Construction of a species-specific vector for improved plastid transformation efficiency in *Capsicum annuum* L.

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Abstract

In the present study, we focused on designing a species-specific chloroplast vector for *Capsicum annuum* L. and finding out its transformation efficiency compared to a heterologous vector. The plastid transformation vector (CaIA) was designed to target homologous regions *trnA* and *trnI* of IR region. A selectable marker gene *aadA*, whose expression is controlled by *psbA* promoter and terminator, was cloned between two flanking regions. A heterologous vector pRB95, which targets *trnFM* and *trnG* of LSC region along with *aadA* driven by *rrn* promoter and *psbA* terminator, was also used for developing plastid transformation in *Capsicum*. Cotyledonary explants were bombarded with stabilized biolistic parameters: 900 psi pressure and 9 cm flight distance, and optimized regeneration protocol (0.7 mg/L TDZ + 0.2 mg/L IAA) was used to obtain transplastomic lines on selection medium (300 mg/L spectinomycin). The *aadA* integration and homoplasmy were confirmed by obtaining 1.2 and 3.7 kb amplicons in CaIA transformants and subsequently verified by Southern blotting, whereas in pRB95 transformants, integration was confirmed by PCR with 1.45 kb and 255 bp amplicons corresponding to *aadA* integration and flanks, respectively. The transformation efficiencies attained with two plastid vectors were found to be 20%, i.e., 10 transplastomic lines in 50 bombarded plates, with CaIA and 2%, i.e., 1 transplastomic line in 50 bombarded plates, with heterologous pRB95, respectively.

Keywords *Capsicum annuum* L. · CaIA · pRB95 · Insertional sequences · *aadA* · Bombardment · Cotyledonary explants

Introduction

Plastid transformation has become a very practical tool that relies on site-specific insertion of foreign sequences by homologous recombination that eventually develops stable homoplasmic transplastomes. Chloroplast transformation has its own unique advantages over nuclear transformation, which includes high-level protein expression (Chiyoda et al.

2007; Lelivelt et al. 2005; Ruf et al. 2001), transgene stacking (Ruf et al. 2001; Staub and Maliga 1995; Wang et al. 2009), transgene containment, lack of position effect, and lack of epigenetic effects (Gottschamel et al. 2013; Maliga 2004).

The first ever high-frequency plastid transformation was successfully established in *Nicotiana tabacum* (Svab et al. 1990) and many other reports revealed its suitability as model experimental plant system for chloroplast transformation (Gottschamel et al. 2013). Apart from tobacco, chloroplast transformation was also reported in other plants such as potato (Sidorov et al. 1999), rapeseed (Cheng et al. 2010), tomato (Ruf et al. 2001), cabbage (Liu et al. 2007), lettuce (Lelivelt et al. 2005), rice (Lee et al. 2006), *Cedrela odorata* (López-Ochoa et al. 2015), scoparia (Muralikrishna et al. 2016), and bitter melon (Narra et al. 2018). However, the development of chloroplast transformation has been extended to many other plant systems, until today, this technology remains challenging and limited to achieve due to

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technical aspects in some species of food and economic crops (Bock 2014).

Proficient vector systems are an important prerequisite for plastid transformation and are designed by identifying transcriptionally inactive regions and regulatory sequences that accelerate the expression of the transgenes (Madanala et al. 2012). Construction of chloroplast vector requires two flanking regions [left (INSL) and right (INSR) flanks], derived from chloroplast genome (Venkatesh and Park 2012), and each flank should be around 1–2 kb in size for improved transformation efficiency (Venkatesh and Park 2012; Wang et al. 2009). In higher plants, 14 targeting sites of LSC and IR regions were mostly suggested to achieve plastid transformation (Maliga 2004). The *trnfM* and *trnG* sites of LSC region were used to design the chloroplast transformation vector in tobacco (Bock and Maliga 1995; Madanala et al. 2012) and tomato (Ruf et al. 2001). In addition, *trnA* and *trnI* fragments from IR region were used to develop new chloroplast transformation vector in tobacco (Bock and Maliga 1995; K Mühlbauer et al. 2002; Staub and Maliga 1992), and other species such as lettuce (Lelivelt et al. 2005), *Marchantia polymorpha* L. (Chiyoda et al. 2007), and *Cedrela odorata* (López-Ochoa et al. 2015). Construction of species-specific plastid vectors is not always necessary, as the plastid genome is very conserved among different plant species, but they will significantly increase transformation efficiency (Maliga 2004; Svab et al. 1990).

The choice of selectable marker genes is significant for successful and efficient plastid transformation and among which aminoglycoside 3'-adenylyltransferase (*aadA*) for spectinomycin resistance is commonly used. A few alternative selectable markers have been extensively employed apart from *aadA* that includes, chimeric *nptII* genes encoding neomycin phosphotransferase, conferring resistance to kanamycin (Carrer et al. 1993), *aphA-6* encoding an aminoglycoside phosphotransferase that confers resistance to kanamycin (Huang et al. 2002), and *cat* gene-encoding chloramphenicol acetyltransferase for chloramphenicol resistance (Li et al. 2011). Very recently, another selectable marker aminoglycoside acetyltransferase (6')-Ie/aminoglycoside phosphotransferase (2'') gene was employed which confers resistance to tobramycin as the replacement of *aadA* in transplastomic selection (Tabatabaei et al. 2017). In engineering native chloroplast genome, the marker gene will be always been side by side to the target gene to decrease the possibility of recombination among the mutant sequence and marker gene (Lutz et al. 2007).

Capsicum annuum L., of Solanaceae family, is one of the major food crops used throughout the world. Although a few advances have been made to improve *Capsicum* through the development of different regeneration and genetic transformation protocols by many researchers (Ellendula et al. 2016; Kothari et al. 2010), its improvement is still protracted. The

present study was mainly aimed to develop a *Capsicum*-specific chloroplast transformation vector to get stable integration and improved transformation efficiency in comparison with the conventional heterologous vector.

Materials and methods

Plastid transformation vectors

Two plastid vectors were used in the present chloroplast transformation studies. The tobacco-based plastid vector, pRB95, that targets large single copy (LSC) of plastome was assessed for its integration and expression. A 1.45 kb cassette consisting of the aminoglycoside 3'-adenylyltransferase (*aadA*), which is under the control of 5' *Prn* promoter and flanked by 3' *psbA* terminator, was inserted in between *trnfM* and *trnG* intergenic regions (Fig. 1b). This plasmid vector is kindly provided by Prof. Ralph Bock, Germany (Ruf et al. 2001). Another vector which is species-specific for *Capsicum*, CaIA was developed here, leads the insertion of *aadA* cassette having *psbA* promoter and terminator towards *trnA* and *trnI* sites of inverted repeat (IR) region (Fig. 2).

Vector construction for *Capsicum* plastid transformation

A capsicum plastid transformation vector, CaIA, was constructed using flanking regions of *Capsicum* chloroplast DNA (Jo et al. 2011). Homologous recombination targeting sequences were chosen and aligned to get common conserved sequences of IR regions from five Solanaceae members having high genome homology (Table 1). The DNA fragments corresponding to the *rrn16-trnI* (INSL) and *trnA-rrn23* (INSR) genes were PCR-amplified from total genomic DNA of *Capsicum annuum* L., using two sets of primers (P1F, P1R, and P2F, P2R), respectively, with suitable PCR conditions for 30 cycles with 94 °C for 3 min, 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 2 min. Distinctive restriction enzyme sites were added to each fragment to facilitate the ligation. The primer pair C1F and C1R were designed to amplify the homologous fragment of *rrn16-trnA* carrying *SacI* and *SacII* restriction sites. Another primer pair C2F and C2R were designed for the amplification of *trnI-rrn23* fragment carrying respective *KpnI* and *SalI* sites. Amplified DNA fragments of the chloroplast homologous region were analyzed on 1% agarose gel, ligated into to T1 vector (Takara) and sequenced for confirmation. The confirmed flanking sequences were ligated individually into the pBluescript (SKII+) plasmid by digesting with respective enzymes and that resulted in

Fig. 1 Schematic representation of two vectors indicating primers and their amplification regions along with utilized probe. **a** Physical map of *Capsicum* chloroplast vector CaIA showing all primers used in the experiment and their amplification regions. C2F and C2R will amplify 1.2 kb size of *rrn16*+*trnI* (INSL) and the same will be used as a probe for southern blotting. *aadA* F and *aadA* R will amplify 1.2 kb size of *aadA* cassette. C1F and C1R amplify 1.3 kb size of *trnA*+*rrn23* (INSR). C1F and C2R amplify 3.7 kb in transplastomes

and 2.5 kb in wild-type plant. Digestion with restriction enzymes *BstEII* and *EcoRI* releases 2.8 kb size (INSL+200 bp of *rrn16* and INSR+100 bp of *rrn23*). **b** Physical map of pRB95 chloroplast transformation vector. *aadA* F and *aadA* R primers amplify 1.2 kb of *aadA* gene cassette. Primers P 70 F and P 70 R will amplify 255 bp in wild-type plant and 1.45 kb in transplastomic lines (1.2 kb *aadA* + 255 bp chloroplast region)

intermediate plastid vectors, i.e., INS1 (pBluescript carrying INSL region) and INSC (pBluescript carrying both INSL and INSR). Ligated plasmids were transformed into DH5 α *E. coli* cells and plated on ampicillin containing medium. Positive colonies were screened by colony PCR using M13 primers and sequenced for proper confirmation and integration. To obtain the complete chloroplast transformation vector, CaIA, the selectable marker gene, i.e., *aadA*, which encodes for aminoglycoside 3'-adenyl-transferase which confers resistance to spectinomycin, was cloned into *ClaI* and *SalI* sites of MCS of INSC to construct the complete vector. The primer pair *aadA* F and *aadA* R were used to amplify the *aadA* gene cassette (controlled by *psbA* promoter and terminator) from pJE NK vector (Prof. Zanmin Hu, IGDB, Beijing, China). All primers and their amplification regions are listed in Table 2. The physical map of the vector shows the complete arrangement of vector elements including expression cassette of the selectable marker.

Establishment of axenic cultures

Seeds of *Capsicum annuum* L. variety G4 were collected from LAM, Guntur (regional agriculture research station). Seeds were sterilized with 0.1% HgCl₂ and inoculated on MS basal medium for raising in vitro cultures. Axenic cultures were maintained on Murashige and Skoog (MS) (Murashige and Skoog 1962) basal medium supplemented with 2% sucrose, and cultures were maintained in the photo-period of 16/8 h under white fluorescent light (65 μ E/m²/s) at 25 $\pm$ 2 $^{\circ}$ C.

Antibiotic sensitivity

Plastid vector with the selectable marker was prerequisite and required to achieve adept transformation method by distinguishing non-transformed lines by gradual sorting out on selection medium. Here, we standardized the optimal levels of spectinomycin concentration at which the cotyledonary

Fig. 2 Schematic representation of CaIA vector construction.

a Wild-type plastid genome map showing *trnI* and *trnA* regions. **b** Ligation of two flanking regions in the pBluescript backbone. **c** *aadA* expression cassette with respective restriction sites. **d** Complete vector map of CaIA

Schematic representation of vector development

Table 1 Solanaceous plants used for the collecting sequencing to design primers for the amplification of flanking regions of *Capsicum annuum*

Name of the plant	Plastome region used for common alignment	Accession number
<i>Lycopersicon esculentum</i>	rrn16-trnI-trnA-rrn23	NC_007898
<i>Nicotiana tomentosiformis</i>	rrn16-trnI-trnA-rrn24	NC_007602
<i>Nicotiana sylvestris</i>	rrn16-trnI-trnA-rrn25	NC_007500
<i>Nicotiana tabacum</i>	rrn16-trnI-trnA-rrn26	Z00044
<i>Solanum tuberosum</i>	rrn16-trnI-trnA-rrn27	NC_007898

explants shows complete bleaching, and likely, it would be the standard parameter of selection pressure throughout the transformation experiments. The 22-day-old in vitro grown cotyledonary explants of *C. annuum* var. G4 were used to

test the antibiotic sensitivity at different concentrations, i.e., 0, 100, 200, 300, and 400 mg/L of spectinomycin.

Transformation of *Capsicum* by biolistic method

In vitro grown cotyledons of *Capsicum annuum* L. were used as the explants for the transformation studies using CaIA and pRB95 vectors. Plasmid DNA of the chloroplast-specific vectors was isolated and coated on 0.6 μ m size gold particle by mixing along with 2.5 M CaCl_2 and 0.1 M Spermidine which can accelerate the DNA to the chloroplast and facilitates the integration (Srinivas et al. 2016; Verma et al. 2008). The whole experiment was carried out using a biolistic apparatus (Biolistic PDS-1000/He Particle Delivery System, Bio-Rad, Hercules, CA). Biolistic genetic transformation of *C. annuum* was employed for the plastid transformation with standardized parameters of 9 cm flight distance, 900 psi pressure by keeping unchangeable 2 cm distance between

Table 2 List of primers and there amplifying regions and size of the amplicon

Primer	Sequence	Amplifying region	Carrying restriction enzyme
P1F	CGCGCCGCGG CCTCTGGCTA CT	1.2 kb rrn16-trnI (INSL)	NA
P1R	GCGCCCCGGG TGCATGCTCC AC		
P2F	CGCGGCGGCC GCCAGATGCT TC	1.38 kb trnA-rrn23 (INSR)	NA
P2R	CGCGGGTACC CTAACCCCAA AA		
C1 F	GCGCCCCGGGTGCATGCTCCAC	1.38 kb trnA-rrn23 (INSR) along with restriction site	Sac I
C1 R	CGCGCCGCGGCCTCTGGCTACT		Sac II
C2 F	CGCGGGTACC CTAACCCCAA AA	1.2 kb rrn16-trnI (INSL) along with restriction site	Sal I
C2 R	CGCGGTTCGACAGCATGCATG AA		KpnI
aadA F	CGCGATCGATAAGCTTCCGATC	1.2 kb aadA along with restriction sites	ClaI
aadA R	CGCGGTTCGACCATGAATAAATG		Sal I
P70 F	GGATTTGGTATAGTTGGCC	255 bp in wild, 1.45 kb in transgenic line	
P70R	CTTGTTTATCTATTAGTTTTTCAGT		

NA not applicable

rupture disc and macrocarrier and 3 mm macrocarrier travel distance. Cotyledons were excised and placed at the center of Petri plate containing a regeneration medium (0.7 TDZ mg/L and 0.2 mg/L IAA) and bombarded with the standardized parameters. Fifty plates for each vector, each plate containing 10 cotyledonary explants were bombarded and cultured for 2 days on the same medium at 25 ± 2 °C, and 16/8 h photoperiod. For the selection of transformants, cotyledonary tissues were made into two pieces and placed on selective *Capsicum* regeneration medium (SCRM: 0.7 mg/L TDZ + 0.2 IAA mg/L along with 300 mg/L spectinomycin). Tissues were subcultured for every 10–15 days on the same SCRM. Resistant shoot buds were developed after 4 weeks of culture and these shoots buds were shifted on to the same media (SCRM) for shoot bud proliferation. Later, proliferated shoot buds were shifted to selective *Capsicum* elongation medium (SCM-MS medium supplemented 1 mg/L zeatin along with 300 mg/L spectinomycin) for elongation; subsequently shifted to half-strength SCM for further elongation. The putative transgenics were maintained under laboratory conditions for further molecular screening.

Molecular analysis of transformed *Capsicum* lines

PCR confirmation

Total leaf DNA was isolated from the spectinomycin-resistant *Capsicum* shoots and wild-type *Capsicum* plant by the CTAB method (Doyle 1991). PCR confirmation of CaIA transgenic lines was done by two sets of primers. The complete *aadA* gene cassette was amplified by the primers *aadAF* (CGCGATCGATAAGCTTCCGATC) and *aadAR* (CGCGGTTCGACCATGAATAAATG) (PCR condition follows for 30 cycles as

95 °C for 3 min, 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1.5 min), whereas the integration (homoplasmic confirmation) of *aadA* gene cassette in between the left- and right-flanking regions was confirmed by C1F and C2R primers (PCR conditions for 30 cycles—95 °C for 3 min, 95 °C for 30 s, 58 °C for 45 s, and 72 °C for 3 min). Amplified PCR products were analyzed on 0.8% agarose gel and documented. For confirmation of pRB95 transplastomic lines, another set of primers pRB70F and pRB70R was used to confirm the integration of *aadA* into flanking plastome regions of *Capsicum*, i.e., *trnFM* and *trnG* (Fig. 1b, Table 2) by performing stabilized PCR conditions: 45 s at 94 °C, 1.5 min at 55 °C, 1.5 min at 72 °C, and 30 cycles.

Southern blot analysis

Homoplasmic nature in *Capsicum* transplastomic lines was confirmed by Southern blotting. About 3 µg of genomic DNA was isolated from wild and transgenic plants and digested with *BstEII* and *EcoRI*. The amplified product of about 1.2 kb INSL segment was labeled with DIG-labeling kit (Roche, USA) and used as a probe for detection. Digested DNA fragments were transferred on to Nylon H+ membrane (Roche, USA) and detected for the hybridized signal with the non-radioactive-labeled probe (Fig. 1a).

Results

Optimization of antibiotic concentration for the screening of transformants

For the development of a proficient protocol for chloroplast transformation in *Capsicum annuum* L., we first optimized an efficient selection system and determined the effective concentration of antibiotic. Initially, at 0 and 100 mg/L of spectinomycin concentrations, cotyledons were regenerated and found to be normal. At 200 mg/L, the green explants turned into pale yellow and regeneration was arrested. Whereas, complete bleaching was observed after 25 days being placed on selection medium at 300 mg/L (Table 3). Therefore, for further screening, regeneration medium amended with 300 mg/L concentration (SCRM) was used throughout the selection of *Capsicum* transplastomes (Fig. 3a).

Construction and confirmation of plastid transformation vector

The plastid vector designed specifically for *Capsicum annuum* L. and targets the expression cassette effectively into *trnI-trnA* region of chloroplast genome by homologous recombination. PCR amplification of a *capsicum* DNA yielded in 1239 bp and 1385 bp fragments that correspond to *rrn 23-trnA* (INSR) and *trnI-rrn 16* (INSL), respectively. Restriction digestion of flanking regions with respective enzymes was ligated into pBluescript SKII+ backbone to construct intermediate vector INSC with right and left borders. In between these two flanking regions, the expression cassette of 1.4 kb (PpsbA-*aadA*-TpsbA) was cloned to form a complete species-specific vector; CaIA and schematic representation of vector development were shown step by step (Fig. 2).

Table 3 Optimisation of spectinomycin concentration for primary screening of transgenic

No. of explants	Conc of anti-biotic	No. of explants survived	Type of response
10	0	10	Green regenerated
10	100	10	Green regenerated
10	200	10	Pale yellow
10	300	0	Black and dead
10	400	0	Black and dead

Recovery of transplastomic lines

For the recovery of the transplastomic line of *Capsicum*, the bombarded cotyledons were cut into 0.5 × 0.5 cm explants that are being placed in the dark for 2 days. The explants were shifted onto Selective *Capsicum* Regeneration Medium (SCRM) for shoot induction along with control. Bombarded explants were subcultured at a regular interval of 10–15 days on the same SCRM (Fig. 3b). 4 weeks after bombardment, shoot bud initiation was observed (Fig. 3c; indicated by arrow), whereas explants in control plates were turned into black and failed to produce shoot buds (Fig. 3c). After repeated subculture on SCRM, these initiated shoot buds were proliferated (Fig. 3d–f). Proliferated shoot buds were turned into rosette structures after 4 weeks being shifted to selective *Capsicum* elongation medium (SCEM) (Fig. 3g, h). These rosette structures were placed on half-strength SCEM. The complete shoot formation was achieved 6 months after bombardment (Fig. 3i); here, most of the rosette structures were failed to develop into shoots. Only two shoots were able to develop from rosette structures on SCEM medium. Subsequently, developed shoots were shifted to MS basal medium along with 300 mg/L spectinomycin for rooting, it took another 3 weeks to establish rooting on the basal medium. Transformation efficiency was calculated as 20%, i.e., 10 transgenic plants were achieved for every 50 bombarded plates, whereas only 2%, i.e., 1 transplastomic line was achieved from every fifty pRB95 bombarded explants and the experiment was repeated thrice (Table 4).

Molecular confirmation

Molecular analysis was carried out for the confirmation of transplastomic lines of *Capsicum* along with wild-type plants. Initially, the spectinomycin-resistant lines of CaIA were screened by PCR with a primer pair; *aadA* F and *aadA* R, which resulted in 1.2 kb amplicon corresponding to Ppsba-*aadA*-Tpsba expression cassette. No reliable banding was observed in wild type with the same set of primers (Fig. 4a). At initial raising stage of transplastomic lines, the heteroplasmic condition has been noticed along with rosettes development, but after repetitive subcultures on selection medium, they turned into the homoplasmic state (Fig. 4b). To confirm the homoplasmic integration, another pair of primers C1F and C2R was used. PCR amplicon of 3.7 kb in transplastomic lines shows an integration of desired *aadA* expression cassette in between two flanking regions and 2.5 kb corresponding to left- and right-flanking regions (*trnI* and *trnA*) in wild type (Fig. 4c, d). PCR confirmation of transplastomic lines of pRB95 was carried out with another primer set P70F and P70R that resulted in 1.45 kb amplicon corresponding to an integration of *aadA* cassette along with

Fig. 3 Recovery of transplastomic lines from transformed *Capsicum* cotyledonary explants. **a** Control explants on selection on selective capsicum regeneration medium (SCRM: 0.7 mg/L TDZ + 0.2 IAA mg/L along with 300 mg/L spectinomycin). **b** Bombarded explants on SCRM. **c** Initiation of shoot bud (indicated by arrow) from transformed *Capsicum* cotyledonary explants after 4 weeks on SCRM after 4 weeks. **d–f** Proliferation of putative transplastomic shoot buds on SCRM. **g, h** Elongation of rosette structures of transplastomic lines on selective capsicum elongation medium (SCEM: 1 mg/L Zeatin along with 300 mg/L spectinomycin). **i** Elongated shoot on half-strength SCEM

Table 4 Transplastomic lines from three independent experiments of *Capsicum* cotyledons bombarded using pRB95 and CaIA vectors

No. of experiment	Bombarded plates	Putative transplastomes	PCR positive
1a	50	2	1
2a	50	3	1
3a	50	2	1
4b	50	11	10
5b	50	12	10
6b	50	12	10

a: pRB95 vector, b: CaIA vector

flanking regions. Whereas wild-type *Capsicum* resulted in 255 bp amplified fragment for flanking regions (Fig. 5).

The proper integration was verified further in PCR-positive CaIA transgenic plants by Southern blot analyses, deploying 1.2 kb labeled INSL amplicon as a probe. The results showed that wild type one had hybridization signal at the position of 2.8 kb, whereas in transplastomic lines, it is found to be at about 4.0 kb position (Fig. 4e). These results demonstrated that the transgenic plants that carried the cassette incorporated into predicted specific sites of the chloroplast genome.

Fig. 4 PCR and Southern blotting confirmation of CaIA *Capsicum* transplastomic lines. **a** Transplastomic lines (1 and 2) showing 1.2 kb *aadA* amplicon and the same amplicon was absent in wild-type plant by *aadA* F and *aadA* R primers; M—marker, Wt—wild-type plant. **b** Heteroplasmic lines (1 and 2) showing 2.5 flanking regions and 3.7 kb amplicon, i.e., 1.2 kb *aadA* integration along with 2.5 kb flanking regions; Wt—wild-type plant showing only 2.5 kb amplicon of flanking regions using primer C1F and C2R; M—marker. **c** Confirmation of homoplasmic lines (1 and 2) indicating 3.7 kb (2.5 kb flanks + 1.2 kb *aadA*) using primer C1F and C2R; Wt—indicating only 2.5 kb of flanks and absence of 1.2 kb *aadA*; M—marker. **d**

Confirmation of homoplasmic lines (1–5) indicating 3.7 kb (2.5 kb flanks + 1.2 kb *aadA*) using primer C1F and C2R; Wt—indicating only 2.5 kb of flanks and absence of 1.2 kb *aadA*; M—marker. **e** Southern blotting confirmation of CaIA transformed *Capsicum* homoplasmic lines. 1 and 2—confirmation of homoplasmic nature indicating 4 kb size including 3.7 vector flanking regions and *aadA* gene along with 300 bp of *rrn16* and *rrn23* regions (beyond vector flanking regions); Wt—wild-type plant showing 2.8 kb flanking regions (2.5 kb vector flanking regions + 300 bp of *rrn16* and *rrn23* beyond vector regions) using dCTP 1.2 kb INSL probe

Fig. 5 PCR confirmation of pRB95 *Capsicum* transplastomic lines. *M* marker, *Wt* wild-type plant showing 255 bp, *1*, *2*, *3* and *4* transplastomic lines showing 1.45 kb size (1.2 kb *aadA* along with 255 bp flanking regions) using P70 F and P70 R

Discussion

Plastid transformation offers a remarkable potential approach for the improvement of agricultural, pharmaceutical, and biotechnological traits in plants with higher expression levels and this report will be the first description on generating stable transplastomic pepper. Chloroplast transformations generally make use of homologous recombination for foreign gene integration by replacing respective border sequences. In this work, we focused on developing improved plastid transformation protocol in *Capsicum annuum* L., by designing species-specific vector that targets IR regions and its transformation frequency was analyzed comparatively with a tobacco-based heterologous vector that targets LSC region.

In this study, the development of species-specific plastid transformation vector was initiated by isolating homologous flanking regions, *trnA* and *trnI* directly by PCR amplification from *Capsicum annuum* L., cultivar G4 with the primers designed based on the chloroplast genome sequences of five closely related plastid sequences of Solanaceae members. This was the same method employed successfully for the construction of plastid vectors in rapeseed and carrot (Cheng et al. 2010; Kumar et al. 2004). Similarly, this kind of approach was also accomplished in the diatom, *Phaeodactylum tricornutum* (Xie et al. 2014) using primers designed on the basis of chloroplast genomes of ten different diatoms.

Every time, species-specific plastid transformation vectors are not necessary to achieve plastid transformation and this could be done by use of heterologous vectors up to a possible extent in attaining plastid transformants successfully. On this point of view, successful plastid transformation in potato, tomato, and scopolia has been achieved with tobacco plastid genome vectors (Muralikrishna et al. 2016; Ruf et al. 2001; Sidorov et al. 1999). However, there could

be a chance for a drastic decrease in transformation efficiency with heterologous vectors (Lutz et al. 2007; Skarjinskaia et al. 2003). In *Arabidopsis*, potato, and tomato, the transformation efficiency was very low when compared with tobacco, i.e., one transplastomic line per 40 bombarded plates, one in 35, and one in 20 bombarded plates, respectively. Even in tobacco transformation, efficiency was also influenced and dropped down to very low when heterologous vector containing flanking sequences of *Petunia* (DeGray et al. 2001) was used. Similar kind of results was observed in our experiments when tobacco-based vector (pRB95) was employed to achieve plastid transformation in *Capsicum*, with a quite low transformation efficiency, i.e., one positive plant from 50 bombarded plates. This may be due to the lack of complete homology of targeting sequences (Kavanagh et al. 1999).

We found that among many target sites used for plastid transformation, the foreign gene inserted at the inverted repeat region showed highest expression levels; probably, this might be because of the location of *oriA* within these sequences. The *trnA* and *trnI* flanking regions were used in plastid transformation successfully in other plants such as cotton, carrot, rapeseed, rice, and cabbage (Cheng et al. 2010; Kumar et al. 2004; Lee et al. 2006; Liu et al. 2007). The newly developed vector, CaIA, with these sites has shown its remarkable identity in transformation frequency with that of the heterologous vector, pRB95 having *trnFM* and *trnG* as flanking regions of large single copy region. The two transformation vectors, CaIA and pRB95, whose *aadA* cassettes were controlled by tobacco plastid regulatory elements. Similar usage of *aadA* cassettes controlled by tobacco-based elements was seen in the transformation of *Arabidopsis* and tomato (Ruf et al. 2001). The *aadA* cassette with tobacco-controlled elements (*rrn* promoter-*aadA-psbA* terminator) acted as the selection marker in plastid transformation of *Capsicum* by the heterologous vector, pRB95. Based on the resources available and apart from our data, it probably seems that a species-specific promoter is not always essential for the construction of plastid transformation vectors.

Comparatively, the transformation efficiencies found in our experimental data are different from both the vectors. Ten positive transplastomics were obtained from 50 bombarded plates using species-specific plastid (CaIA) vector. It resulted in an increase in transformation efficiency, i.e., about tenfolds, with that of the heterologous vector (pRB95), whereas it is found to be one positive transplastomics out of 50 bombarded plates. Even though the regeneration efficiency is low in *Capsicum*, homologous regions of vector helped to achieve improved transformation efficiency. Similarly, improved plastid transformation efficiency was achieved, i.e., 1–2 transplastomes per bombardment in potato, soybean, carrot, and cotton when

heterologous flanking regions were replaced by homologous flanking regions in respective plants (Dufourmantel et al. 2004; Kumar et al. 2004; Valkov et al. 2011). Apart from its own homologous regions of *Capsicum*, the vector CaIA also contains a different promoter (*PpsbA*) and insertion site (*trnA-trnI*), compared to the vector pRB95, these differences may be related to the efficiency of transformation.

Successful establishment of transformation relies on the ability of efficient plant regeneration. In recalcitrant species such as *Capsicum*, regeneration is highly dependent on genotypic nature which is major obstacle to develop a standard regeneration protocol for all cultivars (Balázs et al. 2008; Kothari et al. 2010; Kumar et al. 2012). Several researchers reported organogenesis by employing BAP in combination with IAA using cotyledonary explants (Agrawal et al. 1989; Arroyo and Revilla 1991; Joshi and Kothari 2007; Sanatombi and Sharma 2008). Apart from BAP, TDZ in combination with IAA also initiated enhanced organogenesis in *Capsicum* (Ellendula et al. 2016; Kasula et al. 2008; Venkataiah et al. 2003). Recent findings on *Capsicum* regeneration demonstrated that the addition of AgNO₃, CuSO₄, GA₃, CoCl₂, and Zeatin along with regenerative hormonal combination increased regeneration efficiency by promoting shoot elongation (Gammoudi et al. 2018; Kothari et al. 2010; Kumar et al. 2012; Li et al. 2003; Mythili et al. 2017; Sharma et al. 2008). This improved regeneration efficiency might trigger the recovery of homoplasmic transgenic plants in pepper.

In conclusion, the species-specific vector of *Capsicum* was helpful to increase transformation efficiency at about tenfold increase in comparison with the frequency attained with the heterologous vector. This plastid transformation method may serve as a basis for future to introduce certain agronomically important useful genes into *Capsicum* for its improvement.

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Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interests.

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