Stable plastid transformation in *Scoparia dulcis* L.

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Abstract In the present investigation we report stable plastid transformation in *Scoparia dulcis* L., a versatile medicinal herb via particle gun method. The vector KNTc, harbouring *aadA* as a selectable marker and *egfp* as a reporter gene which were under the control of synthetic promoter pNG1014a, targets inverted repeats, *trnR/trnN* of the plastid genome. By use of this heterologous vector, recovery of transplastomic lines with suitable selection protocol have been successfully established with overall efficiency of two transgenic lines for 25 bombarded leaf explants. PCR and Southern blot analysis demonstrated stable integration of foreign gene into the target sequences. The results represent a significant advancement of the plastid transformation technology in medicinal plants, which relevantly implements a change over in enhancing and regulating of certain metabolic pathways.

Keywords Particle bombardment · Plastid transformation · *aadA* · *egfp* · *Scoparia dulcis* L.

Introduction

The transgene integration and its expression through plastid transformation offer several significant advantages over nuclear transformation. This includes targeted integration of desired foreign genes via homologous recombination

with avoiding uncontrolled position effects, lack of gene silencing and high levels of transgene expression (Bendich 1987; Daniell 2002; Daniell and Dhingra 2002). The chloroplast transformation was successfully established in a unicellular alga *Chlamydomonas reinhardtii* (Boynton et al. 1988; Daniell et al. 1990) and more often in *Nicotiana tabacum* (Svab et al. 1990). The same technology was further extended in other plant systems like *Solanum tuberosum* (Sidorov et al. 1999), *Brassica napus* (Cheng et al. 2010), *Arabidopsis thaliana* (Sikdar et al. 1998), *Solanum lycopersicon* (Ruf et al. 2001), *Glycine max* (Dufourmantel et al. 2005), *Brassica oleracea* (Nugent et al. 2006), *Lactuca sativa* (Kanamoto et al. 2006), *Oryza sativa* (Lee et al. 2006), *Gossypium hirsutum* (Kumar et al. 2004), *Lesquerella fendleri* (Skarjinskaia et al. 2003) and *Solanum melongena* (Singh et al. 2010).

Scoparia dulcis L., is a valuable medicinal herb of Plantaginaceae with enumerating phytochemical and pharmacological properties and higher regeneration efficiency (Aileni et al. 2008; Latha et al. 2006; Murti et al. 2012) has been chosen for the experiment. Traditionally *S. dulcis* is used as a remedy for diabetes mellitus in India and for hypertension in Taiwan (Edeoga et al. 2005). Phytochemical screening showed that the leaf contains tannins, alkaloids, flavonoids, diterpenoids, triterpenes, hexacosonol, ketone-dulcitone and an antidiabetic compound ammelin (Chow et al. 1974; Edeoga et al. 2005; Satyanarayana 1969). Scopadulciol, scopadulcic acid-B and diacetylscopadiol exhibits inhibitory activity on gastric H⁺-K⁺ ATPase enzyme (Asano et al. 1990). Cytotoxic and antitumor activity has been attributed to diterpenoid, scopadulcic acid-B and flavone, hemenoxin (Hayashi et al. 2006). Hispidulin, a steroidal glycoside isolated using an aqueous extract of *S. dulcis* exhibited anti HIV activity (Osei-Safo et al. 2009). HIV type 1 reverse transcriptase

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inhibitory activity of methanolic extracts of leaf was also reported (Porika et al. 2009).

Stable genetic transformation in *S. dulcis* was achieved through *Agrobacterium* using *uidA* and *hpt* genes (Aileni et al. 2011) and efficient biolistic transformation using *uidA* and *npt* II genes (Srinivas et al. 2016). Development of chloroplast transformation in this plant system will provide new opportunities towards plastid based metabolic engineering of certain valuable natural product entities and to delve this technology towards enhanced metabolite production and extension in engineering of biosynthetic pathways by plastid transformation.

Materials and methods

Plastid vector

KNTc, a plastid transformation vector kindly provided by H. U. Koop, Germany and is built in the following way: PNG1014a—*egfp*—*aadA*—3'*rbcL* (Chl) (Fig. 1) to focus on *Nicotiana tabacum* and possibly related species. Homologous flanks in this vector lead to insertion into the ribosomal operon in the inverted repeats. PNG1014a is a synthetic promoter, and it is based on the ribosomal operon promoter elements for transcription by the nuclear-encoded plastid RNA polymerase (*NEP*) as well as the plastid-encoded plastid RNA polymerase (*PEP*). *egfp* is “enhanced *gfp*”, *aadA* is “aminoglycoside adenyl transferase A”, and 3'*rbcL* (Chl) is the 3' UTR of *rbcL* of *Chlamydomonas reinhardtii* and this is a dicistronic operon containing a

synthetic ribosome binding site upstream of *aadA* (Scharff and Koop 2007; Zou et al. 2003).

Plastid transformation procedure

For biolistic transformation, mature green leaves (1 cm) of *S. dulcis* were harvested from in vitro shoot cultures. The particle delivery system (PDS-1000/He, Bio-Rad) was used for bombardment of leaf explants with gold microcarriers coated with desired plasmid DNA. Plasmid coating on to gold microcarriers was carried out according to prescribed protocol and 7 µl of this suspension was used for bombardment (Dheeraj et al. 2008). The optimized bombardment parameters for stable nuclear transformation with 650 psi pressure and 6 cm of distance from stopping plate to target tissue with fixed vacuum pressure of 25 mm of Hg (Srinivas et al. 2016), was employed for plastid transformation.

Following bombardment, the leaf explants were incubated for two days on MS medium without selection pressure. The leaves were cut into small pieces and shifted to standardized direct regeneration medium of 4 mg/L 6-benzylaminopurine (BAP) and 0.2 mg/L Indole-3-acetic acid (IAA) (Aileni et al. 2008) fortified with inhibitory concentration of 75 mg/L of spectinomycin and it will be the final antibiotic dosage throughout the selection procedure. Resistant shootbuds emerged after 3–4 weeks were subjected to a next round of selection under the same conditions for further proliferation upto 3 weeks. The proliferated shoots were transferred for elongation onto hormonal-free MS medium with same quantity of spectinomycin. After 5–6 weeks of elongation, individual shoots with complete mature leaves, were shifted to half strength MS medium supplemented with 1 mg/L Indole-3-butyric acid (IBA) for rooting, without altering in antibiotic concentration. Rooted plants were shifted to the green house for further analysis.

PCR and Southern blot analysis

Total genomic DNA was extracted from the young leaves of putative transgenic lines and non-transformed control plants following the standard CTAB method (Doyle and Doyle 1990). To confirm the plastid integration of expression cassette, PCR was carried out using specific primers of KNTcHF + KNTcR designed against combinations of flanking sequence i.e.; right homologous recombination region (3' end of INSR) and *aadA* expression cassette. A set of primers KNTcF + KNTcR was used to specify the presence of *aadA* along with 3' UTR of *rbcL*. The presence of the *gfp* in spectinomycin-resistant lines were assessed and analyzed by using set of gene specific primers *gfpF* + *gfpR*.

Fig. 1 Physical map of plastid transformation vector

Oligo name	Sequence (5'-3')
(P1) KNTcHF	CGCCCACAACCGGCCCAAAAGGGAAG
(P2) KNTc F	GGCTCGTGAAGCGGTTATCGCCGAAG
(P3) KNTc R	CAATTCCCGG GTACCGAGTCCACCG
(P4) gfpF	GGTGTAGTACCTATTTTGGTAGAATTGG
(P5) gfpR	ATAACCGCTTCACGAGCCATGGATCTCC

The PCR reaction conditions include initial denaturation step at 94 °C for 5-min, then followed by 30 cycles of 94 °C for 30-s, annealing temperature was 63 °C for the combination of primers P2 + P3 and P1 + P3, 60 °C for the combination of P4 + P5 for 40-s respectively, 72 °C for 150-s and final extension at 72 °C for 5-min. The products were separated by electrophoresis on 0.8 % agarose gel and documented.

Southern blot analysis was carried out to confirm stable chloroplast integration, according to manufacturer's instructions (Roche applied sciences, Germany). About 20 µg leaf total genomic DNA from PCR positive transformants was digested with *Not* I and *Eco* 721 and subjected to electrophoresis on 0.8 % agarose gel and transferred onto a Hybond + nylon membrane. DIG labeled *aadA* PCR product was used as probe to confirm the integration through blot analysis.

GFP detection by fluorescent microscopy

Analysis of transgenic lines for visual detection of *gfp* was carried out using a Nikon Eclipse Ni-E microscope. The stomatal cells of young leaves were magnified under excitation filters ranging between 450 and 490 nm for *gfp* fluorescence and 510–560 nm for chlorophyll autofluorescence with barrier filter ranges at 520 and 590 nm respectively. The wild leaf segments were also observed simultaneously at the same ranges of detection.

Characterization of total leaf protein

About 1 g of mature leaves from control as well as transgenic lines were ground in liquid N₂ with 1 ml of extraction buffer (100 mM KPO₄, 1 mM EDTA, 1 % TritonX-100, 10 % Glycerol, PH-7.8) and centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was recovered as total soluble fraction and separated using 12 % polyacrylamide gel for identification of specific protein pattern stained with coomassie blue.

Results

Evaluation of spectinomycin selection conditions

For developing a proficient transformation method through chloroplast engineering in *S. dulcis*, plastid vector that

Fig. 2 Generation of transplastomic *Scoparia dulcis* L. **a** Multiple shoot induction from leaf explants on regeneration medium without spectinomycin (Wild type). **b** Complete suppression of regeneration on selection medium containing 75 mg/L spectinomycin (Wild type). **c** Initial stage of green shoots after 3-weeks of bombardment. **d** Sorting of green spectinomycin resistant shoots in second round of selection. **e** Elongation of shoots into complete plantlet with roots. **f** Reconfirmation of transgenic nature. **g** Plant transferred into the soil and maintained in the green house

harbors a bacterial *aadA* gene encoding amino glycoside 3'-adenylyltransferase which confers resistance to spectinomycin was used as a selectable marker. We have optimized suitable lethal dose by checking out with different concentration levels of spectinomycin (data not shown). The leaf explants were partially bleached at 50 mg/L where as complete bleaching was observed in 75 mg/L (Fig. 2a, b). For further screening, regeneration medium amended with this concentration was used throughout the selection of resistant transgenic lines.

Recovery and molecular analysis of transplastomic plants

Plastid transformation in *S. dulcis* through bombardment of 75 explants conducted in three individual experiments each time with 25 explants yielded totally six independent resistant lines. Green regenerating shootbuds with partial pale tissue were emerged and recovered initially after three weeks of incubation on direct regeneration medium supplemented with appropriate antibiotic selection. Complete sorting into green shoots occurred after second round of selection in the same medium that are maintained for next

Table 1 Results of plastid transformation experiments in *Scoparia dulcis* L

Experiment	No. explants	Spectinomycin resistant shoots	PCR positives	Southern blot positives
1	25	12	4	2
2	25	10	2	2
3	25	10	2	2

2–3 weeks (Fig. 2c). In the first transformation experiment, 12 out of 25 explants were shown to be resistant against spectinomycin initially in first round of selection, finally only two of them showed complete sorting out into green shoots in the second round (Fig. 2d). Regenerated shoots of these transformed plants were resistant to higher concentration up to 100 mg/L. In the second and third experiments, only two of each 10 spectinomycin resistant lines showed stable integration (Table 1). High regeneration efficiency and its minimal time required to complete sorting out of transforming lines have made the selection system easier under stringent antibiotic pressure. Putative transformants were proliferated and elongated within two months of duration (Fig. 2e). A minor experiment that has been conducted by placing the leaf explants obtained from transformed lines for direct shoot organogenesis on spectinomycin fortified medium with increased concentration upto 100 mg/L, as expected the results showed that the complete green shoots emerged from transformed leaf explants (Fig. 2f), whereas wild-type showed complete bleaching. The reconfirmed lines were also verified for

Fig. 3 PCR analysis of transgene integration. **a** Analysis of the *aadA* gene in transformed *S. dulcis* plants using primers P2 + P3 showing 1.2 kb size amplicon. **b** Confirmation of *gfp* using primers P4 + P5 generating 0.9 kb size amplicon. **c** Analysis of the expression cassette

using primers P1 (anneals to 3' end of INSR) & P3 (anneals to 3' end of *aadA*) showing 2.4 kb size amplicon. Lane 1 DNA marker (1 kb, Fermentas). Lane 2 Wild type plant. Lane 3 & 4 transgenic plants in **a** and **c**. Lane 3–7 transgenic plants in **b**

Fig. 4 Fluorescence analysis of *gfp* expression in transplastomic lines

aadA integration and *gfp* expression (data not shown). The transgenic lines were transferred to soil and maintained in the greenhouse (Fig. 2g).

The foreign gene integration was screened and assessed initially in two transgenic lines by PCR with specific primers (KNTcF + KNTcR) that resulted in amplification of 1.2 kb fragment suggesting the insertion of *aadA* gene (Fig. 3a) and this is further verified by sequencing the product with same primers. Second PCR was carried out to confirm the chloroplast integration of *aadA* in the transplastomes with primer KNTcR + KNTcHF, which allows amplification of 2.4 kb fragment which includes part of right insertional sequence along with *aadA* and *gfp* that apparently suggest the proper integration (Fig. 3c). No consistent banding is observed in wild-type with the above set of primers. The *gfp* specific primers yielded 0.9 kb size amplicon from transgenic lines with absence of consistent banding in wild-type (Fig. 3b). Images from microscopic analysis revealed green fluorescence in stomatal chloroplasts of transformed lines upon blue excitation and where as control wild plants showed red autofluorescence (Fig. 4). Both the results regarding expression of *gfp* were represented as reliable indicators of stable transformation. Analysis of extracted protein as total soluble fraction from transgenic lines generated corresponding signals representing the accumulation of foreign protein at 26 and 29 kDa of expected sizes to *gfp* and *aadA* respectively (Fig. 5b) proving foreign protein presence, same thing was absent in wild type. DNA blot analysis of putative transgenics (results from two samples are shown) has proved the stable integration of the expression cassette using the PCR product as a probe (Fig. 6) that resulted from specific primers related to a combination of flanking sequence and *aadA* expression cassette with detectable hybridization signal at 2.4 kb size (Fig. 5a).

The time period taken for the appearance of spectinomycin resistant *S. dulcis* lines on selection medium was 8 weeks, which is almost similar to tobacco transformation studies. Co-expression of *aadA* with *gfp* has facilitated effective screening of the plastid transformants.

Fig. 5 Southern blot and protein analysis. **a** DNA blot confirmation generating 2.4 kb size hybridization signal. Lane 1 Wild type, Lanes 2& 3 Transgenic plants. **b** Protein analysis corresponding to 26 and 29 kDa of the expected sizes to *gfp* and *aadA* respectively. Lane 1 Marker, Lane 2 Wild type, Lane 3& 4 Transgenic plants

Discussion

Plant metabolic engineering has the potential in expanding the progress in genetic manipulation and considerably focused towards expression of proteins. The ability of the chloroplast to integrate and express foreign sequences as operons makes this site an attractive alternative for genetic manipulations which are coexpressed as polycistronic transcripts and are often processed further into monocistronic mRNAs (Lu et al. 2013). Extending chloroplast transformation in medicinal plants seems to be worth full to demonstrate certain metabolic pathways and plant based

Fig. 6 Physical map of the vector showing primers and probe

product entities. This emphasizes the future novel aspects in characterization of natural plant products that have important biological and pharmaceutical functions (Rohmer et al. 1996).

Here we report, the stable plastid integration of *aadA* and *gfp* into the inverted repeats (*trnR* and *trnN*) regions of *Scoparia dulcis* L., plastid genome using KNTc, a dicistronic, universal chloroplast vector. These regions have been used in chloroplast transformation of tobacco that shows significant levels of protein expression (Huang et al. 2002). Many of intergenic regions were found to be target integration sites via homologous recombination (Maliga 2004) and even though the chloroplast DNA sequences are generally highly conserved at nucleotide sequence level (Sriraman et al. 1998), use of heterologous vector in transformation that would reduce the efficiency in non specific species which may be due to lack of complete homology of targeting sequences and this could be the reason for inefficiency found when plastids of tobacco that have been transformed with *Solanum nigrum* (Kavanagh et al. 1999). The transformation frequency in *S. dulcis* using heterologous vector shows significant similarity with frequency observed in *L. fendleri* (Skarjinskaia et al. 2003) and obviously is more efficient than frequency observed in *Arabidopsis* (Sikdar et al. 1998), which was relatively low as one transplastomic clone for 100 bombarded samples. These results may suggest that similar levels of homology may not be required for the construction and using of species specific plastid transformation vectors if they are closely related, that which was conveyed from potato and tomato transformation with tobacco plastid targeting sequences.

In the present investigation, we demonstrated the recovery of spectinomycin resistant lines of *S. dulcis*, with suitable protocol for selection. A strategy of employing the dicistronic format for co-expression of *aadA* with *gfp* in *Nicotiana tabacum* (Jeong et al. 2004), *Solanum tuberosum* (Sidorov et al. 1999) and also in *Oryza sativa* (Lee et al. 2006) was beneficial for effective screening of the plastid transformants. Based on our data, the synthetic promoter PNG1014a has driven successfully the expression of *gfp* in stomatal chloroplasts and spectinomycin resistance in transplastomic lines, suggests likely that species-specific

promoter is not always necessary for designing and construction of species specific plastid transformation vectors.

We have attempted for the development of efficient chloroplast transformation system in *Scoparia dulcis* L., that may consistently focus on attractive advantages and promising future of plastid based metabolic pathway engineering of this species.

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