Transgenic Res (2010) 19:113–119
DOI 10.1007/s11248-009-9290-z

BRIEF COMMUNICATION

Plastid transformation in eggplant (*Solanum melongena* L.)

A. K. Singh · S. S. Verma · K. C. Bansal

Received: 29 January 2009 / Accepted: 26 May 2009 / Published online: 28 June 2009

Abstract We have developed a method for plastid transformation in eggplant (*Solanum melongena* L.), a solanaceous plant species. Plastid transformation in eggplant was achieved by bombardment of green stem segments with pPRV111A plastid expression vector carrying the *aadA* gene encoding aminoglycoside 3″-adenylyltransferase. Biolistic delivery of the pPRV111A plasmid yielded transplastomic plants at a frequency of two per 21 bombarded plates containing 25 stem explants each. Integration of the *aadA* gene in the plastome was verified by PCR analysis and also by Southern blotting using *16S rDNA* (targeting sequence) and the *aadA* gene as a probe. Transplastomic expression of the *aadA* gene was verified by RT-PCR. The development of transplastomic technology in eggplant may open up exciting possibilities for novel gene introduction and expression in the engineered plastome for agronomic or pharmaceutical traits.

Keywords Plastid transformation · Eggplant · Site-specific integration · Aminoglycoside 3″-adenylyltransferase (*aadA*)

Electronic supplementary material The online version of this article (doi:10.1007/s11248-009-9290-z) contains supplementary material, which is available to authorized users.

A. K. Singh · S. S. Verma · K. C. Bansal (✉)
National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi 110 012, India
e-mail: kailashbansal@hotmail.com

Present Address:
A. K. Singh
Department of Plant Pathology, 201F Plant Science Building, University of Kentucky, 1405 Veterans Drive, Lexington, KY 40546-0312, USA

Introduction

Eggplant is an important solanaceous vegetable crop widely cultivated in many tropical, Asian, and some European countries. Nuclear transformation in eggplant has been reported by several workers (Guri and Sink 1988; Filippone and Lurquin 1989; Rotino and Gleddie 1990; Fari et al. 1995; Kumar et al. 1998; Hanyu et al. 1999; Franklin and Sita 2003). However, introduction of transgene(s) into the nuclear genome has led to a growing public concern of the possibility of gene escape through pollen to weedy or wild relatives of the transgenic crops or by causing gene pollution among other crop plants. Since the plastids are maternally inherited in most crop plants, engineering of the plastid genome is gaining momentum as an attractive alternative to nuclear transformation. Plastid transformation technology offers several unique advantages, including a high level of transgene expression and absence of epigenetic transgene instability effects (Bock 2001; Maliga 2004; Grevich and Daniell 2005; Sharma et al. 2005). In addition, increased biosafety associated with transplastomic

plants is of particular relevance to future applications of genetic engineering in agriculture. Because of these advantages, the transplastomic technology could be useful for engineering agronomic traits, and for metabolic engineering and production of biopharmaceuticals in plants.

Despite the numerous merits and potential applications of the technology, plastid transformation is routinely possible only in tobacco. Thus, it is highly desirable to extend this technology to a wide range of plant species. Among higher plants, fertile homoplasmic plants with genetically modified plastid genomes have been reported in *Nicotiana tabacum* (tobacco) (Svab et al. 1990; Svab and Maliga 1993; Koop et al. 1996), *Nicotiana plumbaginifolia* (tex-mex tobacco) (O'Neill et al. 1993), *Solanum lycopersicum* (tomato) (Ruf et al. 2001; Nugent et al. 2005; Wurbs et al. 2007), *Glycine max* (soybean) (Dufourmantel et al. 2004), *Lesquerella fendleri* (bladderpod) (Skarjinskaia et al. 2003), *Gossypium hirsutum* (cotton) (Kumar et al. 2004), *Petunia hybrida* (petunia) (Zubko et al. 2004), and *Lactuca sativa* (lettuce) (Lelivelt et al. 2005; Kanamoto et al. 2006) (reviewed in Koop et al. 2007). Liu et al. (2007) have developed homoplasmic fertile plants of *Brassica oleracea* L. var. *capitata* L. (cabbage).

In the present paper, we report the development of a stable plastid transformation system for eggplant. The development of transplastomic technology may be useful for introducing agronomically and biotechnologically relevant traits into eggplant. The production of transplastomic eggplant with maternal inheritance of the transgene could solve problems related to out crossing between the genetically modified (GM) crops and conventional crops or its wild relatives.

Materials and methods

Plastid transformation vector pPRV111A

Plastid transformation in eggplant was carried out using the pPRV111A vector kindly provided by Pal Maliga (Waksman Institute, Rutgers University). The pPRV111A plastid transformation vector has previously been described in detail (Zoubenko et al. 1994). The plasmid pPRV111A vector contains the *aadA* gene as a selectable marker, and the MCS (multiple cloning site) flanked by *trnV* and *rps7/12* tobacco plastid DNA homologous sequences to target the insertion of the linked transgenes into the plastid genome by homologous recombination. The *aadA* gene encodes resistance to both spectinomycin and streptomycin. The *aadA* gene is expressed from 5′ (P*psbA*) and 3′ (T*psbA*) regulatory regions of the *psbA* plastid gene.

Transformation, selection and regeneration of transplastomic plants

Plants of eggplant (*Solanum melongena* L.) were grown aseptically on agar solidified MS (Murashige and Skoog 1962) medium without any phytohormones. Twenty-five green stem segments (5–7 mm in length) were placed at the centre of a petri-dish containing agar solidified MS medium containing 1.0 mg/l zeatin riboside. In each experiment, repeated thrice, seven petri-dishes were prepared and bombarded with pPRV111A plasmid DNA using the PDS-1000/He Particle Delivery System (Bio-Rad) at 1,100 psi rupture disc pressure and a target distance of 6 cm. For five shots, 10 µl of a 1 µg/µl of plasmid DNA (pPRV111A), 50 µl of 2.5 M $CaCl_2 \cdot 2H_2O$ and 20 µl of 0.1 M spermidine were added sequentially to the 50 µl of 50 mg/ml tungsten particle suspension.

The bombarded stem segments were incubated for 7 days on MS medium containing 1.0 mg/l zeatin riboside and then transferred onto selection medium (MS medium supplemented with 1.0 mg/l zeatin riboside and 300 mg/l spectinomycin) for induction and selection of transformed shoots. The regenerated shoots were sub cultured at intervals of 3 weeks over a period of 9 weeks and finally transferred on MS medium containing 0.5 mg/l zeatin riboside and 300 mg/l each of spectinomycin and streptomycin over a period of 6 weeks. For rooting, the transformed shoots were transferred to half-strength MS medium without any phytohormones. Finally, plants were transferred to a Phytotron, where they grew to maturity and seeds were collected.

Total cellular DNA extraction, PCR and Southern blot analysis

PCR amplification and DNA blot analysis were carried out to verify the integration of the *aadA* gene into the chloroplast genome. Total cellular DNA was

extracted from transplastomic plants and wild-type eggplant leaves based on the CTAB (cetyltrimethyl ammonium bromide) method (Murray and Thompson 1980). A pair of primers [primer P1 (forward): 5′-AACTAAACACGAGGGTTGC-3′ and primer P2 (reverse) 5′-AGTATTAGTTAGTGATCCCGAC-3] was used to detect part of the *16S rDNA/rps7/12* sequence along with the *aadA* gene. One pair of gene-specific primers [primer P3 (forward) 5′-TTAT TTGCCGACTACCTTGGTGAT-3′ and primer P4 (reverse) 5′-ATGAGGGAAGCGGTGATCGCC-3′] was also used to verify the integration of the *aadA* gene. Thirty-five PCR cycles were used for amplification (with denaturation at 94°C for 1 min, an annealing at 58°C for 1 min, and an elongation at 72°C of 2 min and further final extension at 72°C for 10 min), after an initial denaturation step at 94°C for 2 min. The PCR products were electrophoresed on a 1% agarose gel.

Southern blot hybridization was carried out with 10 μg of total cellular DNA digested with *Hind*III (Promega). The digested DNA fragments were separated by electrophoresis at 42 V/cm in a 0.8% agarose gel, before being transferred to a nylon membrane (Hybond N+, Amersham). A 1.78 kb PCR product containing *16S rDNA* and 0.79 kb PCR product of the *aadA* gene were used as a probe for DNA blot hybridization. The PCR product was purified using a PCR purification kit (Qiagen). The probe was labeled with [α-^{32}P]-dCTP using the Random Primer Labeling Kit (Stratagene) and hybridized for 16 h at 65°C with agitation in a hybridization oven. The membrane was washed with 2X SSC + 0.1% SDS, 1X SSC + 0.1% SDS and 0.1X SSC + 0.1% SDS at 60°C. An autoradiogram was obtained after overnight exposure at −80°C.

RNA isolation and RT-PCR

Total RNA was isolated from young leaves collected from the transplastomic plants using RNeasy plant mini kit (Qiagen, USA) combined with an on-column DNase digestion (RNase-Free DNase set, Qiagen Inc.) to ensure DNA-free RNA preparations. DNase-treated RNA samples were tested for genomic DNA contamination by using the minus reverse transcriptase (−RT) controls in parallel to RT-PCR reactions. RT-PCR was performed by using a one-step RT-PCR kit (QIAGEN One-Step RT-PCR Kit). Primers P3 and P4 were used to amplify the *aadA* gene. The amplified PCR products were electrophoresed on a 1% agarose gel.

Genetic stability of plants containing the *aadA* gene

In order to check the transmission of the spectinomycin resistance trait in seed progeny and the stability of the *aadA* gene in transformed plants, the T1 and F1 seed progenies of the transplastomic plants TP3 and TP5 were grown on ½-MS medium containing 300 mg/l spectinomycin. T1 progenies were raised by selfing, whereas F1 progenies were generated by crossing a resistant female parent with a wild-type non transformed male parent. Wild-type seeds were also germinated on ½-MS medium containing 300 mg/l spectinomycin.

Results

Optimization of antibiotic concentration for selection

The most commonly used selectable marker for plastid transformation in higher plants is a bacterial *aadA* gene that encodes aminoglycoside 3″-adenylyltransferase conferring resistance to both spectinomycin and streptomycin. For the development of an efficient protocol for plastid transformation of eggplant, we first standardized an efficient selection system and determined the suitable concentration of spectinomycin and streptomycin. Green stem explants started bleaching at 50 mg/l spectinomycin, 20 days after being placed on selection medium. However, complete bleaching was observed at 300 mg/l spectinomycin, after 35 days of culture. For further screening, this concentration was used to select the transplastomic events.

Recovery of transplastomic plants and their molecular analyses

Plastid transformation in *S. melongena* was achieved by biolistic bombardment of 25 green stem segments per petri plate with plasmid pPRV111A (Supplementary Fig. S1A). After 7 days following bombardment, 15 bombarded stem explants were transferred to a

petri-dish containing MS medium supplemented with 1.0 mg/l zeatin riboside and 300 mg/l spectinomycin (Supplementary Fig. S1B). Spectinomycin-resistant shoot buds appeared from cut ends of the stem explants after a period of 45 days on MS medium supplemented with 1.0 mg/l zeatin riboside and 300 mg/l spectinomycin (Supplementary Fig. S1C). Transplastomic lines were obtained when regenerated shoots were subjected to several rounds of selection (over a period of 9 weeks) on MS medium supplemented with 1.0 mg/l zeatin riboside and 300 mg/l spectinomycin. Homoplasmic transplastomic lines were further confirmed by double selection on MS medium containing 1.0 mg/l zeatin riboside, and 300 mg/l spectinomycin + 300 mg/l streptomycin for 6 weeks (Supplementary Fig. S1D) and grown to maturity in the Phytotron (Supplementary Fig. S1E). The transplastomic plants were fertile and produced seeds (Supplementary Fig. S1F).

Two putative chloroplast transformants were initially identified by PCR. A primer pair P1/P2 was used to amplify part of the *16S rDNA/rps7/12* sequence along with the *aadA* gene to verify the homoplasmic condition of transplastomic plants (Supplementary Fig. S2). A primer pair P3/P4 was also used to amplify the *aadA* gene (Supplementary Fig. S2). The homoplasmic condition of transplastomic plants was verified by PCR analysis and revealed a 3.6 kb fragment in transplastomic plants and a 2.0 kb fragment in wild-type plants using primer pair P1/P2 (Supplementary Fig. S3A). PCR analysis revealed a 0.79 kb fragment amplification of the *aadA* gene using primer pair P3/P4 (Supplementary Fig. S3B). The presence of a 0.79 kb PCR product in two transplastomic lines thus confirmed the integration of the *aadA* gene into the plastid genome, while the wild-type plant did not show any PCR amplification (Supplementary Fig. S3B).

Southern blot analysis was carried out using a *16S rDNA* probe (probe 1) and the *aadA* probe (probe 2) on two transplastomic lines (TP3 and TP5) to verify the homoplasmic condition and transgene integration (Supplementary Fig. S2). Southern blot hybridization analysis shown in the Fig. 1a revealed 5.0 and 7.7 kb fragments in transplastomic and wild-type plants, respectively, when a DNA blot was probed with the probe 1 targeting sequence. Only a 5.0 kb fragment in both transplastomic lines (TP3 and TP5) and no hybridization signal in wild-type non transformed plants was detected when a DNA blot was probed with probe 2 (Fig. 1b).

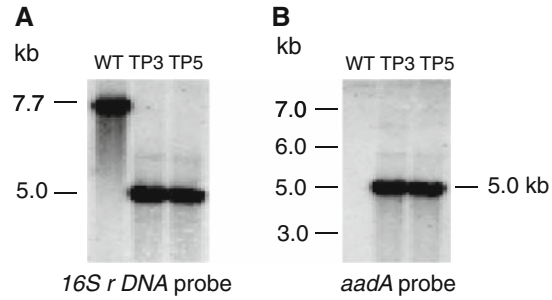

Fig. 1 Southern analysis of two transplastomic plants. Total DNA (10 µg) was digested with *Hind*III. **a** Homoplasmy was examined using *16S rDNA* (Probe 1 as shown in Supplementary Fig. S2) as probe. Transformed plants had one 5.0 kb fragment as expected for homoplasmic transformed plants, whereas the wild-type plant had 7.7 kb fragment. **b** The *aadA* gene-specific probe (Probe 2 as shown in Supplementary Fig. S2) resulted in 5.0 kb fragment in transformed plant, whereas no hybridization signal was detected in wild-type plant. *Lane*-WT: wild-type plant, *Lane* TP3 and TP5: transplastomic lines

In order to test the expression of the *aadA* gene, the transplastomic and wild-type plants were subjected to RT-PCR using *aadA* gene-specific primers. The RT-PCR resulted in an amplification of a 0.79 kb fragment in both transplastomic lines, while no amplification was detected in the wild-type plant (Supplementary Fig. S4).

Genetic stability of plants with spectinomycin resistance trait

Transplastomic plants TP3 and TP5 were transferred to a Phytotron, where they grew to maturity. Seeds were collected following self pollination and crossing the resistant female parent with a wild-type non-transformed male parental plant. The seeds were germinated on spectinomycin containing medium to test whether maintenance of plants under non-selective condition in the Phytotron resulted in the loss of the spectinomycin resistance trait due to elimination of the *aadA* gene. As expected, the seedlings of transplastomic lines were uniformly spectinomycin-resistant (Fig. 2a).

Data on transmission of the spectinomycin resistance trait in seed progeny are summarized in Table 1. Uniform resistance to spectinomycin in selfed seed progeny, and in the cross with the transplastomic plant as the female parent, indicated

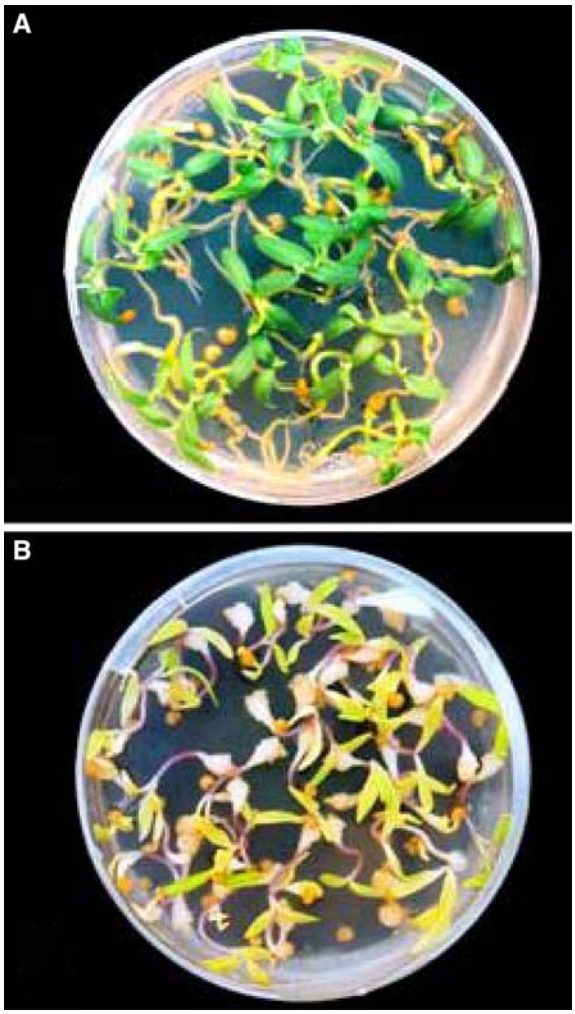

Fig. 2 Screening of seeds for resistance to spectinomycin. **a** Germination of transplastomic T1 seeds on ½-MS medium containing 300 mg/l spectinomycin. T1 seed progeny were generated by selfing. **b** Germination of wild-type seeds on ½-MS medium containing 300 mg/l spectinomycin. The bleaching of seedlings is clearly visible

that integration of the *aadA* marker gene in transplastomic plant was stable. About 500 and 540 seedlings of TP3 transplastomic lines were resistant to spectinomycin, respectively, in the T1 and F1 generations (Table 1). About 480 and 520 seedlings of TP5 transplastomic lines were found resistant to spectinomycin in T1 and in F1 generations, respectively (Table 1). Bleaching was noticed in wild-type seedlings grown on spectinomycin containing medium (Fig. 2b). This suggests that the transplastomic seedlings carried the spectinomycin resistance trait.

Discussion

Plastid transformation in eggplant, an economically important crop plant, represents a major advance in transplastomic research. Taking advantage of the potentially high transgene expression level in the transplastomic plants, this system can now be used to introduce agronomically and biotechnologically relevant traits into eggplant.

In the present investigation, the transgene *aadA* was targeted between *trnV* and *rps7/12* sequence. Homoplasmic transplastomic plants were obtained following several rounds of selection on spectinomycin containing medium. Uniform resistance to spectinomycin in the selfed seed progeny (T1) and in F1 progeny produced by crossing the resistant female parent with a wild-type non-transformed male parent indicates stable integration of the *aadA* gene.

We demonstrated the feasibility of using stem explants to achieve chloroplast transformation in eggplant in contrast to the use of leaf explants in tobacco. The efficiency of plastid transformation in eggplant was lower compared to tobacco. We presume that different transformation efficiencies for eggplant and tobacco could be due to the different explants and vectors used for plastid transformation in these two plant species. In addition, other reasons for lower transformation efficiency in eggplant could be due to a less shoot regeneration frequency (50–55%) from stem explants in eggplant compared to an 80–85% shoot regeneration frequency from leaf explants in tobacco. In eggplant, shoot buds appear from cut ends of the stem explants. We observed that cut ends of the stem explants bear fewer **c**hloroplasts (30–60 chloroplasts per cell) besides the smaller target area as compared to leaf explants (about 100 chloroplasts per cell) that are routinely used for tobacco chloroplast transformation (data not shown), thus lowering the targeting efficiency of particles carrying DNA to the stem explants in the present study. Hou et al. (2003) also observed low chloroplast transformation efficiency in oilseed rape due to smaller and fewer chloroplasts in the cut ends of cotyledon petiole explants, compared with leaf explants used in tobacco transformation. A smaller target area in the oilseed rape explants could also have contributed to the lower transformation efficiency.

Table 1 Transmission of *aadA* gene to the plastid genome of seed progeny as determined by green/white selection of the germinated seedlings on ½-MS medium with and without the antibiotic spectinomycin. T1 progenies were raised by selfing the transplastomic plants, whereas F1 progenies were obtained by crossing antibiotic resistant female parent with a wild-type non transformed male parent

Transplastomic line	Progeny	No spectinomycin		Spectinomycin 300 mg/l	
		Green	White	Green	White
TP3	Self	520	0	500	0
	F1	455	0	540	0
TP5	Self	450	0	480	0
	F1	430	0	520	0

In the wild-type, bleaching was clearly visible in the seedlings when they were grown on ½-MS medium containing 300 mg/l spectinomycin, whereas seedlings were green on media lacking spectinomycin

The plastid transformation method in eggplant is an alternative approach for improving crop performance. The availability of transplastomic technology for transgene expression from the eggplant plastid genome will open up new possibilities for metabolic engineering, and the use of plants as factories for producing biopharmaceuticals. In view of the high capacity of chloroplasts to express and accumulate foreign protein, plants have considerable potential for the production of edible vaccines, antibodies (plantibodies) and therapeutic substances (Bock 2007; Koop et al. 2007). Thus, there is significant interest in developing transplastomic plants as expression factories for biopharmaceuticals, an area commonly referred to as molecular farming (Ma et al. 2005; Daniell 2006; Verma and Daniell 2007; Bock 2007; Koop et al. 2007). For such applications, plastid transformation technology offers solutions to the technical and ecological problems associated with conventional transgenic technologies such as transgene silencing and outcrossing. The technology has also proven useful to achieve high transgene expression levels.

Eggplant is a staple vegetable crop and is extensively damaged by the fruit and shoot borer (*Leucinodes orbonalis*) with losses ranging from 50 to 70%. To reduce pest-linked damage as well as to protect the environment from adverse effects of pesticides, deploying the lepidopteron specific *cry* genes for high level expression of Bt (*Bacillus thuringiensis*) toxins in the plastid genome of eggplant would provide an effective built-in control for fruit and shoot borer. High level of *cry* gene expression can also prove useful for developing insect resistance management strategy. The development of transplastomic Bt eggplant would, hence, eliminate environmental biosafety concerns.

Acknowledgments We would like to thank Dr. Pal Maliga, Waksman Institute, Rutgers University, USA for providing pPRV111A plasmid. We are also grateful to Dr. Randy D. Dinkins, ARS Forage-Animal Production Research Unit, USDA, University of Kentucky, Lexington, USA for critical reading of the manuscript. This work was supported by a grant (9/83(149)2K2/EMR-I) from the Council of Scientific and Industrial Research, New Delhi, India.

References

Bock R (2001) Transgenic plastids in basic research and plant biotechnology. J Mol Biol 312:425–438. doi:10.1006/jmbi.2001.4960

Bock R (2007) Plastid biotechnology: prospects for herbicide and insect resistance, metabolic engineering and molecular farming. Curr Opin Biotechnol 18:100–106. doi:10.1016/j.copbio.2006.12.001

Daniell H (2006) Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. Biotechnol J 1:1071–1079. doi:10.1002/biot.200600145

Dufourmantel N, Pelissier B, Garcon F, Peltier G, Ferullo JM, Tissot G (2004) Generation of fertile transplastomic soybean. Plant Mol Biol 55:479–489. doi:10.1007/s11103-004-0192-4

Fari M, Nagy I, Csanyi M, Mityko J, Andrasfalvy A (1995) *Agrobacterium* mediated genetic transformation and plant regeneration via organogenesis and somatic embryogenesis from cotyledon leaves in eggplant (*Solanum melongena* L. cv. 'Kecskemeti lila'). Plant Cell Rep 15:82–86. doi:10.1007/BF01690259

Filippone E, Lurquin PF (1989) Stable transformation of eggplant (*Solanum melongena* L.) by cocultivation of tissues with *Agrobacterium tumefaciens* carrying a binary plasmid vector. Plant Cell Rep 8:370–373. doi:10.1007/BF00716677

Franklin G, Sita GL (2003) *Agrobacterium tumefaciens*-mediated transformation of eggplant (*Solanum melongena* L.) using root explants. Plant Cell Rep 21:549–554

Grevich JJ, Daniell H (2005) Chloroplast genetic engineering: recent advances and future perspectives. Crit Rev Plant Sci 24:83–107. doi:10.1080/07352680590935387

Guri A, Sink KC (1988) *Agrobacterium* transformation of eggplant. J Plant Physiol 133:52–55

Hanyu H, Murata A, Park EY, Okabe M, Billings S, Jelenkovic G, Pedersen H, Chin CK (1999) Stability of luciferase gene expression in a long term period in transgenic eggplant, *Solanum melongena*. Plant Biotechnol 16:403–407

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

Kanamoto H, Yamashita A, Asao H, Okumura S, Takase H, Hattori M, Yokota A, Tomizawa K (2006) Efficient and stable transformation of *Lactuca sativa* L. cv. Cisco (lettuce) plastids. Transgenic Res 15:205–217. doi:10.1007/s11248-005-3997-2

Koop HU, Steinmuller K, Wagner H, Rossler C, Eibl C, Sacher L (1996) Integration of foreign sequences into the tobacco plastome via polyethylene glycol-mediated protoplast transformation. Planta 199:193–201. doi:10.1007/BF00196559

Koop HU, Herz S, Golds TJ, Nickelson J (2007) The genetic transformation of plastids. In: Bock R (ed) Cell and molecular biology of plastids, Topics Curr Genet, vol 19. Springer-Verlag, Berlin Heidelberg, pp 457–510

Kumar PA, Mandaokar A, Sreenivasu K, Chakrabarti SK, Bisaria S, Sharma SR, Kaur S, Sharma RP (1998) Insect-resistant transgenic brinjal plants. Mol Breed 4:33–37. doi:10.1023/A:1009694016179

Kumar S, Dhingra A, Daniell H (2004) Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. Plant Mol Biol 56:203–216. doi:10.1007/s11103-004-2907-y

Lelivelt CL, McCabe MS, Newell CA, Desnoo 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 JJW, Tseng MJ (2007) Stable chloroplast transformation in cabbage (*Brassica oleracea* L. var. capitata L.) by particle bombardment. Plant Cell Rep 26:1733–1744. doi:10.1007/s00299-007-0374-z

Ma JK-C, Barros E, Bock R, Christou P, Dale PJ, Dix PJ, Fischer R, Irwin J, Mahoney R, Pezzotti M, Schillberg S, Sparrow P, Stoger E, Twyman RM, European Union Framework 6 Pharma-Planta Consortium (2005) Molecular farming for new drugs and vaccines. Current perspectives on the production of pharmaceuticals in transgenic plants. EMBO Rep 6:593–599. doi:10.1038/sj.embor.7400470

Maliga P (2004) Plastid transformation in higher plants. Annu Rev Plant Biol 55:289–313. doi:10.1146/annurev.arplant.55.031903.141633

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325. doi:10.1093/nar/8.19.4321

Nugent GD, Ten Have M, van der Gulik A, Dix PJ, Uijtewaal BA, Mordhorst AP (2005) Plastid transformants of tomato selected using mutations affecting ribosome structure. Plant Cell Rep 24:341–349. doi:10.1007/s00299-005-0930-3

O'Neill C, Horvath GV, Horvath E, Dix PJ, Medgyesy P (1993) Chloroplast transformation in plants: polyethylene glycol (PEG) treatment of protoplasts is an alternative to biolistic delivery systems. Plant J 3:729–738. doi:10.1111/j.1365-313X.1993.00729.x

Rotino GL, Gleddie S (1990) Transformation of eggplant (*Solanum melongena* L.) using a binary *Agrobacterium tumefaciens* vector. Plant Cell Rep 9:26–29. doi:10.1007/BF00232129

Ruf S, Hermann M, Berger IJ, Carrer H, Bock R (2001) Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. Nat Biotechnol 19:870–875. doi:10.1038/nbt0901-870

Sharma RK, Bock R, Bansal KC (2005) Plastid transformation: safer alternative to transgenic plants. Physiol Mol Biol Plants 11:179–185

Skarjinskaia M, Svab Z, Maliga P (2003) Plastid transformation in *Lesquerella fendleri*, an oilseed *Brassicacea*. Transgenic Res 12:115–122. doi:10.1023/A:1022110402302

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. doi:10.1073/pnas.90.3.913

Svab Z, Hajdukiewicz P, Maliga P (1990) Stable transformation of plastids in higher plants. Proc Natl Acad Sci USA 87:8526–8530. doi:10.1073/pnas.87.21.8526

Verma D, Daniell H (2007) Chloroplast vector systems for biotechnology applications. Plant Physiol 145:1129–1143. doi:10.1104/pp.107.106690

Wurbs D, Ruf S, Bock R (2007) Contained metabolic engineering in tomatoes by expression of carotenoid biosynthesis genes from the plastid genome. Plant J 49:276–288. doi:10.1111/j.1365-313X.2006.02960.x

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. doi:10.1093/nar/22.19.3819

Zubko MK, Zubko EI, van Zuilen K, Meyer P, Day A (2004) Stable transformation of petunia plastids. Transgenic Res 13:523–530. doi:10.1007/s11248-004-2374-x