Transgenic Res (2009) 18:17–30
DOI 10.1007/s11248-008-9193-4

ORIGINAL PAPER

# Genetic transformation of the sugar beet plastome

**Francesca De Marchis · Yongxin Wang · Piergiorgio Stevanato · Sergio Arcioni · Michele Bellucci**

Received: 3 April 2008 / Accepted: 21 May 2008 / Published online: 13 June 2008
© Springer Science+Business Media B.V. 2008

**Abstract** It is very important for the application of chloroplast engineering to extend the range of species in which this technology can be achieved. Here, we describe the development of a chloroplast transformation system for the sugar beet (*Beta vulgaris* L. ssp. *vulgaris*, Sugar Beet Group) by biolistic bombardment of leaf petioles. Homoplasmic plastid-transformed plants of breeding line Z025 were obtained. Transformation was achieved using a vector that targets genes to the *rrn16*/*rps12* intergenic region of the sugar beet plastome, employing the *aadA* gene as a selectable marker against spectinomycin and the *gfp* gene for visual screening of plastid transformants. *gfp* gene transcription and protein expression were shown in transplastomic plants. Detection of GFP in Comassie blue-stained gels suggested high GFP levels. Microscopy revealed GFP fluorescence within the chloroplasts. Our results demonstrate the feasibility of engineering the sugar beet chloroplast genome; this technology provides new opportunities for the genetic improvement of this crop and for social acceptance of genetically modified sugar beet plants.

**Keywords** Aminoglicoside 3′-adenylyltransferase (*aadA*) · Biolistic apparatus · Chloroplast DNA transformation · Green fluorescent protein · Spectinomycin · Shoot regeneration

F. De Marchis · Y. Wang · S. Arcioni · M. Bellucci (✉)
Institute of Plant Genetics – Research Division of Perugia, Italian National Research Council (CNR), via della Madonna Alta, 130, 06128 Perugia, Italy
e-mail: michele.bellucci@igv.cnr.it

Y. Wang
Institute of Animal Science, CAAS, Yuan Ming Yuan, West Road, Haidian, Beijing 100094, China

P. Stevanato
Dip. to Biotechnologie Agrarie, Università degli Studi di Padova, v.le dell'Università, 16, Legnaro (PD) 35020, Italy

## Introduction

In the past 20 years, plastids have become attractive targets in the field of plant biotechnology. They offer considerable advantages as compared to conventional transgenic technologies, including high protein expression levels (De Cosa et al. 2001), integration into the plastome via homologous recombination without position effects or gene silencing (Daniell et al. 2001), and the expression of several transgenes in a single transcriptional unit due to the chloroplast's prokaryotic origin (Bock 2001). Moreover, from a biosafety perspective, this technology drastically reduces transgene dissemination by pollen since chloroplasts are maternally inherited in most crops (Bock and Khan 2004). Because of these advantages,

the chloroplast genome has been engineered mainly in three areas of applied research: the engineering of useful agronomic traits, the production of pharmaceuticals in plants (molecular farming), and metabolic engineering, i.e. the modification of metabolic pathways (reviewed in Verma and Daniell 2007; Bock 2007).

Despite these numerous advantages, plastid transformation is routinely used only in tobacco (*Nicotiana tabacum* L.); thus, it is of the utmost importance to extend the range of species in which this technology can be applied. Plastid transformation has been applied in an increasing number of dicotyledonous plant species, including edible crops like tomato (Ruf et al. 2001), potato (Sidorov et al. 1999), soybean (Dufourmantel et al. 2004), lettuce (Lelivelt et al. 2005), carrot (Kumar et al. 2004a), cauliflower (Nugent et al. 2006), cabbage (Liu et al. 2007), industrial crops such as cotton (Kumar et al. 2004b) and oilseed rape (Hou et al. 2003), Brassicaceae species including *Arabidopsis thaliana* (Sikdar et al. 1998) and *Lesquerella fendleri* (Skarjinskaia et al. 2003), and poplar trees (Okumura et al. 2006). There are, however, no efficient protocols for plastid transformation in monocotyledonous species. Recently, fertile transplastomic rice plants have been produced, although they were found to be heteroplasmic (Lee et al. 2006). A highly regenerable tissue culture system has also been developed to study the generation of stably plastid transformed maize plants (Ahmadabadi et al. 2007).

Despite being a dicotyledonous plant and the considerable attention it has received due to its importance as an agricultural crop for sugar production in the Northern Hemisphere, *Beta vulgaris* is considered to be a recalcitrant species with respect to genetic transformation, displaying poor reproducibility and genotype dependency (Ivic-Haymes and Smigocki 2005). This biennial and strongly outcrossing plant species, which contains highly heterogeneous germplasm, has been improved through classical breeding for productive traits such as sugar content and disease resistances (Biancardi et al. 2002; McGrath et al. 2007), but a pressing goal is to broaden the entire sugar beet breeding pool. Genetic engineering is a recent tool for sugar beet geneticists and some lines have been genetically transformed for herbicide (glufosinate, Liberty Link line, and glyphosate, Roundup Ready line) resistance. Though nuclear transformation of sugar beets has been achieved via *Agrobacterium* (Snyder et al. 1999; Hisano et al. 2004; Kishchenko et al. 2005), polyethylene glycol-mediated transformation of guard-cell protoplasts (Hall et al. 1996), and biolistic transformation (Snyder et al. 1999; Ivic-Haymes and Smigocki 2005), a genotype-independent and widely applicable protocol for efficient plant regeneration and genetic transformation is not yet available for sugar beets. Furthermore, there are no reports of sugar beet chloroplast DNA transformation at present, making this field of research open to exploration.

The production of transplastomic sugar beet lines with maternal inheritance of transgenes could solve problems related to outcrossing between genetic modified (GM) varieties and conventional varieties or wild relatives (Daniell 2002). It is well documented that the sugar beet easily inter-crosses with its wild relative, the sea beet (*Beta vulgaris* L. ssp. *maritima* (L.) Arcang.) (Bartsch et al. 1999, 2003; Saeglitz et al. 2000), or with annual weed beets that occur in the field (Darmency et al. 2007). Although *Beta vulgaris* is a biennial crop that is harvested before its reproductive stage and is not expected to flower in the first year, early flowering can occur through occasional vernalization at low spring temperatures. Therefore, unintentional pollination of sea beets or weed beets is possible. The use of chloroplast genetic engineering could drastically reduce the probability of transgenic pollen dispersal.

Here, we present the first report of stable plastid transformation in the sugar beet. To develop a chloroplast transformation system for this species, different methods for plant regeneration were tested for all the varieties analysed. We used the biolistic technique to integrate the *aadA* and *gfp* transgenes into the sugar beet plastome. This work constitutes a first step towards producing GM sugar beet plants that present low risks of gene flow and transgene contamination.

## Materials and methods

### Plant material and media

Twenty-six sugar beet (*Beta vulgaris* L.) breeding lines adapted to the Italian environment were evaluated for their regeneration efficiencies. Seeds of 16 multigerm pollinator lines, derived from Italian and North American germplasm, were kindly provided

**Table 1** Sugar beet lines used in this study

| Breeding line | Letter[a] coding system | Breeding line | Letter[a] coding system |
|---|---|---|---|
| SI26 | A | Z025 | N |
| BZ452 | B | 9933 | O |
| S617566 | C | Y172-2 | P |
| C927-4 | D | R725 | Q |
| KMBRT | E | CZ 25-9 | R |
| 2313B | F | FC727 | S |
| M01 | G | M02 | T |
| M05 | H | M03 | U |
| M08 | I | M04 | V |
| C833-5 | J | M06 | W |
| OT-607 | K | M07 | X |
| S61.75.66 | L | M09 | Y |
| C67-2 | M | M10 | Z |

[a] The breeding lines were identified in this study with a letter coding system

**Table 2** Composition of media for sugar beet tissue culture

| Medium component | Culture medium variants | | | | | |
|---|---|---|---|---|---|---|
| | 1[a] | 2[a] | 5[a] | 7[a] | MS15B2[b] | MSIBA3 |
| MS macro- and microsalts | + | + | + | + | + | + |
| Vitamins B5 | + | + | + | + | + | + |
| BA, mg/l | 1 | 0.25 | 0.5 | 1 | 2 | – |
| NAA, mg/l | – | – | 0.1 | – | – | – |
| TIBA, mg/l | 0.5 | – | – | – | – | – |
| ABA, mg/l | – | – | – | 0.5 | – | – |
| IBA, mg/l | – | – | – | – | – | 3 |
| Sucrose, g/l | 30 | 30 | 30 | 30 | 15 | 30 |
| Phytagel, % | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |

*Note*: ABA, abscisic acid; BA, benzylaminopurine; IBA, indole-3-butyric acid; NAA, α-naphtaleneacetic acid; TIBA, 2,3,5-triiodobenzoic acid. All solutions were adjusted to pH 5.8

[a] Mishutkina and Gaponenko (2006)

[b] Dovzhenko and Koop (2003)

by CRA-CIN (Centro di Ricerca per le Colture Industriali – Sede Distaccata di Rovigo); the remaining 10 varieties consisted of monogerm mother plant seeds from Germany (KWS SAAT AG, Einbeck, Germany). All sugar beet lines (Table 1) were analysed for their regeneration efficiency using both methods described below. Media for sugar beet tissue culture are listed in Table 2.

### Seed sterilization and germination

Sterilization was performed as described by Dovzhenko and Koop (2003). To synchronize germination, seeds were kept at 4°C for 1 week, then soaked overnight in 4°C tap water. Water was removed and the seeds were transferred in sequence to 70% ethanol for 1 min, 35% (v/v) formaldehyde for 1 min, 0.05% (w/v) $HgCl_2$ for 5 min, and 5% (w/v) hypochlorite for 10 min, followed by three 10 min washes in distilled sterilized water. Seeds were germinated in the dark at 25°C on medium 1 for 1 month. Seedlings were used as sources of hypocotyls or apexes for regeneration experiments.

### Callus induction from hypocotyls (indirect regeneration, method A)

Hypocotyl segments approximately 1 cm in length were placed on Petri dishes containing the medium MS15B2 in the dark at 25°C until callus formation was observed (from 6 to 8 weeks). Calli derived from hypocotyl tips were compact and did not regenerate in MS15B2, whereas calli that developed on hypocotyl surfaces were friable and light yellow and regenerated upon culturing in the same medium.

### Shoot formation from petioles (direct regeneration, method B)

After root removal, sugar beet seedling apexes (formed by cotyledons and a hypocotyl segment of 4–5 mm) were placed in the light (16 h photo-period under fluorescent light, 27 μE $s^{-1}$ $m^{-2}$) at 23 ± 1°C in glass pots containing medium 2 for the formation of leaf rosettes as described in Mishutkina and Gaponenko (2006). After 6–9 weeks, the external leaves of the rosette were used as a source of petioles, and the internal leaves were propagated in medium 2. Petioles were cut into pieces 1 cm long and placed in Petri dishes filled with medium 5 for shoot formation by organogenesis. In addition to direct regeneration, we noted that, for two breeding lines, small friable calli arose from brown coloured petioles. These calli were grown in the light and selected for their regeneration activity by somatic embryogenesis on medium 7.

Plant regeneration

Shoots obtained from both regeneration protocols were transferred to glass pots supplemented with MSIBA3 for rooting.

Recombinant DNA

The first plastid transformation vector, pSB1, was constructed by placing an expression cassette containing the spectinomycin-resistance (*aadA*) and the green fluorescent protein (*gfp*) genes in the intergenic spacer between the *rrn16* and *rps12* genes (which are located in the inverted repeat region, IR) of the sugar beet chloroplast genome. PCR amplification was used to clone the *rrn16–rps12* region. Due to the absence in GenBank (http://www.ncbi.nlm.nih.gov) at the time we started this study of some portions of the sugar beet plastid genome (GenBank/EMBL Accession EF534108), primers lying in unsequenced DNA regions were based on the chloroplast sequence of spinach (*Spinacia oleracea* L.), which is evolutionary close to sugar beet (GenBank/EMBL Accession NC002202). A 1.5 kb DNA fragment containing the *rrn16* gene was amplified from total sugar beet DNA using the primers 16SFor (5′-tgtgca**gagctc**attgtagcacg tgtgtcgcc-3′) and 16SRev (5′-tgtgca**gtcgacggcgcgcc**a gtagaatagtggcgttgag-3′). A *Sac*I restriction site was included at the left end and *Asc*I and *Sal*I sites were introduced at the right end of the *rrn16* PCR product (restriction sites are in bold in the primer sequences). The resulting fragment was sequenced, digested with *Sal*I/*Sac*I, and cloned into the pBluescriptII plasmid (Fermentas, Burlington, Ontario, Canada) to obtain the pBS-16S intermediate plasmid. The 1.5 kb DNA fragment containing a portion of the *rps12* gene was amplified from total sugar beet DNA using the primer pair rps12For (5′-tgtgca**gtcgacttaattaa**ttcttgtcttgaacaactt-3′) and rps12Rev (5′-tgtgca**ggtacc**taccaggtata taagcagtg-3′). A *Sal*I and *Pac*I restriction sites were included at the left end and *Kpn*I site was introduced at the right end of the *rps12* PCR product (restriction sites are in bold in the primer sequences). The PCR fragment was sequenced, digested with the enzymes *Sal*I and *Kpn*I and cloned into vector pBS-16S. The resulting plasmid was named pBS-16Srps12. The *aadA-gfp* cassette was constructed as follows: the *gfp* coding region was amplified from the pDHA$\gamma$zein-GFP vector (unpublished) containing the gfp.S65C gene (Reichel et al. 1996) using primers 5′GFP (5′-ga cctagtacc**attaat**gggtaaaggagaagaac-3′) and 3′GFP (5′-cagcgggcat**gcggccgc**ttatctagatccggac-3′), which added *Ase*I and *Not*I restriction sites (in bold) at the left end and at the right end of the PCR product, respectively. The *gfp* gene was sequenced, digested with *Ase*I and *Not*I, and cloned into a pCR 2.1-5′UTR (Watson et al. 2004) intermediate plasmid digested with *Nde*I, which generates compatible ends with *Ase*I, and *Not*I enzymes, producing pCR2.1-5′UTR-GFP, in which the *gfp* gene is under the control of the tobacco *psbA* promoter/5′UTR. pCR 2.1-5′UTR-GFP was digested with *Eco*RV and *Not*I, and the 5′UTR-*gfp* cassette was subcloned into *Eco*RV/*Not*I restriction sites of the chloroplast transformation vector pLD-CtV (Dhingra et al. 2004) downstream of the *aadA* coding region. The final construct was designated pLD-CtV-aadA-GFP. In the pLD-CtV-aadA-GFP vector, *aadA* gene expression is controlled by the *Prrn* promoter, and the spectinomycin resistance gene lacks its own terminator which is placed at the end of the *gfp* sequence (TpsbA). The entire Prrn-aadA-PpsbA-GFP-TpsbA cassette was amplified from the pLD-CtV-aadA-GFP vector using primers PrrnAscI (5′-cgatt**ggcgcgcc**gctcccccgccgtcgttcaatgagaatg-3′) and PsbAAscI (5′-ggcagttg**ggcgcgcc**cccaaa caaatacaaaatc-3′) to obtain a PCR product with *Asc*I sites at both ends. After sequencing, the cassette was subcloned into pBS-16Srps12 digested with *Asc*I to obtain the final sugar beet chloroplast transformation vector pSB1 (Fig. 4a).

A second transformation vector, pSB2, was designed choosing the *accD* and *rbcL* genes and their intergenic spacer (located in the large single copy region, LSC) as the homologous recombination region of the *Beta vulgaris* plastome. The 1.6 kb *accD* DNA region, amplified using primers accDFor (5′-tgtgca**gtcgacttaattaa**ttcttatacatattccgtgg-3′) and accDrev (5′-tgtgca**ggtacc**agtagtaggagatgtaaggat-′3) containing the *Pac*I and *Sal*I restriction sites at the left end and *Kpn*I site at the right end of the *accD* PCR product (restriction sites are in bold in the primer sequences), was sequenced and cloned into pBluescriptII digested with *Sal*I and *Kpn*I. The resulting intermediate plasmid, pBS-accD, was digested with *Sac*I and *Sal*I and ligated into the *rbcL* sugar beet DNA region. This region was amplified with primers rbcLFor (5′-tgtgca**gagctc**atgtcaccacaaacagagact-3′) and rbcLRev (5′-tgtgca**gtcgacggcg**

**cgcc**taccaattctaccaatccta-3′), which introduced *Sac*I and *Asc*I/*Sal*I restriction sites at the left end and at the right end of the 1.6 kb PCR product, respectively, to obtain the pBS-rbcLaccD vector. The Prrn-aadA-PpsbA-GFP-TpsbA expression cassette was than subcloned into the pBS-rbcLaccD plasmid using *Asc*I sites as described above for pSB1.

## Bombardment and selection of transplastomic plants

To choose the correct shooting parameters, transient transformations were performed in which sugar beet petioles were bombarded with the pCK.gfp.S65C vector (Reichel et al. 1996), which carries a *gfp* gene under control of the CaMV 35S promoter. GFP fluorescence was visualized with a Zeiss Axiophot microscope and a filter set comprising an excitation filter (450–490 nm), a dichroic mirror (510 nm), and a barrier filter (LP 520 nm). The light source was an HBO 100 W mercury lamp. Images were processed using Adobe Photoshop software (Mountain View, CA, USA). To test *Beta vulgaris* petiole explant resistance to antibiotics, which is necessary to select transplastomic cells, spectinomycin and streptomycin were added at concentrations of 0, 50, 100, 250, 500, and 1,000 mg/l to regeneration medium 5.

To obtain transplastomic plants, sugar beet petioles cut into 0.5 cm pieces were placed on Petri dishes with medium 5 and bombarded with gold particles of 0.6 μm diameter coated with pSB1 or pSB2 vectors using the DuPont PDS1000He biolistic gun (BioRad Laboratories, Hercules, CA, USA). Bombardments to obtain stable transformants were performed at a pressure of 1,350 psi. Samples were held at a vacuum of 28″ mercury and placed 6 cm from the micro-projectile stopping screen. After particle bombardment, petioles were incubated in the dark for 48 h, transferred to medium 5 containing 50 mg/l spectinomycin, and incubated in the light for 4 months. Samples were transferred onto fresh selection medium every 30 days. Small friable calli developing from brown petioles were transferred to medium 7 containing 50 mg/l spectinomycin. Primary spectinomycin-resistant calli appeared after 1 month of selection on medium 7 and were identified as pale green growing calli. After 3 months, due to the lack of regeneration in the presence of spectinomycin, we first reduced the antibiotic concentration to 12.5 mg/l (which is the lowest antibiotic concentration that induces bleaching of sensitive cells, data not shown) and then eliminated it completely. Regenerated embryos obtained were left on medium 7 for another 3 months until they formed leaflets, and shoots were transferred into glass pots containing MSIBA3 with 12.5 mg/l spectinomycin for rooting. These plantlets were subjected to two additional rounds of regeneration on medium 5 with 12.5 mg/l spectinomycin to obtain homoplasmic tissues. Transgenic sugar beet plants were transferred to soil in pots for further development in the greenhouse. Fourteen months were required to produce the first transplastomic plants.

## Nucleic acid analyses

Total DNA was isolated from 100 mg of sugar beet seedling leaves derived from pSB1 transformants using the GenEluteTMPlant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, USA). PCR amplification was performed by amplifying 50 ng of DNA with the following primers: T2, 5′-ctagggttcctcgaacaatgtg-3′, T1, 5′-cgattactagcgattccggcttc-3′, aadArev, 5′-tcgtcgtgcacaacaatggt-3′, Efor 5′-cattccaaggcataacttgt-3′, Hrev, 5′-gaaagcatgtgctatggctc-3′, GFP1, 5′-cgacgggaactacaagacac-3′. For Southern blot analysis, total DNA (2 μg) was digested overnight with *Sac*II, electrophoresed on 0.8% agarose gel, and transferred to a Hybond-XL nylon membrane (GE Healthcare, Chalfont St. Giles, UK) according to the manufacturer's instructions. Two DNA sequences were used as probes (probe 1 and probe 2, Fig. 4a). Total RNA was extracted with the RNAeasy Plant Mini Kit (QIAGEN N.V., Venlo, The Netherlands). RNA (2.5 μg) was electrophoretically fractionated in 1.4% formaldehyde agarose gels and transferred to Hybond-N nylon membranes (GE Healthcare, Chalfont St. Giles, UK) according to the manufacturer's instructions. The *gfp* gene was used as a probe. Hybridization was performed for both DNA and RNA filters as indicated by the membrane supplier with $^{32}$P-labelled probes and the Ready-To-GoTM kit (GE Healthcare, Chalfont St. Giles, UK).

## Western blots

Leaves (0.15 g) of transplastomic sugar beet plants were ground in liquid nitrogen and homogenized in 0.5 ml extraction buffer [100 mM Tris–HCl pH 7.8,

200 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, 4% (v/v) 2-mercaptoethanol, 1 mM PMSF, 1× COMPLETE protease inhibitor mix (Roche Diagnostics GmbH, Mannheim, Germany)]. After centrifugation for 15 min at 17,000*g* at 5°C, the supernatant was recovered and protein extracts were measured by Bradford assay (Bradford 1976). Forty micrograms of total protein samples were fractionated by 10% SDS-PAGE and transferred onto nitro-cellulose membrane (Schleicher and Schuell GmbH, Dassel, Germany). GFP was visualized using a 1:400 dilution of polyclonal anti-GFP A.v. Peptide Antibody (Clontech Laboratories, Mountain View, CA, USA). Protein bands were visualized with peroxidase-linked goat anti-rabbit secondary antibody diluted 1:10,000 (Pierce Chemical, Rockford, USA) using SuperSignal West Pico chemiluminescent substrates (Pierce Chemical, Rockford, USA).

## Results

### Selection of an efficient regeneration protocol

Sugar beet transformation is particularly difficult, above all due to the low regeneration efficiency of cultured explants either through organogenesis (direct regeneration) or somatic embryogenesis (indirect regeneration). Moreover, plant regeneration is strictly dependent on the genotype used. Thus, to perform sugar beet plastome transformation, we needed to develop a highly efficient regeneration procedure for sugar beet breeding lines. Two regeneration methods were initially tested.

#### *Indirect regeneration (method A)*

Hypocotyl segments of 26 sugar beet varieties were tested for regeneration efficiency using the indirect regeneration method described by Dovzhenko and Koop (2003), which utilises hypocotyl segments as explants. At least 400 explants per line were used. The percentage of explants forming callus varied greatly between lines, from 2.3% to 47.6%, with no clear distinction between multigerm or monogerm sugar beet lines (data not shown). These calli rarely evolved into plant-regeneration competent calli, i.e., friable, light-yellow calli with a coarse-grained structure (Fig. 1a) that generate plantlets (Fig. 1b, c). The frequency of shoot regeneration from calli of all varieties analysed, except for KMBRT (line E) for which the frequency was 42%, was less than 5%

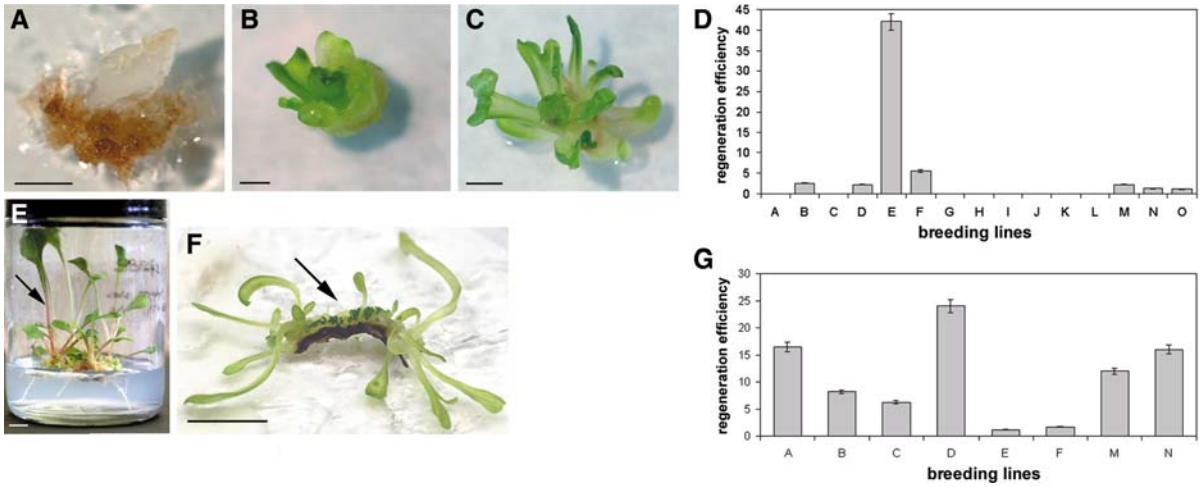


**Fig. 1** Plant regeneration methods in sugar beet and comparison of their regeneration efficiency. (**a**–**c**) Callus formation from hypocotyls (**a**) and plantlet differentiation from this callus via somatic embryogenesis (**b**, **c**). (**d**) Regeneration efficiency (r.e.) values with the indirect regeneration method (callus induction from the hypocotyls) of 15 sugar beet breeding lines. The other 11 lines examined are not shown because their r.e. values are zero. (**e**) Excision of regenerable leaf petioles from young sugar beet seedlings grown in sterile culture. (**f**) After a few weeks, the formation of the first shoots can be observed (direct regeneration method). (**g**) Values of r.e. with the direct regeneration method of 8 sugar beet breeding lines. The other 18 lines analysed have r.e. values of zero. Bars represent 95% confidence interval. Scale bars in **a**–**c**: 100 μm. Scale bars in **e** and **f**: 1 cm

(Fig. 1d). Unfortunately, the KMBRT multigerm pollinator line, due to its low efficiency in callus formation from hypocotyl segments (less than 5%), was not a good source of regenerating material for sugar beet genetic modification.

*Direct regeneration (method B)*

The frequency of direct shoot regeneration from leaf rosette-isolated petioles (Fig. 1e, f) was determined in all 26 *Beta vulgaris* varieties using the method developed by Mishutkina and Gaponenko (2006). At least 400 explants (petioles) for each line were tested with this method. We identified four breeding lines (A, D, M, and N) as the most responsive to shoot induction media. In these varieties the regeneration frequency (percentage of petioles producing one or more shoots) ranged from 16% to 24% (Fig. 1g). In addition to direct shoot formation from petioles, we noted that small friable calli arose from almost all the petioles in two of the four lines, C67-2 (line M) and Z025 (line N). These calli formed all along the petiole length and generally appeared after 1 month of culture on medium 5 (Fig. 2). Once these calli grew, they were transferred to medium 7 for shoot regeneration, which occurred at a frequency of 80% for both lines. Interestingly, petioles in which direct shoot regeneration occurred generally remained green and thick, in contrast to petioles that turned soft and deep brown before starting callus dedifferentiation (Fig. 2).

Generation and analysis of plastid transformants

On the basis of the results obtained with the two regeneration methods, we decided to use method B and petiole explants from the C67-2 and Z025 lines for chloroplast transformation experiments. It was first necessary to test whether sugar beet petioles are amenable to biolistic transformation. We performed a series of transient assays using a nuclear-expressed *gfp* gene (pCK.GFP.S56C plasmid). Transformation was detected by fluorescence microscopy. The highest fluorescent cell number was obtained with a shooting pressure of 1,350 psi and a distance of 6 cm from the stopping screen. Long epidermal cells and stomatal guard cells were transiently transformed along the petiole using these parameters (Fig. 3a–d). The latter have been demonstrated to be the only progenitors of totipotent protoplasts in sugar beet leaf protoplast populations (Hall et al. 1997), so they represent good targets for biolistic chloroplast transformation. Mesophyll cells were also transiently transformed (data not shown).

To select for transplastomic plants, we used the *aadA* gene, which inactivates spectinomycin and streptomycin. Before commencing bombardments, the optimal antibiotic concentration in culture medium was determined. We screened untransformed sugar beet petiole explants on medium 5 under light conditions at different concentrations of spectinomycin and streptomycin. In tobacco, 500 mg/l spectinomycin was required for effective bleaching, whereas, in sugar beet, only 50 mg/l spectinomycin caused clorosis of tissues and inhibited shoot formation after 5 weeks (Fig. 3e). The growth of friable calli isolated from untransformed petiole explants and cultured on medium 7 with 50 mg/l spectinomycin was slightly delayed and they did not regenerate (data not shown). Therefore, 50 mg/l spectinomycin was chosen for selection of sugar beet transformants. Contrastingly, *Beta vulgaris* was found to be naturally resistant to streptomycin up to 1,000 mg/l (Fig. 3f), as already reported for soybean (Dufourmantel et al. 2004).

Plastid transformation was carried out by bombarding sugar beet petioles with gold particles coated

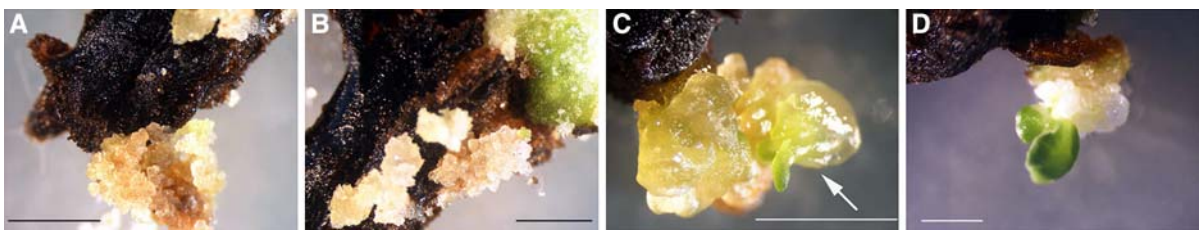


**Fig. 2** Highly regenerable callus from brown petioles. (**a**, **b**) Small friable calli arising along the petiole. (**c**) A somatic embryo (arrow) is formed from a callus. (**d**) The embryo developed into a plantlet. Scale bars: 100 μm

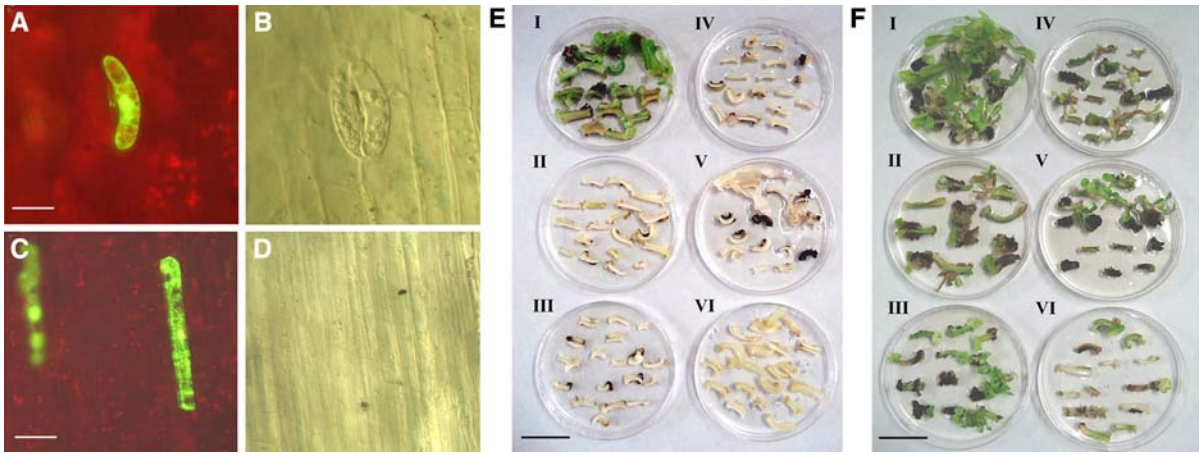


**Fig. 3** Optimization of sugar beet chloroplast DNA transformation parameters. (**a**–**d**) Images were taken with a conventional fluorescence microscope. (**a**) Visualization of GFP in a transiently transformed sugar beet stomatal guard cell. (**b**) Bright-field image of **a**. (**c**) GFP expression in two long epidermal cells. (**d**) Bright-field image of **c**. (**e**) Spectinomycin resistance of leaf petioles on medium 5 containing antibiotic concentrations of 0 mg/l (I), 50 mg/l (II), 100 mg/l (III), 250 mg/l (IV), 500 mg/l (V), or 1,000 mg/l (VI). (**f**) The same assay as in **e**, replacing spectinomycin with streptomycin. Scale bars in **a** and **c**: 15 μm. Scale bars in **e** and **f**: 3 cm

with pSB1 or pSB2 (Fig. 4a). These two sugar beet-specific plastid transformation constructs target the insertion of the transgene expression cassette to two different plastome regions: pSB1 targets the IR region, and pSB2 targets the LSC region. In both vectors, the transgene cassette is designed as an operon with two promoters (a P*rrn* promoter driving *aadA* gene expression and a *psbA* promoter/5′ UTR driving *gfp* gene expression) and a single 3′ termination region (the *psbA* 3′UTR). Because of the presence of these two promoters, the *gfp* gene should be transcribed as a monocistron in addition to the *aadA*/*gfp* dicistron. The capacity of the plastid for dicistronic expression of the *aadA* and *gfp* genes has been shown previously (Jeong et al. 2004). For each construct we performed six bombardment experiments on approximately 1,700 explants (36–40 bombarded plates). We selected three spectinomycin-resistant, putatively transformed calli after 5 months of selection in regeneration medium containing 50 mg/l spectinomycin (see Materials and methods). These calli resulted from petioles of Z025 line bombarded with the pSB1 vector, whereas no transformants were obtained with the pSB2 vector. We were not able to transform the C67-2 line. The transplastomic origin of these calli was confirmed by PCR amplification with the T2/aadArev primer pair (Fig. 4b) and by microscopy in which GFP fluorescence was evident within chloroplasts (Fig. 4d). Primer T2 binds on the native chloroplast genome adjacent to the integration point and primer aadArev binds to the *aadA* gene (Fig. 4a). We prepared two samples for each callus, isolating cells from two different parts of the regenerative structure. Chloroplast transformed calli should produce a 2.1 kb PCR fragment that is absent in nuclear transgenic cells or mutants. The presence of a PCR product of 2.1 kb confirmed site-specific integration of the transgene cassette into the plastome, except in one part of callus C2 (Fig. 4b, lane 4). The same results were obtained using another combination of primers (T1/GFP1, Fig. 4a) internal and external to the transgene cassette (data not shown). Homoplasmy of transplastomic calli was determined by PCR using two primers external to the flanking sequences that produced an amplification product of 2.5 kb, indicating the presence of the *aadA*-*gfp* cassette, or a product of 480 bp for the wild-type DNA (Fig. 4c). Calli C2 and C3 were clearly heteroplasmic, but we did not obtain the 2.5 kb fragment for callus C1. Taken together, the results shown in Fig. 4b and c indicate that the three calli are transplastomic, heteroplasmic, and they have different characteristics. Callus C1, for example, likely has very few transformed plastomes that we were not able to amplify with primers Efor/Hrev due to their preference for the amplification of the short wild-type PCR fragment. Moreover, only a portion of callus C2 is transplastomic, whereas callus C3 seems

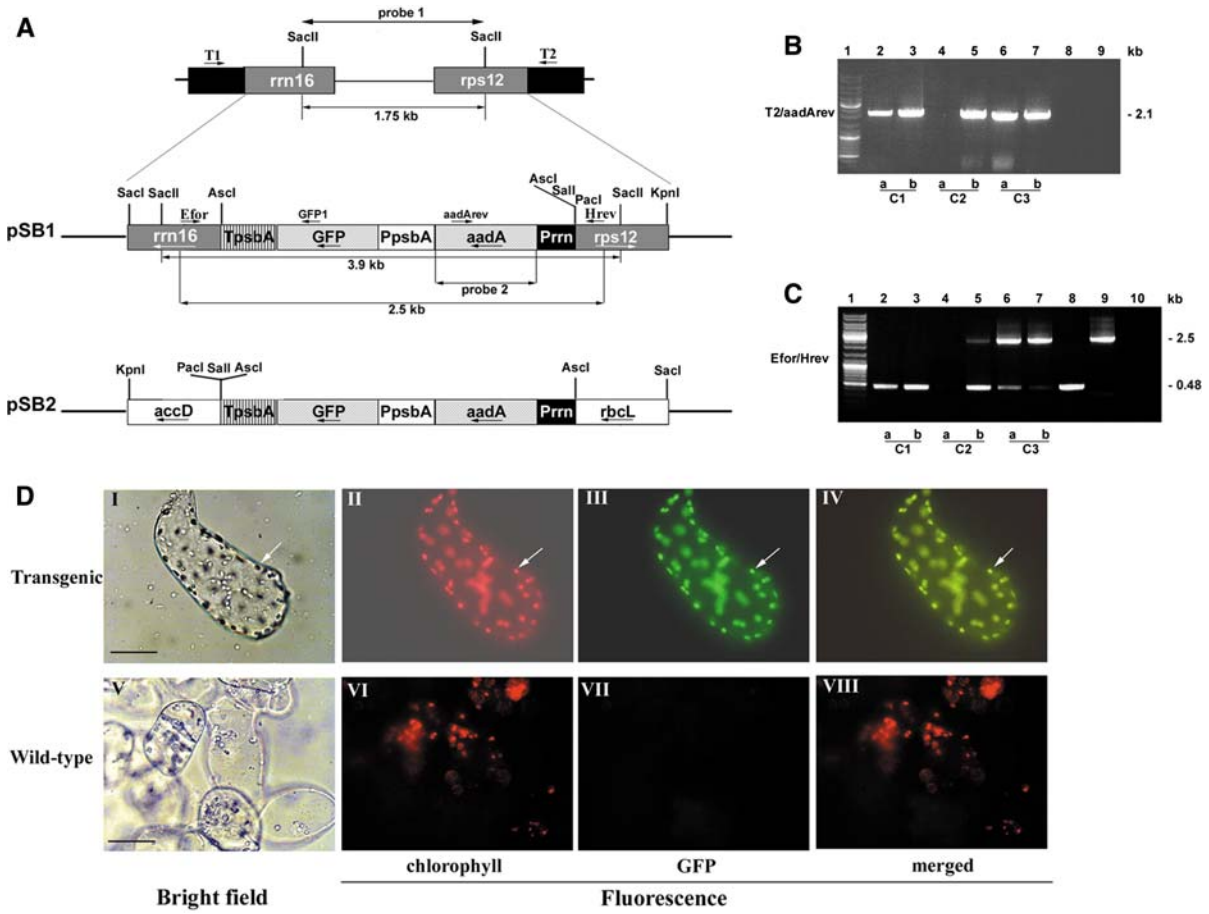


**Fig. 4** Vectors for plastome transformation and analysis of pSB1 integration and expression at the callus stage. (**a**) Schematic map (not drawn to scale) of the *rrn16*/*rps12* region of the sugar beet chloroplast genome used to construct the pSB1 vector targeting the transgene cassette to this region. The two black boxes flanking the *rrn16* and *rps12* genes indicate the sugar beet chloroplast genome not included in the pSB1 vector. The annealing positions of primers T2/aadArev, T1/GFP1 and Efor/Hrev are shown. The PCR product of 2.5 kb originated using primers Efor/Hrev is shown. Probe 1 flanking the cassette and probe 2 corresponding to the *aadA* gene used for Southern blot analysis are shown. The predicted hybridizing fragments (3.9 and 1.75 kb) are indicated in kb when total DNA digested with *Sac*II is probed with probe 1. Probe 2 detects a 3.9 kb fragment. Vector pSB2 harbours the same transgene cassette as pSB1 inserted in the intergenic region between the *accD* and *rbcL* genes. Arrows within the genes indicate the direction of transcription. (**b**) PCR analysis using total genomic DNA of three spectinomycin-resistant calli (C 1–3) to investigate their transplastomic state with primer pair T2/aadArev. Each callus is split into two samples (**a**, **b**). Lane 8 is wild-type sugar beet DNA and in lane 9 there is no DNA. (**c**) PCR analysis of the same calli to verify their homoplasmic state with primer pair Efor/Hrev. Lanes are loaded in the same way as in **b**, except for lane 9 where there is pSB1 DNA and lane 10 where there is no DNA. (**d**) Bright-field and fluorescent images of one cell (I–IV) belonging to the callus C3 expressing GFP in the chloroplast (white arrow) and several wild-type callus cells (V–VIII) taken with a conventional fluorescence microscope. The red channel (chlorophyll) and the green channel (GFP) are shown both separately and merged. *aadA*, aminoglycoside 3′-adenylyl transferase gene; GFP, green fluorescent protein; Prnn, *Prnn* promoter; PpsbA, *psbA* promoter/5′UTR; TpsbA, *psbA* 3′UTR. Scale bars: 10 μm

to be heteroplasmic but with very few wild-type plastome copies. Interestingly, callus C3 developed into embryos after culturing in regenerative medium 7, whereas calli C1 and C2 did not regenerate. Callus C3 started to regenerate only after the removal of spectinomycin from medium 7. The resulting shoots were placed in medium MSIBA3 with 12.5 mg/l spectinomycin for rooting. Microscopy to detect GFP fluorescence in these shoots confirmed their heteroplasmic state. Indeed, when callus C3 derived shoots were examined the majority of cells contained only GFP-fluorescent chloroplasts, and only few shoots

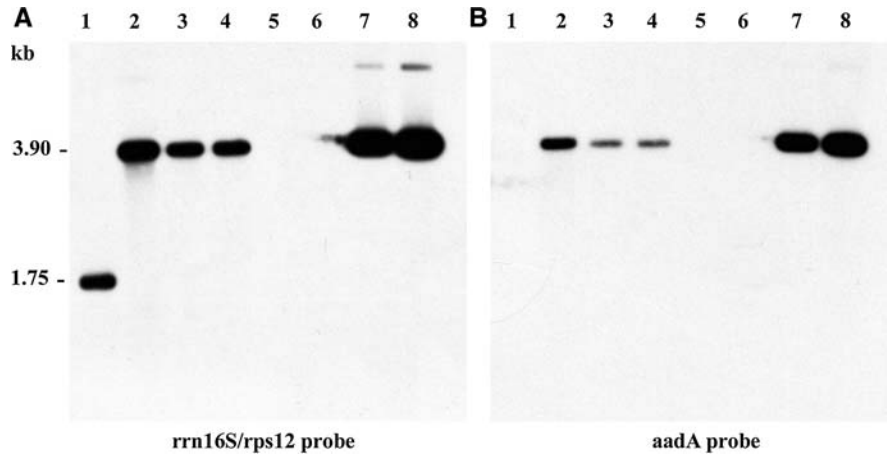


**Fig. 5** Southern blot analysis of three sugar beet transplastomic plants. (**a**) Total DNA (2 μg) and plasmid pSB1 (2 and 4 ng, lanes 7 and 8, respectively) were digested with *Sac*II. Plant homoplasmy was examined using part of the *rrn16/rps12* region as a probe (probe 1, Fig. 4a). The transformed plants (lanes 2–4) had one 3.9 kb fragment as expected for homoplasmic transformed plants, whereas the wild-type plant (lane 1) has a smaller fragment of 1.7 kb. Lanes 5 and 6 are empty. (**b**) The same filter as in A after removal of probe 1 and reprobing with the *aadA* gene (probe 2, Fig. 4a). Note that the wild-type DNA has no signal as expected. Molecular masses in kb (numbers at left) are indicated. wt, wild-type DNA

had chimeric leaves with small tissue sections lacking GFP-fluorescent chloroplasts (data not shown). Transgenic plantlets were regenerated two additional times by direct shoot formation from petiole explants on medium 5 with spectinomycin 12.5 mg/l. Southern blot analysis of DNA extracted from these transgenic plants demonstrated homoplasmy when the *rrn16/rps12* probe was used (probe 1, Fig. 4a). Total leaf DNA cut with *Sac*II produced only one 3.9 kb fragment in transformed plants, as expected for transplastomic DNA (Fig. 5a, lanes 2–4). Wild-type plants showed a 1.7 kb fragment (Fig. 5a, lane 1). The homoplasmy of transgenic plants was further verified by PCR using the Efor/Hrev primer pair (data not shown). The same filter shown in Fig. 5a was stripped and reprobed with the *aadA* gene (probe 2, Fig. 4a), revealing the same 3.9 kb fragment for all transformed plants (Fig. 5b, lanes 2–4) and thus further verifying the integrity of the inserted transgene cassette and that all of the plants were transplastomic.

Transplastomic plants were also analysed for *gfp* expression. Transcription of the *gfp* gene was analysed by Northern blot analysis of total RNA extracted from the leaves of homoplasmic sugar beet plants. Probing of the blot with the *gfp* gene showed an abundant transcript of about 1.5 kb and a minor transcript of approximately 3.1 kb (Fig. 6a). The 1.5 kb mRNA is the expected monocistronic *gfp* transcript. The 3.1 kb mRNA should be the dicistronic *aadA*/*gfp* transcript. Alternatively, the presence of this minor transcript is consistent with read through transcription of adjacent genes downstream of the monocistronic *gfp* expression cassette. Similar results were obtained inserting a desaturase gene in the tobacco *rrn16–rps12* region (Craig et al. 2008). Leaf tissues were found to produce green fluorescence localized in the chloroplast (Fig. 6c), and anti-GFP antibody recognized a peptide of 27 kDa by Western blot (Fig. 6e). The band corresponding to GFP could be easily detected in Coomassie blue-stained gels (Fig. 6d), suggesting that GFP levels were high in the sugar beet chloroplasts.

## Discussion

We report the stable transformation of sugar beet plastids using the biolistic technique, resulting in homoplasmic transgenic plants. To develop a protocol for sugar beet plastid transformation, we needed to study different aspects of this technology including regeneration systems, selection conditions and DNA delivery strategies. Hence, we first examined the regeneration efficiency of several sugar beet varieties using different target tissues and media compositions,

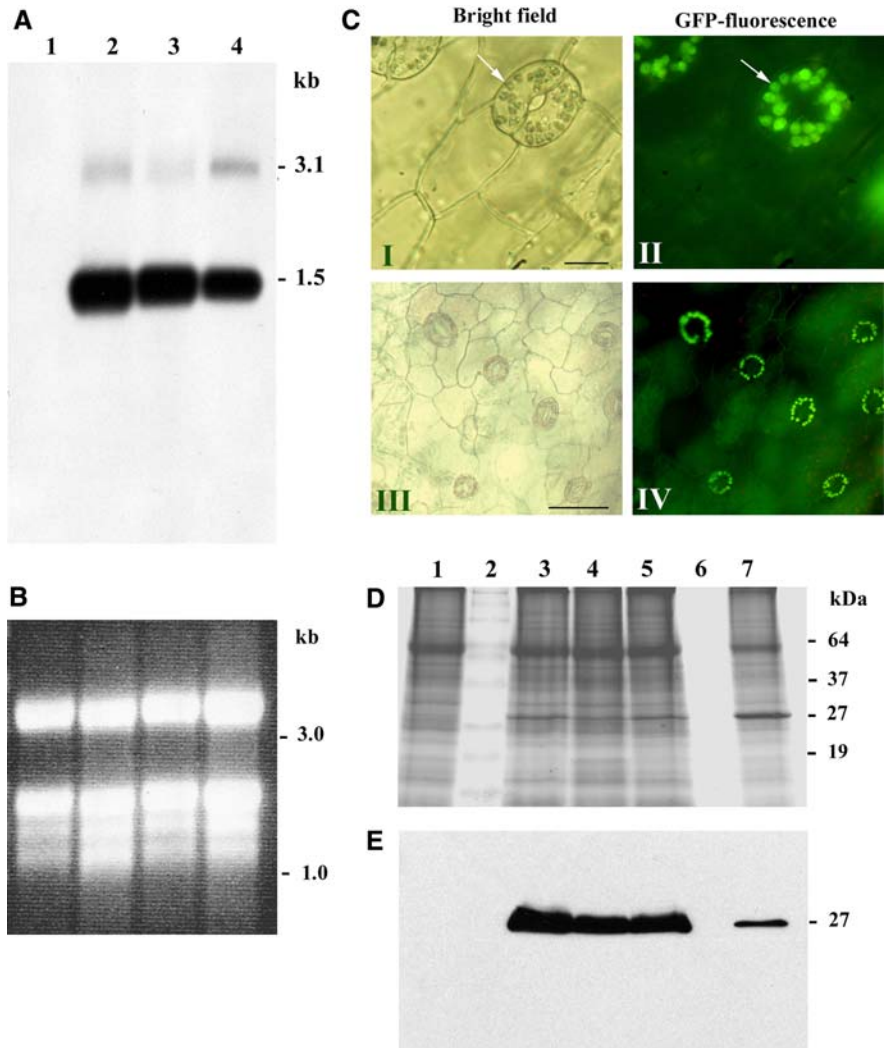


**Fig. 6** GFP expression in sugar beet transplastomic plants. (**a**) Northern blot analysis of *gfp* transcript accumulation in three transformed plants (lanes 2–4) and in the wild-type plant (lane 1). The filter, containing 2.5 µg RNA in each lane, was hybridized with a PCR fragment corresponding to the *gfp* coding region. Molecular masses in kb (number at right) are indicated. (**b**) Ethidium bromide staining of the gel in **a** as a control for RNA loading differences. Molecular masses in kb (number at right) are indicated. (**c**) Bright-field (I and III) and fluorescence (II and IV) images from leaves of a transplastomic plant. Chloroplasts of stomatal guard cells show intense fluorescence. In panels I and II the white arrow indicates a chloroplast. (**d**) Protein (40 µg) was extracted from leaves of plants expressing GFP (lanes 3–5) or of a wild-type plant (lane 1) and analysed by SDS-PAGE. The gel is stained with Coomassie blue. Lane 2, molecular weight marker. Lane 6 is empty. In lane 7, 40 µg of wild-type proteins are loaded together with 1 µg of recombinant GFP (Clontech). Numbers at right indicate molecular mass in kDa. (**e**) Protein blot of the same proteins as in **d**, except that in lane 7 where only 20 ng of recombinant GFP are added to 40 µg of wild-type proteins, using anti-GFP antiserum. Scale bar in **c** I: 10 µm. Scale bar in **c** III: 40 µm

because a genotype-independent and widely applicable protocol for sugar beet plant regeneration has not been reported. The percentage of sugar beet hypocotyl explants forming callus obtained in this work (method A) were comparable with values reported by Dovzhenko and Koop (2003) using the same protocol, but the regeneration efficiencies of these calli were dramatically lower than previously reported. This means that method A is not suitable for plant regeneration in our sugar beet breeding lines. Thus, we used another method (method B), based on the protocol developed by Mishutkina and Gaponenko (2006), of

direct shoot regeneration from leaf rosette-isolated petioles. In this case as well, our best values of regeneration frequency (from 16% to 24%) were lower than those reported by these authors (from 73% to 97%), but we modified the original method because friable yellow calli, which regenerated adventitious shoots at a frequency of roughly 80%, were obtained from petioles of lines C67-2 and Z025. Shoot formation from friable callus of different origins is a well-described phenomenon in sugar beet tissue culture and has been widely reported (Catlin 1990; Jacq et al. 1992; Ivic-Haymes and Smigocki 2005).

Regarding selection conditions, the *aadA* gene was used as a selection marker due to its efficacy in numerous plant species, despite the fact that selection conditions using this gene vary greatly among species (reviewed in Verma and Daniell 2007). In this work, selection of sugar beet transformants was initially performed using 50 mg/l spectinomycin for the first 8 months; the spectinomycin concentration was then reduced to 12.5 mg/l because the three transgenic calli we obtained did not regenerate in the presence of 50 mg/l spectinomycin. These pale green calli were not able to regenerate even at this low antibiotic concentration in the culture medium, and shoots grew from these calli only when spectinomycin was removed. After transgenic shoots developed into plantlets, we applied antibiotic again and these plantlets were rooted in 12.5 mg/l spectinomycin. Based on these results, alternatives to *aadA* should be tested for selection of transplastomic sugar beet plants. The use of suitable selection markers has previously been shown to be critical for success in the transformation of plastids (Kumar et al. 2004b; Svab and Maliga 1993). The kanamycin resistance genes *neo* (Carrer et al. 1993) and *aphA6* (Huang et al. 2002) could be alternative markers for sugar beet plastid transformation, although repression of transgenic plant regeneration has been described in sugar beet using kanamycin as a nuclear-selectable marker (Zhang et al. 2001; Ivic-Haymes and Smigocki 2005). In these studies, stably transformed plants were recovered only from bombarded or Agrobacterium-inoculated explants in medium lacking kanamycin.

We designed two *B. vulgaris*-specific transformation vectors. The pSB1 vector contains the sugar beet *rrn16* and *rps12* genes as target sites for homologous recombination into the IR region of the sugar beet plastome. The region between the *rrn16* and *rps12* genes is one of the most frequently used sites for transgene insertion (Zoubenko et al. 1994; Lutz and Maliga 2007). The pSB2 vector was modelled on the tobacco plastid vector pZS197 (Svab and Maliga 1993) and targets the insertion of the transgenes to the LSC region. Targeted integration of the *aadA*/*gfp* expression cassette was only achieved in the Z025 sugar beet line using the pSB1 vector. The frequency of sugar beet plastid transformation was estimated at 1 transplastomic line per 36 bombarded plates. This is significantly lower than results achieved in tobacco, where 1–14 transgenic lines per bombardment can be obtained (Svab and Maliga 1993; Daniell et al. 2001), but it is comparable with transformation frequencies reported for *Arabidopsis* or potato (Sikdar et al. 1998; Sidorov et al. 1999). Our calculation of sugar beet transformation frequency is based on six independent bombardment experiments that generated three spectinomycin-resistant calli. These transgenic calli were found to be heteroplasmic by PCR. This is common in plastid transformations where chloroplast transformants are subsequently purified to homoplasmy by passing them through additional regeneration cycles under antibiotic selection. For this reason, we subjected the primary regenerates resulting from one of these calli (C3) to two additional rounds of regeneration by organogenesis on medium 5 with 12.5 mg/l spectinomycin. The transplastomic plants obtained appeared to be homoplasmic by Southern blot analysis (Fig. 5a).

GFP has been widely used as a versatile marker for monitoring gene expression in plants, and there are examples of its use in transformed sugar beet tissue (Zhang et al. 2001). GFP is also valuable as a plastid-expressed marker for transformation (Sidorov et al. 1999). We used GFP expression in sugar beet to visually screen plastid transformants at callus and shoot/plant stages, greatly facilitating our work. Moreover, a band corresponding to GFP can be detected in Coomassie blue-stained gels (Fig. 6d), suggesting that there is a significant accumulation of recombinant protein in sugar beet chloroplasts. We are now growing the sugar beet transplastomic plants at cold temperatures in the greenhouse to induce vernalization, which promotes flowering and seed production. The *aadA* and *gfp* transgenes will be used to confirm homoplasmy of the transgenic plants and to study transgene maternal inheritance.

In conclusion, we selected heteroplasmic sugar beet calli on spectinomycin-containing medium that

were induced to regenerate into heteroplasmic shoots by somatic embryogenesis only when spectinomycin was removed from the culture medium. Stable homoplasmic plants were obtained after subsequent rounds of regeneration by organogenesis in low spectinomycin concentrations. Overall, it took about 14 months to obtain transplastomic plants. The developed procedure could likely be improved by using a new selection scheme since spectinomycin hampered transgenic callus regeneration. An alternative strategy might be to use protoplasts as explants to deliver transformation vectors to chloroplasts by polyethylene glycol treatment (Golds et al. 1993). Indeed, successful shoot regeneration from callus protoplasts (Dovzhenko and Koop 2003) or leaf protoplasts (Hall et al. 1997) has been reported in sugar beet. The results of our work, however, indicate that production of sugar beet plastid transformants is feasible, and this might open new opportunities for genetic improvement of this crop and social acceptance of GM sugar beet plants.

**Acknowledgements** This article is contribution no. 130 from the Institute of Plant Genetic, Research Division of Perugia. This work was supported by the European Framework VI Specific Target Research Project "Transcontainer". We are grateful to Dr. Gunter Diener (KWS SAAT AG) for providing us the monogerm sugar beet seeds and Dr. Marco De Biaggi and Dr. Enrico Biancardi (CRA – CIN Centro di Ricerca per le Colture Industriali Sede di Rovigo) for the other sugar beet breeding lines. We thank Professor Henry Daniell for providing pCR 2.1-5′UTR and pLD-CtV, and Dr. Phil Dix and Dr. Matthew McCabe for their helpful comments.

## References

Ahmadabadi M, Ruf S, Bock R (2007) A leaf-based regeneration and transformation system for maize (*Zea mays* L.). Transgenic Res 16:437–448

Bartsch D, Lehnen M, Clegg J, Pohl-Orf M, Schuphan I, Ellstrand NC (1999) Impact of gene flow from cultivated beet on genetic diversity of wild sea beet populations. Mol Ecol 8:1733–1741

Bartsch D, Cuguen J, Biancardi E, Sweet J (2003) Environmental implications of gene flow from sugar beet to wild beet—current status and future research needs. Environ Biosafety Res 2:1–11

Biancardi E, Lewellen RT, De Biaggi M, Erichsen AW, Stevanato P (2002) The origin of rizomania resistance in sugar beet. Euphytica 127:383–397

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

Bock R (2007) Plastid biotechnology: prospects for herbicide and insect resistance, metabolic engineering and molecular farming. Curr Opin Biotechnol 18:100–106

Bock R, Khan MS (2004) Taming plastids for a green future. Trends Biotechnol 22:311–318

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal Biochem 72:248–254

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

Catlin DW (1990) The effect of antibiotics on the inhibition of callus induction and plant regeneration from cotyledons of sugarbeet (*Beta vulgaris* L.). Plant Cell Rep 9:285–288

Craig W, Lenzi P, Scotti N, De Palma M, Saggese P, Carbone V, McGrath Curran N, Magee AM, Medgyesy P, Kavanagh TA, Dix PJ, Grillo S, Cardi T (2008) Transplastomic tobacco plants expressing a fatty acid desaturase gene exhibit altered fatty acid profiles and improved cold tolerance. Transgenic Res. doi: 10.1007/s11248-008-9164-9

Daniell H (2002) Molecular strategies for gene containment in transgenic crops. Nat Biotechnol 20:581–586

Daniell H, Lee SB, Panchal T, Wiebe PO (2001) Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. J Mol Biol 311:1001–1009

Darmency H, Vigouroux Y, Gestat De Garambé T, Richard-Molard M, Muchembled C (2007) Transgene escape in sugar beet production fields: data from six years farm scale monitoring. Environ Biosafety Res 6:197–206

De Cosa B, Moar W, Lee SB, Miller M, Daniell H (2001) Overexpression of the Bt cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. Nat Biotechnol 19:71–74

Dhingra A, Portis AR Jr, Daniell H (2004) Enhanced translation of a chloroplast-expressed RbcS gene restores small subunit levels and photosynthesis in nuclear RbcS antisense plants. Proc Natl Acad Sci USA 101:6315–6320

Dovzhenko A, Koop HU (2003) Sugarbeet (*Beta vulgaris* L.): shoot regeneration from callus and callus protoplasts. Planta 217:374–381

Dufourmantel N, Pelissier B, Garcon F, Peltier G, Frullo JM, Tissot G (2004) Generation of fertile transplastomic soybean. Plant Mol Biol 55:479–489

Golds T, Maliga P, Koop HU (1993) Stable plastid transformation in PEG-treated protoplasts of *Nicotiana tabacum*. Nat Biotechnol 11:95–97

Hall RD, Riksen-Bruinsma T, Weyens G, Lefebvre M, Dunwell JM, Krens FA (1996) Stomatal guard cells are totipotent. Plant Physiol 112:889–892

Hall RD, Riksen-Bruinsma T, Weyens G, Lefebre M, Dunwell JM, van Tunen A, Krens FA (1997) Sugar beet guard protoplast demonstrate a remarkable capacity for cell division enabling applications in stomatal physiology and molecular breeding. J Exp Bot 48:255–263

Hisano H, Kimoto Y, Hayakawa H, Takeichi J, Domae T, Hashimoto R, Abe J, Asano S, Kanazawa A, Shimamoto Y (2004) High frequency *Agrobacterium*-mediated transformation and plant regeneration via direct shoot formation from leaf explants in *Beta vulgaris* and *Beta maritima*. Plant Cell Rep 22:910–918

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

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

Ivic-Haymes SD, Smigocki AC (2005) Biolistic transformation of highly regenerative sugar beet (*Beta vulgaris* L.) leaves. Plant Cell Rep 23:699–704

Jacq B, Tétu T, Sangwan RS, De Laat A, Sangwan-Norreel BS (1992) Plant regeneration from sugarbeet (*Beta vulgaris* L.) hypocotyls cultured in vitro and flow cytometric nuclear DNA analysis of regenerants. Plant Cell Rep 11:329–333

Jeong SW, Jeong WJ, Woo JW, Choi DW, Park YI, Liu JR (2004) Dicistronic expression of the green fluorescent protein and antibiotic resistance genes in the plastid for selection and tracking of plastid-transformed cells in tobacco. Plant Cell Rep 22:747–751

Kishchenko EM, Komarnitskii IK, Kuchuk NV (2005) Production of transgenetic sugarbeet (*Beta vulgaris* L.) plants resistant to phosphinothricin. Cell Biol Int 29:15–19

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

Kumar S, Dhingra A, Daniell H (2004b) Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. Plant Mol Biol 56:203–216

Lee SM, Kang K, Chung H, Yoo SH, Xu XM, Lee SB, Cheong JJ, Daniell H, Kim M (2006) Plastid transformation in the monocotyledonous cereal crop rice (*Oryza sativa*) and transmission of transgenes to their progeny. Mol Cells 21:401–410

Lelivelt CL, McCabe MS, Newell CA, de Snoo CB, van Dun KM, Birch-Machin I, Gray JC, Mills KH, Nugent JM (2005) Stable plastid transformation in lettuce (*Lactuca sativa* L.). Plant Mol Biol 58:763–774

Liu CW, Lin CC, Chen JJ, Tseng MJ (2007) Stable chloroplast transformation in cabbage (*Brassica oleracea* L. var. *capitata* L.) by particle bombardment. Plant Cell Rep 26:1733–1744

Lutz KA, Maliga P (2007) Transformation of the plastid genome to study RNA editing. Methods Enzymol 424: 501–518

McGrath JM, Saccomani M, Stevanato P, Biancardi E (2007) Beet. In: Kole C (ed) Genome mapping and molecular breeding in plants, volume 5 vegetables. Springer-Verlag, Berlin, pp 135–151

Mishutkina IaV, Gaponenko AK (2006) Sugar beet (*Beta vulgaris* L.) morphogenesis in vitro: effects of phytohormone type and concentration in the culture medium, type of explants, and plant genotype on shoot regeneration frequency. Genetika 42:210–218

Nugent GD, Coyne S, Nguyen TT, Kavanagh TA, Dix PJ (2006) Nuclear and plastid transformation of *Brassica oleracea* var. *botrytis* (cauliflower) using PEG-mediated uptake of DNA into protoplast. Plant Sci 170:35–142

Okumura S, Sawada M, Park YW, Hayashi T, Shimamura M, Takase H, Tomizawa K (2006) Transformation of poplar (*Populus alba*) plastids and expression of foreign proteins in tree chloroplasts. Transgenic Res 15:637–646

Reichel C, Mathur J, Eckes P, Langenkemper K, Koncz C, Schell J, Reiss B, Maas C (1996) Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. Proc Natl Acad Sci USA 93:5888–5893

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

Saeglitz C, Pohl M, Bartsch D (2000) Monitoring gene flow from transgenic sugar beet using cytoplasmic male-sterile bait plants. Mol Ecol 9:2035–2040

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

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

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

Snyder GW, Ingersoll JC, Smigocki AC, Owens LD (1999) Introduction of pathogen defense genes and a cytokinin biosynthesis gene into sugarbeet (*Beta vulgaris* L.) by *Agrobacterium* or particle bombardment. Plant Cell Rep 18:829–834

Svab Z, Maliga P (1993) High-frequency plastid transformation in tobacco by selection for a chimeric aadA gene. Proc Natl Acad Sci USA 90:913–917

Verma D, Daniell H (2007) Chloroplast vector systems for biotechnology applications. Plant Physiol 145:1129–1143

Watson J, Koya V, Leppla SH, Daniell H (2004) Expression of *Bacillus anthracis* protective antigen in transgenic chloroplasts of tobacco, a non-food/feed crop. Vaccine 22:4374–4384

Zhang CL, Chen DF, McCormac AC, Scott NW, Elliot MC, Slater A (2001) Use of GFP reporter as a vital marker for *Agrobacterium*-mediated transformation of sugar beet (*Beta vulgaris* L.). Molecular Biotechnol 17:109–117

Zoubenko OV, Allison LA, Svab Z, Maliga P (1994) Efficient targeting of foreign genes into the tobacco plastid genome. Nucleic Acids Res 22:3819–3824