendleri* (Skarjinskaia et al. 2003). All of these investigators used biolistics to deliver DNA into chloroplasts. Direct DNA uptake into protoplasts is an alternative means of plastid transformation for *Nicotiana* species (Golds et al. 1993; O'Neill et al. 1993; Kavanagh et al. 1999) but has not been reported for any other genera.

Resistance to spectinomycin, which is conferred by the bacterial *aadA* gene, is most commonly used in the selection of plastid transformants (e.g. Maliga 2002) in both biolistic and protoplast delivery systems of *N. tabacum* (Koop et al. 1996; Kofer et al. 1998). Selection for kanamycin resistance with *nptII* (Carrer et al. 1993) and *aphA-6* genes (Huang et al. 2002) has also been used. Excision of antibiotic resistance genes from tobacco plastid transformants has been demonstrated, either simply through recombination between directly repeated sequences (Iamtham and Day 2000) or by using the Cre/*lox* marker-excision system (Corneille et al. 2001; Hajdukiewitz et al. 2001). More recently, this has been achieved through the use of the reconstitution of mutant lines to generate marker-free lines during the first round of selection, where the selectable marker gene is not stably integrated into the plastid genome (Klaus et al. 2004). This system is the quickest and most refined developed to date for marker excision from tobacco, as additional recombination events in plastid DNA were not detected, and extra rounds of selection or transformations or crosses to introduce the *cre* gene into the nucleus were unnecessary. Binding-type markers, where cloned plastid genes contain point mutations conferring antibiotic insensitivity, offers an alternative means of selection, at least in *Nicotiana*, without the need for foreign antibiotic resistance genes (e.g. Svab et al. 1990; Golds et al. 1993; O'Neill et al. 1993; Kavanagh et al. 1999). However, when binding-type markers are used, the plastid transformation frequency of tobacco is much lower in both biolistic transformation and direct DNA uptake experiments than when dominant selectable markers such as *aadA* (Koop et al. 1996) are used. Selection based on binding-type markers, however, has the advantage that primary transformants are usually homoplasmic, where all plastome copies in cells contain the introduced DNA (Dix and Kavanagh 1995). The binding-type markers are therefore a good alternative when using PEG-mediated transformation compared to biolistics, given that a large number of shots are needed to recover transformants with these plasmids (Svab et al. 1990; Staub and Maliga 1992). An efficient protoplast regeneration system is required irrespective of the selection system used. An additional advantage of binding-type markers is that the selection of nuclear transformants through the integration of the *aadA* gene in the nuclear genome, which has often been observed (own unpublished results), is excluded.

The study reported here extends the utility of binding-type markers to tomato plastid transformation by using plasmids containing homeologous *Nicotiana* and *Solanum* cloned ptDNA sequences that harbour point mutations conferring spectinomycin and streptomycin insensitivity. Transplastomic tomato plants have been obtained with cloned ptDNA of both species. Multiple recombinations were evident in the two transformants analysed in more detail.

Materials and methods

Transformation vectors

Vector pTB116 (Fig. 1a) was kindly provided by P. Medgyesy. It contains a 3.27-kb *Bam*HI ptDNA fragment containing the entire *16S rRNA* gene from the *Nicotiana tabacum* SR-SPR3 plastid mutant (O'Neill et al. 1993), with point mutations within the *16S rRNA* gene conferring spectinomycin and streptomycin insensitivity. Vector pSSH1 (GenBank accession no. Y18934) (Fig. 1b) was kindly provided by T. Kavanagh (Kavanagh et al. 1999). It contains a 7.8-kb *Hin*dIII fragment containing the *16S rRNA* and the *rps12*(3′) genes cloned from a *Solanum nigrum* plastid mutant (McCabe et al. 1989; Kavanagh et al. 1999). The respective single-basepair mutations in both plasmids (see below) conferred the so-called "binding type" (Dix and Kavanagh 1995) antibiotic resistance (or antibiotic insensitivity) to both streptomycin and spectinomycin.
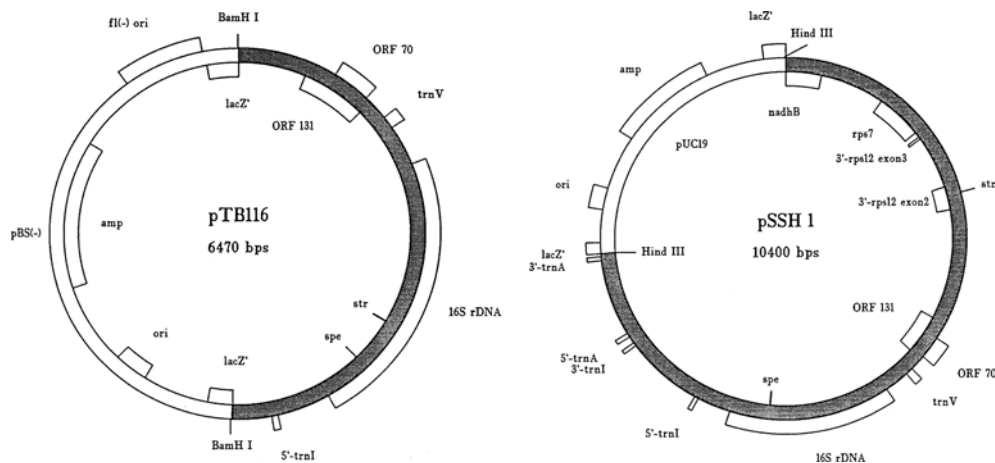
Fig. 1 a,b Plastid transformation vectors. **a** Plasmid pTB116 containing a 3.27-kb *Bam*HI ptDNA fragment with the entire *16S rRNA* gene from the *Nicotiana tabacum* SR-SPR3 plastid mutant. Locations of spectinomycin (*spec*) and streptomycin (*strep*) mutations are indicated. **b** Plasmid pSSH1 containing a 7.8-kb *Hin*dIII fragment with the *16S rRNA* and the *rps12*(3′) genes cloned from a *Solanum nigrum* plastid mutant. Locations of mutations conferring spectinomycin (*spec*) and streptomycin (*strep*) insensitivities are indicated. *ORF* Open reading frame

Protoplast isolation

Cultured shoots of the tomato processing breeding line T1783 were maintained on solidified [0.65% (w/v) agar] MS medium (Murashige and Skoog 1962) containing 20 g/l sucrose at pH 5.8 (MS20). For the isolation of protoplasts, leaves from cultured shoots were cut into small strips and pre-plasmolysed for 10 min. in CCP pre-plasmolysis solution [CCP medium (Dirks et al. 1996) with 75 g/l mannitol]. For enzymatic digestion, the pre-plasmolysis solution was replaced by enzyme solution EK-1 (0.6% cellulase R10 Onozuka, 0.6% macerozyme, 0.15% driselase, 0.2% cellulysin, 0.75% glycine, 0.1% $CaCl_2 \cdot 2H_2O$, 6% mannitol; pH 5.6; all w/v). Digestion took place overnight in the dark at 27°C.

The enzyme solution was diluted 1:1 with W5 wash medium (as described by Menczel et al. 1981), and protoplasts were separated from undigested tissue by passage through an 80-μm stainless steel sieve. The protoplast suspension was transferred to centrifuge tubes, pelleted (10 min, 60g) and washed twice with W5. The pellet was finally resuspended in 9 ml CCP medium (Dirks et al. 1996) with 110 g/l sucrose, overlaid with 1 ml W5 and centrifuged (5 min, 100g). The protoplasts were recovered from the interface, diluted with W5 and counted.

Protoplast transformation

Aliquots of 5×10^5 protoplasts were centrifuged (5 min, 60g) and resuspended in 0.2 ml MaMg-solution (72 g/l mannitol, 25 mM $MgCl_2$, 1 g/l MES; pH 5.8), and 50 μg of plasmid DNA (without carrier DNA) was added (O'Neill et al. 1993). Ten droplets of PEG solution [40% (w/v) PEG 6000 in 0.4 M mannitol, 0.1 mM $Ca(NO_3)_2$, 21 mM HEPES; pH 7.1] were added and mixed gently and incubated for 10 min on ice. The protoplasts were then washed twice in W5, resuspended in 1.6 ml CPP medium (Dirks et al. (1996), supplemented with 60 g/l glucose, 0.1 mg/l 2,4-D and 0.2 mg/l zeatin riboside) and kept at 4°C. The following day the protoplasts were embedded in alginate according to Dirks et al. (1996) and cultured in 3 ml CPP medium (as above) in the dark at 27°C. At days 9 and 16, 1 ml CPP medium with 0.2 mg/l zeatin riboside was added (at day 16, CPP medium with only 6 g/l glucose).

At day 23 the alginate was dissolved with 5 mM Na-citrate in 60 g/l mannitol (pH 5.8). The resulting microcalli were washed twice in 5% (w/v) mannitol and embedded in agarose (0.2%, w/v) in CPP medium (including 0.1 mg/l 2,4-D, 0.2 mg/l zeatin riboside, 35 g/l glucose, 0.4 g/l sucrose and 300 or 600 mg/l spectinomycin) in the dark at 27°C. At day 35 the agarose slides were spread on tomato greening medium (TGM; 350 mg/l KCl, 440 mg/l $CaCl_2 \cdot 2H_2O$, 170 mg/l KH_2PO_4, 370 mg/l $MgSO_4 \cdot 2H_2O$, MS micro-elements and FeEDTA, Nitsch vitamins, 100 mg/l inositol, 1 g/l casein hydrolysate, 40 mg/l adenine sulfate, 3,6% mannitol, 0.25% sucrose, 0.5 mg/l BAP, 0.05 mg/l NAA, 300 or 600 mg/l spectinomycin, 0.7% agarose; pH 5.8). Petri dishes were trans-ferred to dim light for the greening of calli. Surviving green calli were transferred several times to tomato regeneration medium (TRM: 350 mg/l KCl, 440 mg/l $CaCl_2 \cdot 2H_2O$, 170 mg/l KH_2PO_4, 370 mg/l $MgSO_4 \cdot 2H_2O$, 830 mg/l NH_4NO_3, MS micro-elements and FeEDTA, Nitsch vitamins, 100 mg/l inositol, 10 g/l mannitol, 10 g/l sucrose, 2.0 mg/l zeatin riboside, 0.01 mg/l NAA, 0.35% (w/v) agar and agarose each; pH 5.8) for shoot regeneration. Calli regenerated up to two shoots. Regenerated shoots were transferred to MS20 medium supplemented with 300 mg/l spectinomycin for rooting. Spectinomycin selection worked well during callus development and the rooting of regenerated shoots. Selection on streptomycin, however, did not have a selective effect at concentrations up to 500 mg/l or was too toxic at higher concentration, thereby killing all micro-colonies and making selection impossible with this antibiotic. In order to try to determine resistance to streptomycin in a later developmental phase, leaf discs and cuttings were cultured on MS20 that was supplemented with various concentrations of streptomycin (see Results) with and without 2 mg/l zeatin riboside, respectively. After about 10 months of culture on spectinomycin-containing media (as callus or shoot), rooted shoots (putative transplastomic plants) were transferred to the soil and grown to maturity in the greenhouse. The inheritance of the transplastomic trait was analysed in self and reciprocal crosses. Seeds were harvested from one or two fruits per plant, and in the latter case the seeds were pooled.

Molecular analysis

The mutation conferring spectinomycin resistance is a G to A transition at nucleotide 1,140 of the *16S rRNA* gene in both plasmids. This nucleotide change causes the loss of an *Aat*II restriction site. In order to analyse rooted putative transplastomic plants, we isolated total DNA from leaves of in vitro shoots of putative transformants, and an approximately 763-bp PCR product spanning this restriction site was amplified with SSH1spec-forward (5′-AAAGAAGACTTCCATCTCCAAGTT-3′) (position 138,173–138,197 of the tobacco chloroplast genome, GenBank accession Z00044 S54304; position 1,963–1,986 of pSSH1) and SSH1spec-reverse (5′-GGAGTACGTTCGCAAGAATGAA-3′) (position 138,937–138,916 of the tobacco chloroplast genome; position 2,726–2,705 of pSSH1) (Fig. 4a) primers and digested with *Aat*II. The presence of this restriction site in the amplified PCR product represents the wild-type situation. Therefore, shoots with an *Aat*II-digested PCR product are either escape shoots (untransformed) or could have survived the selection pressure due to an alternative mutation in the plastome conferring resistance to spectinomycin. The absence of the *Aat*II site demonstrates the transplastomic nature through the incorporation of the plasmid DNA into the plastome by homologous recombination or a spontaneous mutation at this site. Putative transplastomic plants transformed with pSSH1 were tested for streptomycin resistance in a similar way.

The streptomycin resistance mutation in pSSH1 is an A to C transversion at position 5,645 of pSSH1 in the *rps12*(3′) gene (Kavanagh et al. 1999) eliminating an *Mse*I restriction site. A 589-bp PCR product was amplified with the SSH1strep-forward (5′-AGAGTACGATTAACCTCTGG-3′, position 5,562–5,543) and SSH1strep-reverse (5′-TTGAGTATCCGTTTCCCTCC-3′, position 6,132–6,113) primers and digested with *Mse*I. In accordance with the spectinomycin resistance, the absence of the restriction site reveals homologous recombination (or spontaneous mutation at this site). The mutation at nucleotide 1,190 in pTB116 conferring streptomycin resistance is in the *16S rRNA* gene and does not result in a gain or loss of a restriction site and could therefore not be mapped by restriction digestion.

Sequence analysis

For sequence analysis, total genomic DNA was isolated from wild-type tomato, two putative transformed lines and a third line believed to be a spontaneous mutant that had re-generated from protoplast-derived calli selected on spectinomycin. DNA was dissolved in sterile distilled water to a concentration of 5–15 ng/µl. Aliquots of 10–50 ng of DNA were used per PCR, with 1.5 U of *Taq* polymerase (Promega, Madison, Wis.). PCR cycles were optimised for each primer pair in a reaction volume of 25 µl. Primers used for PCR and sequencing and their nucleotide position on the 7.8-kb pSSH1 vector are shown below. The two external primers LRP1 and LRP2 (Kavanagh et al. 1999) are shown by their position on the tobacco chloroplast genome (GenBank accession Z00044; S54304) and are approximately 170 bp and 40 bp external to the pSSH1 plasmid sequence on the chloroplast genome, respectively.

(1) LRP2 [144,131-144,098] 5′-ACGTCAGGAGTCCATTGATGAGAAGGGGCTGGGG-3′
(2) INS5TT10 [6,772-6,793] 5′-GAATGGCAGAGGCAAATAGAGC-3′
(3) rps7opp [6,445-6,427] 5′-TCCGTGTTTCAGAATACGG-3′
(4) rps12opp [5,964-5,944] 5′-GTTGACACGGACAAAGTCAGG-3′
(5) STR1 [5,455-5,479] 5′-TCTGTAGAGTGGCAGTAAGGGTGAC-3′
(6) SSHREV [5,200-5,180] 5′-TCTGAATCAATCCCTTTGCC-3′
(7) SSH [4,716-4,737] 5′-CTCGGAGACCCACCAAAGTACG-3′
(8) INS10-21 [4,378-4,398] 5′-GAAAGAGGTTGACCTCCTTGC-3′
(9) INS10-21opp [4,398-4,378] 5′-GCAAGGAGGTCAACCTCTTT-3′
(10) trnV [3,821-3,841] 5′-TGACTTCCACCACGTCAAGGT-3′
(11) 16SRNAend [3,437-3,455] 5′-CAAGGGCAGGTTCTTACGC-3′
(12) SPC1: [2,247-2,272] 5′-GTATGGCTGACCGGCGATTACTAGC-3′
(13) trnIii: [1,678-1,690] 5′-CACATTGAACTATCCATGTGGC-3′
(14) 16SRNAopp [2,161-2,141] 5′-AGGAGGGGGATGCCTAATTCA-3′
(15) trnAFii [836-855] 5′-TGGACAATTAGACATCCCAAC-3′
(16) INS100 [419-435] 5′-GGACCGACCATAGAACCCTG-3′
(17) LRP1 [136,250–136,283] 5′-TCGTCTCTGGGTGCCTAGGTATCCACCGTAAGCC-3′.

Eight of these primers were used in an earlier study (Kavanagh et al. 1999), but additional primers were designed for the present investigation. The approximate positions of the primers on pSSH1 are shown in Fig. 4a. For sequencing, PCR products were purified (StrataPrep PCR Purification kit, Stratagene, La Jolla, Calif.), quantified by gel electrophoresis, precipitated and sequenced at MWG-Biotech (Ebersberg, Germany). BLAST searches and pairwise or multiple sequence alignments were carried out between tomato and vector sequences using the BESTFIT or GAP programmes via ANGIS (Australian National Genomics Information Service).

Results

Protoplast culture

About 2 weeks after embedding of the protoplasts, micro-colonies of about 20 cells had formed. Following their transfer to spectinomycin-containing medium, most of the calli turned brown and died (Fig. 2). Sixty-two green calli were recovered after spectinomycin selection, from which 29 also regenerated at least one shoot. Upon transfer to spectinomycin-containing rooting medium, 30 shoots derived from 21 calli rooted (regenerated from 12 calli after transformation with pTB116 and nine calli after
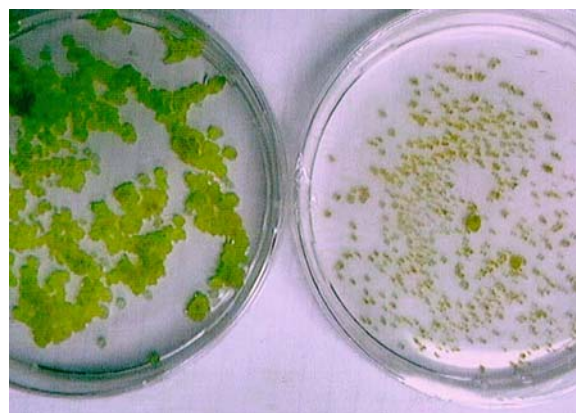
Fig. 2 Protoplast-derived calli on medium without spectinomycin (*left*) and spectinomycin-resistant calli 2 months after protoplast isolation [5 weeks selection on 300 mg/l spectinomycin (*right*)]

transformation with pSSH1). Shoots from ten calli were transferred to the greenhouse and flowered.

In order to determine resistance to streptomycin, cuttings from 20 shoots selected on spectinomycin and from wild-type controls were transferred to MS20 medium with 0 mg/l to 300 mg/l streptomycin. Both controls and putative transplastomics rooted on 0 mg/l and 50 mg/l streptomycin, while they did not form roots on higher concentrations. As a further test, leaf discs were transferred to MS20 medium with 2 mg/l zeatin riboside containing 0–600 mg/l either spectinomycin or streptomycin. On all the spectinomycin concentrations leaf discs of shoots selected on spectinomycin stayed green and formed callus, while untransformed control discs turned yellow or brown with increasing spectinomycin concentration (data not shown). With increasing streptomycin concentration callus development was reduced, however no difference could be observed between the reaction of controls and spectinomycin-resistant material (data not shown). With these tests it was not possible to demonstrate streptomycin resistance in any of the lines, including line 53 (see below).

Molecular analysis

Restriction analysis of an approximately 763-bp PCR product including the G to A transition causing resistance against spectinomycin was performed. Of the 21 shoots from the pSSH1 and pTB116 experiments, 16 had lost an *Aat*II restriction site (Table 1) suggesting the presence of the resistance mutation introduced by homologous recombination with the transforming plasmid. Alternatively, a spontaneous mutation in this *Aat*II restriction site might have occurred, leading to the absence of this site. The method used does not distinguish between these two possibilities. However, in none of the control experiments was any spectinomycin-resistant callus found. In the cases where two shoots were regenerated from the same callus they gave an identical result. Significantly more pTB116 putative transformants had lost the *Aat*II site than plants obtained after pSSH1 transformation (Table 1). Only one line (line 53) out of nine lines tested (from which five were positive for loss of the *Aat*II site) was also carrying the additional streptomycin mutation [loss of the *Mse*I restriction site (Table 1)]. These results strongly suggest that

homologous recombination of the plasmid DNA had occurred, resulting in antibiotic-resistant tomato lines. Based on these data we calculate a maximum plastid transformation efficiency of 1.5×10^{-6}. Since we cannot exclude the occurrence of spontaneous mutations, the number might be an overestimate.

Inheritance of transplastomic traits

Shoots from ten lines (five each after transformation with pTB116 and pSSH1) that had rooted on spectinomycin-containing medium were transferred to the greenhouse. Reciprocal crosses of all lines with wild-type plants were performed in order to demonstrate maternal inheritance, and seeds were also collected after self-pollination. Most of the crosses failed or produced only a low seed number due to reduced pollen production and low pollen fertility, which could not be linked to the transplastomic nature of the plants. Seeds were germinated on MS20 medium supplemented with either 300 mg/l spectinomycin or 600 mg/l streptomycin. Control (sensitive) seedlings on spectinomycin stopped growing immediately after germination and died (Fig. 3a–c). The growth of sensitive seedlings on streptomycin was initially similar to that of wild-type seedlings on control medium (Fig. 3d), but growth was subsequently reduced, and bleaching of tissue around leaf veins became visible (Fig. 3e). Tests on spectinomycin were scored after 2 weeks, while those on streptomycin needed about 4 weeks for a clear difference between sensitive and tolerant phenotypes to become apparent.

Seed germination tests clearly demonstrated maternal inheritance of the introduced traits and suggested that homoplasmy had been achieved, as no segregation of spectinomycin resistance was detected (Table 2). All crosses with tolerant plants as the maternal parent yielded 100% tolerant seedlings against spectinomycin and, in the case of line 53, also against streptomycin. On the other hand, the cross of the wild type × line 38 (spectinomycin tolerant) yielded 100% sensitive seedlings (Table 2). Line 9 maternally inherits the tolerance against spectinomycin, but it has been shown that the desired mutation conferring this tolerance is not present (Table 1). This suggests strongly that another mutation has occurred in the plastome, thereby conferring spectinomycin tolerance.

Table 1 Results of molecular analysis[a] of 21 putative chloroplast-transformed tomato lines. The absence of restriction sites demonstrates the transplastomic nature of the plants (homologous recombination) (*n/a* not applicable). Demonstrated is the number of lines of the respective categories

Plasmid used	Streptomycin resistance: *Aat*II site		Spectinomycin resistance: *Mse*I site	
	Present (no homologous recombination)	Absent (homologous recombination)	Present (no homologous recombination)	Absent (homologous recombination)
Control	1	0	1	0
pTB116	1	11 (e.g. line 38)	n/a	n/a
pSSH1	4 (e.g. line 9)	5 (e.g. line 53)	8	1 (e.g. line 53)

[a]For details see Materials and methods. Examples of the line numbers of plants in each major category used in the sequence analysis are listed

Table 2 Segregation analysis of T_1 seedlings of chloroplast-transformed tomato lines

Maternal line		T_1 as result of pollination with paternal line		
Number (plasmid)	Molecular character[a]	Wild-type (T1783)[b]	Self pollination[b]	38
Wild-type	spec^{M-}		100% spec^S (128)	100% spec^S (69)
	strep^{M-}		100% strep^S (124)	
9 (pSSH1)	spec^{M-}		100% spec^T (88)	
	strep^{M-}			
53 (pSSH1)	spec^{M+}	100% spec^T (66)	100% spec^T (24)	
	strep^{M+}	100% strep^T (66)	100% strep^T (24)	
13 (pTB116)	spec^{M+}	100% spec^T (33)		
37 (pTB116)	spec^{M+}	100% spec^T (15)	100% spec^T (100)	
38 (pTB116)	spec^{M+}	100% spec^T (28)	100% spec^T (200)	
			100% strep^S (24)	

[a]Strep, streptomycin; spec, spectinomycin; supercript M+ orM−, mutation corresponding to tolerance present or absent, respectively, as analysed by restriction enzyme analysis of PCR products

[b]In brackets, number of seeds tested; superscript S, sensitive; superscript T, tolerant, as analysed by the germination test on selective media
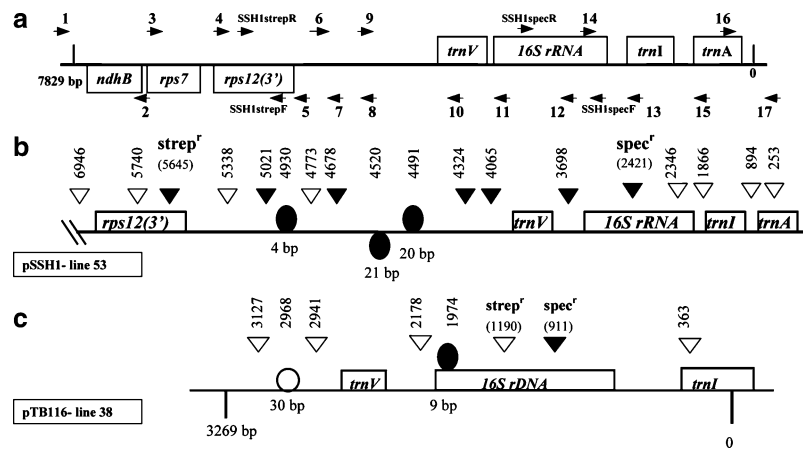
Sequence analysis

In order to get further evidence that regenerants are true plastid transformants PCR products of between 400 kb and 2.2 kb, covering 80% of the region covered by pSSH1 and 100% of pTB116 were generated and sequenced. In the *rps12-trnV* intergenic region, sequence identity between tomato and *Solanum* was around 97%, while elsewhere it was around 99–100%. A series of genetic markers were identified in wild-type tomato compared to pSSH1 or pTB116. Tomato wild-type ptDNA sequence in the regions corresponding to plasmid pSSH1 (GenBank Y18934) and pTB116 revealed a number of single base-pair differences/insertions or deletions or 4- to 30-bp DNA stretches only present in either tomato or vector DNA. For example, there are 14 single base-pair differences or deletions between tomato and *Solanum* between the *rps7-rps12* intergenic region and the *trnA* end of the vector, including the two point mutations for spectinomycin and streptomycin, respectively (Fig. 4b). There are also two insertions in tomato (4 bp and 20 bp) and one insertion in *Solanum* (21 bp) in the *rps12-trnV* intergenic region (Fig. 4b). Three regenerated tomato lines were analysed in more detail. The presence of tomato- or vector-specific markers in the three putative transformants was determined from sequence alignments. This analysis revealed that lines 53 (pSSH1) and 38 (pTB116) (Fig. 4b,c) were plastid transformants, and line 9 (pSSH1) was a spontaneous mutant as no *Solanum* genetic markers were present (data not shown). Interestingly, there were no *Solanum*

Fig. 3 a–e Segregation analysis of offspring of chloroplast-transformed tomato plants on spectinomycin- (**a–c**) and streptomycin- (**d**, **e**) containing media 2 weeks (**d**) and 4 weeks (**a–c**, **e**) after sowing. Two sensitive wild-type seedlings, marked by *arrows*, are located on the *upper left corner* (**a–d**). **a** Sensitive seedlings of the cross wild-type × line 38, **b** resistant seedlings of reciprocal cross line 38 × wild-type, **c** resistant seedlings line 53 selfed, **d** resistant seedlings line 53 × wild-type, **e** comparison of two sensitive wild-type seedling (*left*, see *arrows*) and one resistant seedling line 53 × wild-type. Note that after 2 weeks the difference between resistant and sensitive seedlings on streptomycin is difficult to judge (**d**) while it is clear after 4 weeks (**e**)

Fig. 4 Schematic representation of sequence and PCR analyses. **a–c** Cloned *Solanum* ptDNA in vector pSSH1 and location of primers on both strands used for sequence and PCR analyses of wild-type tomato and putative transformants (lines 53 and 38) and positions of single-base differences and insertions or deletions. **a** Positions of primers on pSSH1 used for sequencing (for detailed information of primers, see Materials and methods). *Vertical lines* mark the boundary of cloned *Solanum* ptDNA in the vector. Primers 1 (LRP2) and 17 (LRP1) anneal to plastid sequences outside the vector. (**b**,**c**) Position of single base (*triangles*) and longer sequence differences (*circles*) between transformation vectors pSSH1 (**b**) and pTB116 (**c**) and the corresponding region of wild-type tomato ptDNA. Positions of tomato-specific DNA stretches not present in either vector are marked with *circles above the line*, and vector-specific DNA stretches (pSSH1, **b**; pTB116, **c**) not present in the tomato wild type are marked with *circles below the line*. *Unfilled triangles* or *circles* represent wt tomato DNA markers in the transformant, *filled triangles* or *circles* represent vector DNA markers present in the transformants

markers present in line 53 outside of the two point mutations (Fig. 4b). Clearly, given the pattern of *Solanum* markers interspersed with tomato genetic markers, this line is a result of multiple recombinations. Line 38 also showed evidence for multiple recombinations due to the pattern of tobacco and tomato genetic markers present (Fig. 4c). The majority of the sequence in this region, however, was tomato genotype, with only two tobacco markers being identified (Fig. 4c). The sequence data confirmed the restriction mapping and inheritance data for the three analysed lines.

Discussion

Plastid transformation of tomato, an important vegetable fruit crop, has been obtained by the PEG-mediated transformation of protoplasts by means of homologous recombination with plasmids containing homeologous *Nicotiana* and *Solanum* cloned ptDNA sequences. A point mutation in the transforming ptDNA, conferring resistance to spectinomycin, was used to select transformants. The maternal inheritance and lack of segregation of spectinomycin resistance, the presence of the unselected streptomycin resistance marker from the transforming plasmid and other *Solanum*-specific markers in line 53, and two of the tobacco genetic markers in line 38 showed that these were true plastid transformants. The presence of streptomycin sensitivity in seedling progeny of line 38 confirms the sequence data, thereby showing that the unselected streptomycin resistance marker was absent in this line. This indicates that the marker was not present after the initial integration event into tomato ptDNA during protoplast culture, which is not surprising in view of the multiple recombination events associated with plastid transformation in Solanaceae (Kavanagh et al. 1999).

The transformation frequency in our system was calculated to be up to 1.5×10^{-6} based on mapping the restriction enzyme sites. This frequency could be an overestimate since it might include false positives in the form of spontaneous mutations at the used restriction site, leading to spectinomycin resistance without integration of foreign ptDNA. In control experiments, however, no calli or shoots resistant to spectinomycin were regenerated. Therefore, mutations in this particular site should be rare. However, sequence analysis of line 9 demonstrated that spontaneous mutations do occur, in this case at an undetermined location, giving rise to maternally inherited spectinomycin resistance. The transformation frequency of 1.5×10^{-6} is around tenfold lower than that reported for *Nicotiana* in similar protoplast/plastid transformation studies with vectors containing binding-type markers (Kavanagh et al. 1999) or *aadA* as a dominant antibiotic resistance marker (Kofer et al. 1998).

Ruf et al. (2001) reported successful tomato plastid transformation using the biolistic method for DNA delivery into leaf discs with selection based on the introduced *aadA* resistance gene. These authors also found that the transformation frequency in tomato was lower than that obtainable with tobacco.

Staub and Maliga (1992) showed that almost all of the 6.2-kb homologous cloned ptDNA in the same ptDNA inverted repeat region as that targeted by pSSH1 integrated in two tobacco plastid transformants. However multiple recombinations were evident when a larger number of transformants were analysed in a homeologous (*Solanum/Nicotiana*) system with a 7.8-kb target with pSSH1 (Kavanagh et al. 1999). The current data in tomato concurs with this result, as multiple recombinations were

evident in both confirmed transformants, but more notably with line 53 as pSSH1 contains a much larger region of homeologous ptDNA than pTB116.

In tobacco transformants obtained with pSSH1, the unselected streptomycin resistance mutation was always present in a number of the transplastomic plants analysed, and a peripheral genetic marker located closer to the vector-insert border was less likely to be co-integrated in transformants than markers more distant from the border (Kavanagh et al. 1999). The sequence data from the single pSSH1 tomato transformant agrees with these two observations, especially as no *Solanum* markers were detected peripheral to the two antibiotic resistance markers. However, for line 38, the unselected streptomycin resistance marker was absent, and no streptomycin resistant seedling progeny were obtained. This is despite the close proximity (279 bp) between the mutations conferring spectinomycin and streptomycin resistance in pTB116 compared to pSSH1 (in which they are located in different genes).

The molecular analysis combined with the lack of leakiness of maternal inheritance strongly suggest that these transplastomic lines are homoplasmic, as is consistent with other studies using the "binding-type" markers for selection (O'Neill et al. 1993; Kavanagh et al. 1999). Absolute confirmation of homoplasmy, however, would require further analysis. This study provides further evidence that species-specific vectors may not be necessary in targeting this region of the plastome. In addition, vectors with naturally occurring recessive point mutations may be more acceptable than dominant bacterial antibiotic resistance genes and obviate the need for marker-excision technologies (e.g. Iamtham and Day 2000; Hajdukiewitz et al. 2001; Corneille et al. 2001; Klaus et al. 2004). However, the point mutation conferring antibiotic insensitivity cannot be subsequently removed, as it has become part of an essential plastid gene associated with the production of functional ribosomes. The versatility of derivatives of pSSH1 has also been demonstrated in targeted inactivation of the plastid *ndhB* gene (Horvath et al. 2000) and integration and expression of genes for β-glucuronidase (GUS) and the *bar* gene for bialophos resistance in tobacco (McGrath-Curran et al. 2003). Transgenes carried on one vector can also be targeted to another region of the plastid genome with a co-integration strategy with a vector similar to pSSH1 targeted to the *16S rRNA* region (Rumeau et al. 2004).

The present study demonstrates the regeneration of plastid transformants directly free of a dominant antibiotic resistance gene in a significant food crop. This can be considered as a further important step towards the practical application of plastid transformation technology, opening the way to the production of valuable recombinant protein in the leaves (chloroplasts) and fruit (chromoplasts) of tomato.

References

Bock R (2001) Transgenic plastids in basic research and plant biotechnology. J Mol Biol 312:425–438

Carrer H, Hockenberry TN, Svab Z, Maliga P (1993) Kanamycin resistance as a selectable marker for plastid transformation in tobacco. Mol Gen Genet 241:49–56

Corneille S, Lutz K, Svab Z, Maliga P (2001) Efficient elimination of selectable marker genes from the plastid genome by the CRE-*lox* site-specific recombination system. Plant J 27:171–178

Dirks R, Sidorov V, Tulmans C (1996) A new protoplast culture system in *Daucus carota* L and its applications for mutant selection and transformation. Theor Appl Genet 93:809–815

Dix PJ, Kavanagh TA (1995) Transformation the plastome: genetic markers and DNA delivery systems. Euphytica 85:29–34

Golds TJ, Maliga P, Koop HU (1993) Stable plastid transformation in PEG-treated protoplasts of *Nicotiana tabacum*. Bio/Technology 11:95–97

Hajdukiewitz PTJ, Gilbertson L, Staub JW (2001) Multiple pathways for Cre/lox-mediated recombination in plastids. Plant J 27:161–170

Heifetz PB (2000) Genetic engineering of the chloroplast. Biochemie 82:655–666

Horvath EM, Peter SO, Joet T, Rumeau D, Cournac L, Horvath GV, Kavanagh TA, Schafer C, Peltier G, Medgyesy P (2000) Targeted inactivation of the plastid *ndhB* gene in tobacco results in an enhanced sensitivity of photosynthesis to moderate stomatal closure. Plant Physiol 123:1337–1349

Hou BK, Zhou YH, Wan LH, Zhang ZL, Shen GF, Chen ZH, Hu ZM (2003) Chloroplast transformation in oilseed rape. Transgenic 12:111–114

Huang F-C, Klaus S, Herz S, Zou Z, Koop H-U, Golds T (2002) Efficient plastid transformation in tobacco using the aphA-6 gene and kanamycin selection. Mol Genet Genomics 268:19–27

Iamtham S, Day A (2000) Removal of antibiotic resistance genes from transgenic tobacco plastids. Nat Biotechnol 18:1172–1176

Kavanagh TA, Thanh ND, Lao NT, McGrath N, Peter SO, Horváth EM, Dix PJ, Medgyesy P (1999) Homeologous plastid DNA transformation in tobacco is mediated by multiple recombination events. Genetics 152:1111–1122

Khan MS, Maliga P (1999) Fluorescent antibiotic resistance marker to track plastid transformation in higher plants. Nat Biotechnol 17:910–915

Klaus SMJ, Huang F-C, Golds TJ, Koop H-U (2004) Generation of marker-free plastid transformants using a transiently cointegrated selection gene. Nat Biotechnol 22:225–229

Kofer W, Eibl C, Steinmüller K, Koop H-U (1998) Review: PEG-mediated plastid transformation in higher plants. In Vitro Cell Dev Biol- Plant 34:303–309

Koop H-U, Steinmüller K, Wagner H, Rössler C, Eibl C, Sacher L (1996) Integration of foreign sequences into the tobacco plastome via polyethylene glycol-mediated protoplast transformation. Planta 199:193–201

Kumar S, Dhingra A, Daniell H (2004) Plastid-expressed *Betaine Aldehyde Dehydrogenase* gene in carrot cultured cells, roots, and leaves confers enhanced salt tolerance. Plant Physiol 136:2843–2854

Maliga P (2002) Engineering the plastid genome of higher plants. Curr Opin Plant Biol 5:164–172

Maliga P (2003) Progress towards commercialization of plastid transformation technology. Trends Biotechnol 21:20–28

Maliga P (2004) Plastid transformation in higher plants. Annu Rev Plant Physiol Plant Mol Biol 55:289–313

McCabe PF, Timmons AM, Dix PJ (1989) A simple procedure for the isolation of streptomycin-resistant plants in Solanaceae. Mol Gen Genet 216:132–137

McGrath-Curran N, Shiel K, Nugent J, Kavanagh T, Dix P (2003) Chloroplast transformation of tobacco using non-bacterial selectable marker genes. In: 7th Int Congr Plant Mol Biol., Barcelona, Abstr S23–28. Available at: http://www.ispmb2003.com/

Menczel L, Nagy F, Kiss ZS, Maliga P (1981) Streptomycin resistant and sensitive somatic hybrids of *Nicotiana tabacum* and *Nicotiana knightiana*: correlation of resistance to *N. tabacum* plastids. Theor Appl Gen 59:191–195

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant 15:473–498

O'Neill C, Horváth GV, Horváth E, Dix PJ, Medgyesy P (1993) Chloroplast transformation in plants: polyethylene glycol (PEG) treatment of protoplasts is an alternative to biolistic systems. Plant J 3:729–738

Ruf S, Hermann M, Berger IJ, Carrrer H, Bock R (2001) Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. Nat Biotechnol 19:870–875

Rumeau D, Bécuwe-Linka N, Beyly A, Carrier P, Cuiné S, Genty B, Medgyesy P, Horváth E, Peltier G (2004) Increased zinc content in transplastomic tobacco plants expressing a polyhistidine-tagged Rubisco large subunit. Plant Biotechnol J 2:389–399

Sikdar SR, Serino G, Chaudhuri S, Maliga P (1998) Plastid transformation in *Arabidopsis thaliana*. Plant Cell Rep 18:20–24

Sidorov VA, Kasten D, Pang S-Z, Hajdukiewicz PTJ, Staub JM, Nehra NS (1999) Stable chloroplast transformation in potato: use of green fluorescent protein as a plastid marker. Plant J 19:209–216

Skarjinskaia M, Svab Z, Maliga P (2003) Plastid transformation in *Lesquerella fendleri*, an oilseed Brassicacea. Transgene Res 12:115–122

Staub JM, Maliga P (1992) Long regions of homologous DNA are incorporated into the tobacco plastome by transformation. Plant Cell 4:39–45

Sugita M, Svab Z, Maliga P, Sugiura M (1997) Targeted deletion of *sprA* from the tobacco plastome indicates that the encoded small RNA is not essential for pre-16S rRNA maturation in plastids. Mol Gen Genet 257:23–27

Svab Z, Hajdukiewicz P, Maliga P (1990) Stable transformation of plastids in higher plants. Proc Natl Acad Sci USA 87:8526–8530