Construction of chloroplast transformation vector and its functional evaluation in *Momordica charantia* L.

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Abstract

Chloroplast transformation vectors require an expression cassette flanked by homologous plastid sequences to drive plastome recombination. The *rrn16-rrn23* plastome region was selected and using this region, a new species-specific plastid transformation vector CuIA was developed with pKS⁺II as a backbone by inserting the *rrn16-trnI* and *trnA-rrn23* sequences from *Cucumis sativus* L. An independent expression cassette with *aadA* gene encoding aminoglycoside 3'-adenylyltransferase with *psbA* controlling elements is added into the *trnI-trnA* intergenic region that confers resistance to spectinomycin. An efficient plastid transformation in bitter melon (*Momordica charantia* L.) was achieved by bombardment of petiole segments. The frequency of transplastomic plants yielded using standardized biolistic parameters with CuIA vector was two per 15 bombarded plates, each containing 20 petiole explants. Integration of *aadA* gene was verified by PCR analysis in transplastomes. Transplastomic technology developed may be a novel approach for high level expression of pharmaceutical traits.

Keywords Chloroplast transformation vector · Expression cassette · Flanking regions · Plastome sequence

Introduction

Over the years, the advances in chloroplast transformation technology in higher plants have revolutionized the investigation on regulatory mechanisms of gene expression at all possible levels. The high degree of genome conservation has extended the plastid genetic engineering to various economically important agriculture and horticulture crops (Clarke and Daniell 2011; Maliga and Bock 2011; Bock 2014). This platform also has become a powerful tool for basic research in plastid biogenesis and construction of suitable expression cassettes for stable expression of heterologous proteins in chloroplasts (Ruhlman et al. 2010). The key step in the development of species-specific chloroplast transformation vector is determined by the selection of site for intergenic insertion sequences, flanking the marker gene and the gene of interest (Wang et al. 2009). So far only a few of these sequences were made convenient for cloning

and constructing vectors, especially that targets large single copy region (LSC) and inverted repeats (IR) regions (Maliga 2004).

The foreign gene inserted at the inverted repeat region of the chloroplast genome showed highest expression levels (Cosa et al. 2001), probably because *oriA* located within the *trnI* flanking region that facilitates replication of foreign genes within the chloroplasts and any gene inserted into one of the repeats is rapidly copied into the second repeat copy by gene conversion (Lugo et al. 2004). Hence, the *trnI/trnA* intergenic space regions were mostly selected for construction of chloroplast vector. The interspecies sequence diversity has set positive effect on high transformation efficiency using homologous flanking sequences rather than limiting the concept of using a universal chloroplast transformation vector containing heterologous sequences (Verma and Daniell 2007). The use of heterologous plastid sequences derived from *Nicotiana tabacum* that targets principle sites of integration shows comparable transformation frequencies in various plants like cotton, potato and petunia (Wang et al. 2009). Probably, this could be the answer for the necessity of not constructing specific vector for wide range of species which are closely related at genomic level. However, these events will be low efficient when compared to transformation

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with species-specific vectors (Kavanagh et al. 1999). The feasibility and function of species-specific chloroplast transformation were studied indeed in different regions of plastid genomes that include inverted repeat region (*trnI/trnA*) in *Brassica napus* (Cheng et al. 2010) and *oryza sativa* (Lee et al. 2006) and large single copy regions in *Lactuca sativa* L. (*rbcL/accD*) (Kanamoto et al. 2006).

At the preface with the development towards constructing specific chloroplast vector, a successful attempt was done for future execution of transplastomic technology by constructing plastid-specific vector with available *Cucumis sativus* L., plastid genome sequence. Similar work has been carried out in constructing species-specific plastid vector in timber tree, *Cedrela odorata* L. (Lopez-ocha et al. 2015). This may even open up the exciting possibilities for novel gene introduction and its expression in the engineered plastome of all possible and closely related plant systems.

Bitter melon (*Momordica charantia* L.) is an important cucurbitaceous vegetable, medicinal and horticultural crop widely cultivated in many tropical and subtropical areas of Asia (Grover and Yadav 2004). The development of novel techniques enhances the potential applications of genetic engineering in the fields of medicine, agriculture and industries. Very few reports regarding genetic improvement of bitter melon have been achieved through *Agrobacterium*-mediated transformation of immature cotyledonary nodes (Sikdar et al. 2005) and leaf discs (Thiruvengadam et al. 2012). However, an efficient protocol for biolistic-mediated genetic transformation of bitter melon using petiole segments was also reported (Muralikrishna et al. 2018). Since the plastids are maternally inherited, engineering of the plastid genome is an attractive alternative to nuclear transformation in achieving successful desired traits.

The unique advantages from plastid transformation were significant in carrying out the particular relevance to future applications in agriculture through genetic engineering. These include a high level of transgene expression and absence of epigenetic transgene effects (Maliga 2004). Despite its numerous advantages and applications, the plastid transformation technology has been extended to several higher plants and succeeded in generating homoplasmic fertile transplastomes. A number of selectable markers are employed in attaining stable plastid transformants that includes the *aadA* gene (encodes aminoglycoside 3'-adenylyltransferase) that confers resistance to spectinomycin (Goldschmidt-Clermont 1991). Other selectable marker genes used in plastid transformation are *nptII* (Carrer et al. 1993), *codA* (Serino and Maliga 1997), *bar* (Iamtham and Day 2000), *EPSPS* (Ye et al. 2001), *gfp* (Khan and Maliga 1999), and *uidA* (Staub and Maliga 1995).

In the present investigation, we describe designing and construction of species-specific chloroplast transformation vector for *Cucumis sativus* L., to target the inverted repeat

regions for attaining stable plastid transformation system. The efficient functionality of this newly developed vector was evaluated in bitter melon, an important cucurbitaceae species with high regeneration ability. Establishment of transplastome technology in bitter melon may bring the advantages of plastid engineering which can improve the trait characteristics of this plant in showing high level of gene expression and maternal inheritance.

Materials and methods

Elements of chloroplast expression vector

The total genomic DNA was extracted from the leaf tissues of *Cucumis sativus* L. according to the described protocol of Doyle (Doyle and Doyle 1987) and used as template for amplification of selected flanking sequences. From the available nucleotide sequence data of the *Cucumis sativus* L. cv. Baekmibaekdadagi (Accession no: DQ119058) (Kim et al. 2006), vector targeting inverted repeat regions (IRa and IRb) of chloroplast genome specifically *rrn16-trnI* and *trnA-rrn23* was selected as insertion targets for homologous recombination. The cucumber plastid DNA sequences spanning nucleotides 135741–137241 and 137242–138742 of plastid genome were selected as flanking regions, i.e., *rrn23-trnA* and *trnI-rrn16*, respectively. The independent expression cassette is introduced into the *trnI-trnA* intergenic region that consists of the spectinomycin-resistant gene *aadA*, flanked by both *psbA* promoter and terminator of tobacco plastid genome (*PpsbA:aadA:TpsbA*). pKS⁺II plasmid was used as the backbone to carry the elements necessary for site-specific integration (Fig. 1).

Cloning of homologous flanking sequences

The chloroplast-specific regions *rrn16-trnI* and *trnA-rrn23* of *C. sativus*, were amplified from total genomic DNA using primers that are designed on the basis of the available sequence data of chloroplast genome. As well, suitable restriction sites were introduced to facilitate the gene assembly.

Insertion of restriction cut sites into flanking sequences

To clone the *rrn16-trnI* region, a PCR was set up using 10 pM of each primer, TISacI F and TISacII R, which carries the restriction sites *SacI* and *SacII*, respectively. PCR mixture was prepared with 20 ng of *C. sativus* genomic DNA, 2.5 mM dNTPs, 10 × amplification buffer, 2 mM MgCl₂, and 1 U *Taq* DNA polymerase for a final volume of 25 µL. The thermal cycler program consisted of 1 cycle at

Fig. 1 Schematic representation of plastid vector construction. **a** Physical map of inverted repeat region of *C.sativus* chloroplast genome used for vector construction. **b** Flanking sequences *trnI/trnA* genes with respective restriction sites at their cut ends inserted into pKS⁺II. **c** *aadA* expression cassette having promoter *PsbA* and terminator *PsbA*. **d** Chloroplast transformation vector carrying a selectable marker with two flanking regions

95 °C for 3 min, 35 cycles at 95 °C for 40 s, 55 °C for 1 min, 72 °C for 3 min, and a final cycle of 72 °C for 5 min. The resulting amplicon is column purified and digested with *SacI* and *SacII* and ligated independently into the pKS⁺II vector, which was already digested with same enzymes. Ligations were performed with a 2:1 insert: vector ratio with 2 U of *T4 ligase* in a final volume of 20 µL. Then, 10 µL each ligation reaction was added to 100 µL of competent DH5α *Escherichia coli* cells for standard thermal shock transformation followed by selection on LB media plates supplemented with 40 mM isopropyl β-D-1-thiogalactopyranoside, 80 mg/mL X-gal and 75 mg/L ampicillin and the resultant plasmid were named as pLI1. To introduce the *rrn23-trnA* region

in pLI1, primers pair *TAsalI* F and *TAKpnI* R, carrying the *SalI* and *KpnI* restriction sites, respectively, were used (Table 1). Polymerase chain reactions, ligation and selection were performed as discussed before. The resulted plasmid named it as pLIA2 having *trnI-trnA* intergenic regions, carrying *SacII* and *KpnI* at the ends. All the recombinant colonies were verified by colony PCR, restriction digestion and sequencing at the end.

Expression cassette design

An expression cassette containing 5' and 3' UTR sequences is commonly used to regulate high levels of chloroplast

Table 1 Name, primer sequence, orientation, and target gene are shown

Name	Primer sequence (5'–3')	Orientation	Restriction sites added	Target
TISacI F (P1)	CGCGGAGCTCTAACTCCAAAAACC	FW	<i>SacI</i>	<i>trnI-rrn16</i>
TISacI R (P2)	CGCGCCGCGGTGCTTCTTCTATTC	RV	<i>SacII</i>	<i>trnI-rrn16</i>
TASalI F (P3)	CGCGGTCGACTCTGACTCCTTCAT	FW	<i>SalI</i>	<i>rrn23-trnA</i>
TAKpnIR (P4)	CGCGGGTACCGTTCTTTTCACCCT	RV	<i>KpnI</i>	<i>rrn23-trnA</i>
TaadAF (P5)	CGCGATCGATAAGCTTCCGATC	FW	<i>ClaI</i>	<i>aadA</i>
TaadAR (P6)	CGCGGTCGACCATGAATAAATG	RV	<i>SalI</i>	<i>aadA</i>
M13F	GTAAACGACGTCCAGT	FW	–	MCS
M13R	CAGGAAACAGCTATGAC	RV	–	MCS

Letters in bold underlined in the sequence of primers, TISacIF to M13R correspond to nucleotides introducing new restriction sites

expression of structural genes. Thus, the *psbA* promoter was used to drive constitutively the expression of the *aadA*, which encodes aminoglycoside adenine transferase that confers resistance to spectinomycin, followed by the regulatory element *psbA* terminator.

Insertion of restriction enzyme sites at the end of *aadA* gene

The *aadA* from its carrying vector, which contains *psbA* promoter and terminator, is amplified by 1 µL of each primer, TaadA F and TaadA R (10 pmols), and is capable of inserting *ClaI* and *SalI* cut sites, respectively, at the end of terminals. PCR was performed using 30 µL of reaction mixture containing 1 µL (1 unit per reaction) of *Taq* DNA polymerase, 2 µL of 10 × PCR buffer, 1 µL of 2.5 mM dNTPs, 1 µL of extracted DNA (0.5 ng) and 23 µL of milliQ water. PCR conditions carried out for amplification consist of an initial denaturation step at 95 °C for 3 min, 40 cycles of 1 min at 95 °C, 40 s at 55 °C and 2 min at 72 °C, followed by final extension of 10 min at 72 °C. This amplified PCR product of *aadA* was used to clone as a selectable marker in between the flanking sequences of the chloroplast transformation vector that results in a new species-specific vector for *C. sativus*, which was named as CuIA.

Evaluation of spectinomycin selection conditions

For developing a proficient transformation method through chloroplast engineering in *Momordica charantia* L., plastid vector with selectable marker was required to achieve uniform transformation by distinguishing and gradual sorting out of nontransformed lines. Here, we standardized the optimal and suitable lethal dose by checking out with different concentration levels of spectinomycin on petiole explants. The concentration at which the petioles were completely bleached would be the standard parameter of selection pressure.

Transformation and selection of transformants

The standardized bombardment parameters, i.e., a flight distance of 6 cm and 650 psi acceleration pressure, were employed in attaining efficient plastid transformation from petiole explants (Muralikrishna et al. 2018). Bombarded explants were transferred to selection medium after 2 days of incubation on regeneration medium. Growth regulators for efficient regeneration were optimized with 8.9 µM BAP along with 1.14 µM IAA and 0.34 µM GA₃ (Yashodhara et al. 2016). In addition to growth regulators, a selection pressure of spectinomycin was included in the medium and the tissues were regularly subcultured for every 2 weeks for the generation of putative transformants.

Polymerase chain reaction

PCR amplification was carried out to verify the integration of the *aadA* gene into the chloroplast genome. The total cellular DNA was extracted from transplastomic plants and wild-type bitter melon leaves by CTAB method (Doyle and Doyle 1987). One pair of gene specific primers, (P5) TaadAF (forward) 5'-CGCGATCGATAAGCTTCCGATC-3' and (P6) TaadAR (reverse) 5'-CGCG GTCG ACCA TGAAT AAATG -3' was used to verify the integration of *aadA* gene. A second set of primers, TAKpnIR (forward): 5'CGCGGGTACCGTTCTTTTCACCCT-3' and TISacIIR (reverse) 5'-A CGGCGCGCGGTGCTTCTTCTATTC-3' was used to detect two flanking sequences along with the *aadA* gene. Amplification reactions were carried out with an initial denaturation step at 94 °C for 2 min followed by 35 PCR cycles used for amplification with denaturation at 94 °C for 1 min, an annealing at 55 °C for 1 min for *aadA* and 58 °C for integration primers, and an elongation step at 72 °C of 2 min and further final extension at 72 °C for 10 min. The PCR products were electrophoresed on 1% agarose gel.

Results

Amplification of cucumber *trnI* and *trnA* sequences

The flanking regions, *rrn16-trnI* and *trnA-rrn23*, were amplified as 1.530 and 1.570 kb fragments with primers sets P1/P2 and P3/P4, respectively (Fig. 2a).

Ligation and confirmation of flanks into pKS⁺ vector

The ligated plasmid with targeting site, *trnI* of *Cucumis sativus* L., (pLI1), is subjected to double digestion with respective enzymes *SacI* and *SacII* that resulted in generation of 1.5 and 3 kb fragments (Fig. 2c, d). The plasmid with second targeting region, *trnA* (pLIA2), is double digested with *SalI* and *KpnI* that resulted in 1.5 and 4.2 kb fragments (Fig. 2e, f).

Amplification and ligation of *aadA*

Using P5 and P6 primers, *aadA* is amplified as 1.16 kb PCR product (Fig. 2b). According to vector plan, the *aadA* sequence results confirmed that the presence of *ClaI* and *SalI* restriction sites at the cut ends of *aadA*. The confirmed *aadA* fragment was digested with respective restriction enzymes and further it was used to ligate into the pBluescript carrying confirmed flanking sequences of *trnA* and *trnI*. Confirmation of *aadA* in between two flanks was confirmed by PCR that resulted in 4.2 kb amplicon with primers M13 F and M13 R. The orientation of *aadA* in between two flanking inserts, *trnA* and *trnI*, of plastid-specific vector was confirmed by restriction digestion (Fig. 2g, h).

CuIA plasmid assembly and confirmation of vector

The CuIA vector was designed for plastid transformation of *Cucumis sativus* L., and related species, that specifically targets inverted repeats and was built in the following way: *rrn23-trnA*—*PpsbA:aadA:TpsbA*—*trnI-rrn16* (Fig. 3a). The flanking genes, *rrn23-trnA* and *trnI-rrn16* used in cloning, were confirmed by sequencing analysis of final plasmid, CuIA (Fig. 3b, c).

Optimization of antibiotic concentration for selection

For the development of an efficient protocol for plastid transformation of *Momordica charantia* L., we first standardized an effective selection system by determining the suitable concentration of spectinomycin antibiotic. Initially at 250 mg/L, the green petiole explants were partially

bleached, whereas complete bleaching was observed at 300 mg/L, 35 days after being placed on selection medium. For further screening, regeneration medium amended with this concentration was used throughout the selection of resistant transgenic lines.

Recovery of transplastomic plants

Plastid transformation in *M. charantia* was achieved by biolistic bombardment of 20 green petiole segments per petriplate with gold coated CuIA plasmid (Fig. 4a). After 2 days following bombardment, the explants were transferred to regeneration medium supplemented with 300 mg/L spectinomycin (Fig. 4b). Spectinomycin-resistant shoot buds appeared from cut ends of the petiole explants after a period of 6–7 weeks (Fig. 4c). Explants were regularly subcultured on selection medium for every 2 weeks. After 2 months, shoots of 2 cm length from putative transformants were cultured on same medium for further elongation with same concentration of spectinomycin (Fig. 4d). The regenerated shoots were subjected to several rounds of selection to generate homoplasmic cells lacking residual copies of wild-type plastid genome at a frequency of two per fifteen bombarded plates with 20 explants each (Table 2). Well-developed transgenic lines were maintained on selection medium for further molecular screening.

Molecular analysis of plastid transformants

The putative plastid transformants were initially identified by PCR (Fig. 5). Amplification of *aadA* gene using primer pair *TaadAF/TaadAR* confirmed its presence as 1.1 kb PCR product in transplastomic lines, while the wild-type plants did not show any amplification (Fig. 6a). The homoplasmic condition of transplastomic plants was verified as a 4.2 kb size amplicon in transplastomic plants and a 3.0 kb fragment in wild-type plants using primer pair *TAKpnIR/TISacIIR* (Fig. 6b).

Discussion

The applications of species-specific plastid vector have been extended successfully to various valuable plant species to express the foreign proteins in chloroplasts as an alternative to existing universal vectors. In the present investigation, we explained the cloning of inverted repeated regions, *trnI/trnA* from *C. sativus* and construction of a pKS⁺-based chloroplast transformation vector: CuIA containing *aadA* expression cassette with tobacco plastid regulatory elements. Similar consecutive studies for transformation of foreign gene cassette controlled by tobacco plastid regulatory elements were also used in the

Fig. 2 CuIA plasmid assembly and confirmation of vector. **a** Amplified products of *trnA* (lane: 1) and *trnI* (lane: 2) flanking sequences with 1.570 and 1.530 kb sizes, respectively. **b** Amplified product of *aadA* (lane: 2) with 1.1 kb size. **c** Double restriction digestion of *trnI* flank (pLI1), Lane: 1 Undigested pKS + plasmid, Lane: 2 Restriction digestion of pKS + plasmid with *SacI* resulting in 3.0 kb fragment, Lanes: 3 & 4 Double restriction digestion of *trnI* flank with *SacI* and *SacII* resulted in 3.0 & 1.5 kb fragments. **d** Colony PCR confirming the ligation of *trnI*, resulted in 1.5 kb fragment with M13 primers. **e** Restriction digestion of pLIA2 plasmid having *trnI* and *trnA* flanks, Lane: 1 Undigested pKS+backbone vector, Lane: 2 pKS + digested with *SacI* resulting in 3.0 kb size fragment, Lane: 3 Plasmid pLI1 digested with *SacI* resulting in 4.2 kb size fragment, Lane: 4 Plasmid

pLIA2 digested with *SalI* resulting in 6.0 kb size fragment, Lane: 5 pLI1 digested with *SacI* & *SacII* (*trnI*) resulting in 3.0 & 1.5 kb size fragments, Lane: 6 pLIA2 digested with *SalI* & *KpnI* (*trnA*) resulting in 4.2 & 1.5 kb size fragments, Lane: 7 pLIA2 digested with *SacI* & *SacII* (*trnI*) resulting in 4.2 & 1.5 kb size fragments. **f** Colony PCR confirming the ligation of *trnI* & *trnA*, resulted in 3.0 kb fragment. **g** Restriction digestion of CuIA plasmid having *aadA* flanked by *trnI* and *trnA* sequences, Lane: 1 pKS⁺ digested with *SacI* resulting in 3.0 kb size fragment, Lanes: 2 & 4 Single digestion of CuIA plasmid vector with *SacI* resulted in 7.1 kb size fragment, Lane: 3 CuIA plasmid digested with *SacII* and *KpnI* resulted in 3.0 & 4.2 kb size fragments. **h** Colony PCR confirming the ligation of *aadA* along with *trnI* and *trnA* flanks, resulted in 4.2 kb fragment

Fig. 3 Map of CuIA plasmid transformation vector and sequence analysis of flanking genes. **a** Physical map of species-specific vector showing position of different restriction sites. **b** blastn alignment results of cloned *rrn16-trnI* flanking gene into the vector showing maximum similarity with genome of *Cucumis sativus* L. **c** blastn alignment results of cloned *trnA-rrn23* flanking gene into the vector showing maximum similarity with genome of *Cucumis sativus* L.

Fig. 4 Generation of transplastomic plants. **a** Petiole segments prior to bombardment. **b** After 2 days of bombardment, petiole explants were transferred to MS regeneration medium supplemented with 300 mg/L spectinomycin. **c** Regeneration of spectinomycin-resistant shoot from petiole segment after 45 days of culture on same selection medium. **d** Elongation of transplastomic shoots on MS regeneration medium containing 300 mg/L spectinomycin

Table 2 Results of plastid transformation experiments in *Momordica charantia* L. using CuIA vector

Experiment	No. of bombarding plates (each with 20 explants)	Spectinomycin-resistant shoots	PCR positives
1	15	5	2
2	15	6	2
3	15	4	2

transformation of *Arabidopsis*, Tomato and *L. fendleri* (Sikdar et al. 1998; Ruf et al. 2001; Skarjinskaia et al. 2003). Based on our experimental data and other available examples for plastid transformation, it seems likely that a species-specific promoter is not always required for construction of selection marker cassette in plastid vector development.

The use of specific vector that leads to precise transgene integration estimates the transformation efficiency with

Fig. 5 Vector map and position of primers used for PCR amplification

Fig. 6 PCR analysis of plastid transformants of *Momardica charantia* L. **a** Confirmation of *aadA* resulted in 1.1 kb size amplicon fragment. **b** Confirmation of plastome integration resulting in 3.0 kb in wild and 4.2 kb fragment in transformed lines

comparison to frequency abilities of heterologous vectors. These considerable differences suggest that the lack of homology in flanking sequences may affect the recombination rates (Daniell et al. 2006; Rao et al. 2013). The relevant use of specific vector was best studied in targeting inverted repeat region (IR) *trnI/trnA* of *Brassica napus* (Cheng et al. 2010) and *Oryza sativa* (Lee et al. 2006) and large single copy region (LSC) *rbcL/accD* genes of *Lactuca sativa* L (Kanamoto et al. 2006). In contrast, the use of heterologous vectors in plastid transformation attained appreciable frequency rates in species that are closely related at genome level, including that vectors used for transformation of the tobacco plastid genome have been successfully used for potato (*rbcL/accD*), tomato (*trnM/trnG*) and scopolia (*trnR/trnN*) plastid transformation (Sidorov et al. 1999; Ruf et al. 2001; Muralikrishna et al. 2016). As well, the vectors employed for chloroplast transformation of tobacco and lesquerella (*rrn16S/trnV*) contained the flanking sequences from petunia, lettuce or arabidopsis (DeGray et al. 2001; Skarjinskaia et al. 2003; Ruhlman et al. 2010). The chloroplast transformation vector thus developed here will be useful in transforming the desirable traits into plastid genome of closely related and certain higher crop plants of Cucurbitaceae family.

Although there is availability of reproducible regeneration protocols for several cucurbitaceous members where the regeneration rate remains low when compared to herbaceous model plant like tobacco (Svab and Maliga 1993; Svab et al. 1990). Therefore, it is relevant to use flanking sequences from closely related species like *Cucumis sativus* L., for attaining comparative high plastidic transformation efficiency in bitter melon which has superior regeneration capability. In the present investigation, the feasibility of using petiole explants was utilized to achieve efficient chloroplast transformation for obtaining homoplasmic

plants after several rounds of selection on spectinomycin containing medium.

Previous studies in higher plants for plastid transformation used many target sites that include *trnH-psbA*, *trnG-trnM*, *ycf3-trnS*, *rbcL-accD*, *petA-psbJ*, *trnV-rrn16*, *rrn16-trnI*, *trnI-trnA*, *trnR-trnN* and *rpl32-trnL* intergenic regions (Maliga 2004). Considering the major advances in transplastomic research, plastid transformation in bitter melon can now be used for introduction of agronomically and pharmaceutically important traits for hyper expression. However, the efficiency of plastid transformation in this system was noted as two events for fifteen bombarded plates and is lower when compared to tobacco in which one event per bombardment plate. The difference in transformation efficiencies for bitter melon and tobacco could be due to the different explants and vectors employed for plastid transformation. In addition, a less shoot regeneration frequency from cut ends of the petiole explants that provide the smaller target area may possibly be the reason for lower transformation efficiency. As such, the transformation efficiency rate found to be lower in case of oilseed rape as the target area of explants is smaller (Hou et al. 2003). Finally, a successful attempt was executed towards plastid transformation in bitter melon with the chloroplast vector having close sequence identity.

Conclusion

The application of this plastid transformation vector may be extended to other valuable cucurbitaceous plant species with high sequence similarity to express foreign proteins successfully in the chloroplast as an alternative to existing universal chloroplast transformation vectors. The plastid transformation method in bitter melon is an attractive approach for improving crop performance and attaining the new possibilities for metabolic engineering and plant-based production of biopharmaceuticals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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