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Brief Communication

Chloroplast genome transformation of medicinal plant *Artemisia annua*

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Artemisia annua produces lifesaving antimalarial drug artemisinin. The biosynthetic pathway of artemisinin is intercalated with chloroplasts of *A. annua*. Therefore, it will be imperative to optimize its chloroplast transformation for increasing the yield of artemisinin, which has not been achieved to desired levels via nuclear genome transformation (Ikram and Simonsen, 2017). The low yield has limited its current global demand for the artemisinin-based combination therapies (ACTs).

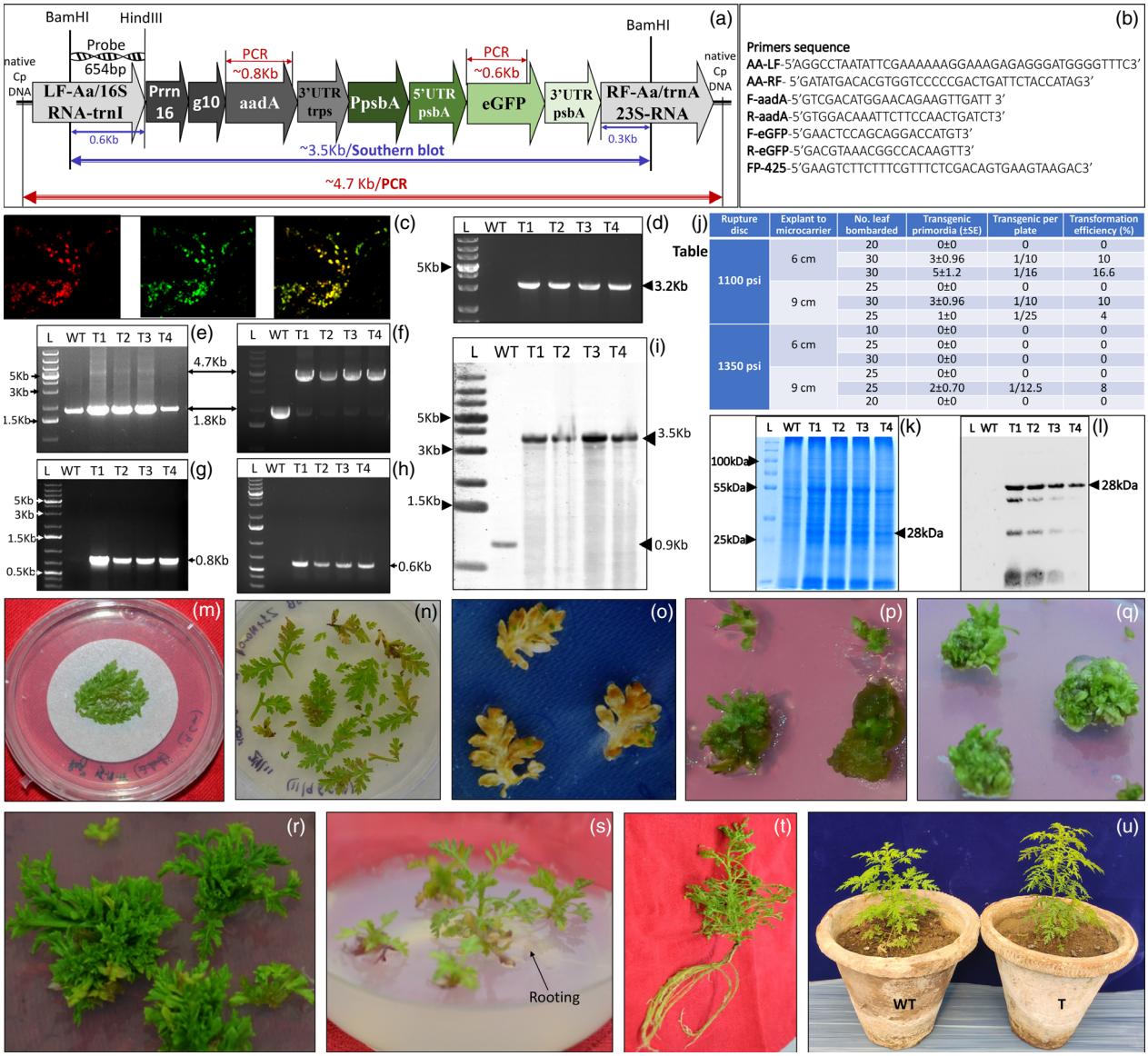
For optimizing the chloroplast transformation, species-specific flanking sequences were amplified from the total genomic DNA of *A. annua*. Vector was named as CP-AA (LF:Prrn16S-g10-aadA-3′UTR-Trps::Ppsba-5′UTRpsba-eGFP-3UTRpsbA:RF) (Fig. 1a), (GenBank Accession # MT096403) was designed in silico and synthesized by vendor (BioBasic Inc., Canada). It was integrated between the intergenic spacer region of rrn16S-trnI and trnA–rrn23S via homologous recombination, without impacting the functionality of other genes (Jin and Daniell, 2015). The *aad*A (*aminoglycoside adenylyltransferase*) gene conferring resistance to spectinomycin was codon optimized (GenBank Accession # MT096403). Its expression was regulated by 5′ ribosome-binding site region of the bacteriophage T7 gene10 (Olins *et al.*, 1988) and 3′UTR rps16 from tobacco (NC_001879). The second ORF encodes the enhanced green fluorescence protein eGFP (Zhang *et al.*, 1996) driven by light-regulated PEP promoter psbA, along with 5′ and 3′ UTRs of psbA of tobacco (NC_001879). The eGFP reporter gene was used for early detection of transgenic cultures and optimization of chloroplast transformation (Fig. 1c).

Seeds were germinated as described (Alam and Abdin, 2011), and cultures were maintained in light conditions (16h light:8h dark), with 40-50 μmol m^{-2} s^{-1} intensity and temp 25 ± 2°C. The pinnately compound, sessile leaves (Fig. 1m), excised from aseptically grown 3-4 weeks old *A. annua,* were bombarded with 4 μg CP-AA DNA containing ~ $1.35x10^{11}$ copies of the plasmid coated on 0.6 μm gold particles (Seashell Technology, La Jolla, CA, USA) using a biolistic DNA delivery system as reported (Kumar and Daniell, 2004; Malhotra *et al.*, 2016). After 48 hours of dark incubation, bombarded leaves were excised into small pieces and placed on selection medium (Fig. 1n). Several independent transgenic shoot primordia were observed after repetitive subculture on every two weeks for 2–3 months on MS medium containing plant growth regulators (1.25 mg/L BAP and 0.05 mg/L NAA) and 500 mg/L spectinomycin (Fig. 1o-p). These primordia were progressively proliferated into distinct shoots after 20 days on MS medium supplemented with reduced spectinomycin (250 mg/L) (Fig. 1q). Transgenic shoots were multiplied and elongated in 3–4 months (subculture after 2 weeks intervals) on MS medium when spectinomycin was reduced to one fourth (125 mg/L) (Fig. 1r). Rooting was induced only in elongated shoots after 5–7 weeks on MS medium containing 125 mg/L spectinomycin and 0.01 mg/L NAA (Fig. 1s-t). Four independent transplastomic plants with proper root were recovered. These plantlets after analysing for transgenes integration by PCR, Southern blot and eGFP protein expression by Western blot analysis were transferred to greenhouse. The transplastomic plants were further clonally propagated as reported (Wetzstein *et al.*, 2018). Transplastomic plants thrive well in the greenhouse, and growth was comparable to WT plants (Fig. 1u).

The early-stage transgenic green primordia screening on selection medium (Fig. 1o) was confirmed by eGFP expression (Fig. 1c). The site-specific integration of CP-AA vector cassette into the chloroplast genome was confirmed by primers AA-LF and AA-RF (Fig. 1b) that landed outside the flanking regions (LF-RF), yielding ~4.7Kb PCR amplicon in all transgenic shoot primordia in first round of selection (Fig. 1e). After repeated subculture of transgenic primordia on antibiotic selection, homoplastomic shoots were produced yielding PCR amplicon 4.7 Kb while heteroplastomic shoots also contained a faint signal of 1.8 Kb (Fig. 1f). The gene-specific PCR primers F-aadA and R-aadA; F-eGFP and R-eGFP (Fig. 1b) yielded amplicons ~0.8 Kb (Fig. 1g) and ~ 0.6 Kb (Fig. 1h), respectively, confirming integration of transgenes aadA and eGFP into the chloroplast genome.

The homoplastomy status of integrated transgenes was confirmed by Southern blotting. Genomic DNA of transgenics T1-T4 and WT was digested with BamHI. The probe (0.65 Kb) was excised from CP-AA vector for Southern blot hybridization as shown (Fig. 1a). The presence of only ~ 3.5 Kb signal in transgenic plants (T1-T4) and absence of WT chloroplast background (0.9 Kb) has confirmed the homoplastomic status of plants (Fig. 1i).

The fluorescent eGFP was used for a preliminary screening to distinguish the transgenics from WT, under the Nikon-A1 confocal microscopy. The eGFP micrographs of transgenics (Fig. 1c) showed auto red fluorescence of chlorophyll (excitation: 565 nm), (left), a green fluorescent micrograph expressing the eGFP (excitation: 485 nm) (middle), and merged micrograph of first two showed the colocalization of GFP expression in chloroplasts (right). Further, localization of eGFP in chloroplast genome was also confirmed by FP-425 primer (land 425 base pair outside the left flank) and R-eGFP primer bind to eGFP gene that yielding ~ 3.2 Kb PCR amplicon (Fig. 1d). For eGFP analysis, total



Rupture disc	Explant to microcarrier	No. leaf bombarded	Transgenic primordia (±SE)	Transgenic per plate	Transformation efficiency (%)
1100 psi	6 cm	20	0±0	0	0
		30	3±0.96	1/10	10
		30	5±1.2	1/16	16.6
	9 cm	25	0±0	0	0
		30	3±0.96	1/10	10
		25	1±0	1/25	4
1350 psi	6 cm	10	0±0	0	0
		25	0±0	0	0
		30	0±0	0	0
	9 cm	25	0±0	0	0
		25	2±0.70	1/12.5	8
		20	0±0	0	0

Figure 1 (a) Schematic representation of the chloroplast transformation vector CP-AA (GenBank Accession # MT096403) containing trnI and trnA flanks for homologous recombination with native chloroplast DNA of *Artemisia annua*. The complete cassette contains AALF-Prrn16S-g10-aadA-3'UTR-Trps:: Ppsba-5'UTRpsba-eGFP-3UTRpsbA-RFAA. (b) List of PCR primers used for the amplification of transplastomic genomic DNA. (c) The preliminary screening of transgenic cultures using eGFP protein with chlorophyll autofluorescence (left), fluorescent green colour (middle) and merged micrograph (right) with excitation wavelengths at 565 and 485 nm, respectively. (d) eGFP localization in chloroplasts confirmed using FP-425 primer, landed 425 base pair upstream of left flank and R-eGFP primer bind eGFP gene internally to yield ~ 3.2 Kb PCR amplicon. (e) PCR amplification of transgenics with site-specific primes, landed outside the flanking (LF-RF) regions, yielded 1.8 Kb and 4.7 Kb amplicons respectively in wild and transplastomic plants. (f) AA-LF and AA-RF primers landed on flanking regions have confirmed the partial homoplastomy status in transgenics by yielding 4.7 Kb PCR amplicon of integrated cassette while 1.8 Kb amplicon observed due to untransformed chloroplast genome in heteroplastomic cultures. (g) Integration of *aad*A and (h) e*GFP* genes was confirmed using gene-specific primers in lanes T1, T2, T3 and T4. No PCR product was observed in WT. (i) Homoplastomy status of transplastomic plants was confirmed by Southern blot. Transplastomic genome produced a ~ 3.5 Kb signal while untransformed 0.9Kb on hybridization with a probe, generated based on left flanking region. (j) Maximum transformation efficiency was observed 16.6% using 1100 psi rupture discs. (k) In SDS-PAGE Coomassie-blue stained gel, 28KDa band was observed in transplastomic plants (T1, T2, T3 and T4). (l) It was reconfirmed by Western blot using a polyclonal GFP antibody. The 28KDa signal was absent in WT plants. (m) The pinnately compound and sessile leaves of aseptically grown *A. annua* were arranged in a stack on a filter paper prior to bombardment with CP-AA vector using a Biolistic DNA delivery system. (n) Targeted explants after 48 hrs of dark incubation (without any antibiotic selection) were excised and placed on selection medium. (o) Transgenic green primordia appeared after 2 months from bombarded explants, initially confirmed by eGFP expression. (p-t) Progression of shoot growth and root induction in transplastomic when spectinomycin amount was reduced. (u) Growth of transplastomic plant compared with WT in greenhouse.

40 μg protein from transplastomic and WT plants was electrophoresed on 12% SDS-Polyacrylamide gel and stained with Coomassie Brilliant Dyes, yielding a 28 kDa signal only in transgenics lanes (Fig. 1k). The eGFP expression was confirmed by Western blot using a polyclonal GFP antibody conjugated with HRP. About 28 kDa signal was observed in all the four transgenic

lines (T1, T2, T3 and T4). No signal was detected in WT lane (Fig. 1l).

The maximum transformation efficiency (16.6%) was observed when explants were bombarded using 1100 psi rupture disc at 6 cm distance from microcarrier (Fig. 1j). No transgenics were recovered using rupture discs below 1100 psi or above 1350 psi. The use of 1800 psi rupture disc was deleterious to bombarded explants, causing an internal damage to tissues, leaching phenolic compounds, and cultures died in two weeks after browning.

The optimization of chloroplast transformation in agronomically important recalcitrant species is a major challenge due to poor tissue culture response and inhibitory impact of antibiotics on the shoot and root induction in transplastomic tissues (Bock, 2015). In potato, only 3 lines were regenerated out of 104 plates subjected to bombardment, in *Arabidopsis*, only two transplastomic plants were obtained out of 201 bombarded leaves. In tomato, 3 transplastomic lines were recovered out of 30 leaf samples. However, obtaining a lesser number of chloroplast transgenic should not be a concern as chloroplast transformation is highly site specific via homologous recombination. All transplastomics as well as their clonally propagated plants produced a similar level of artemisinin when compared to WT plants, which indicated that there was no deleterious impact of aadA and eGFP transgenes on plants. In brief, we have optimized a stable chloroplast genome transformation in medicinal plant *A. annua*, which could be explored further for expressing the non-glycosylated proteins of artemisinin biosynthetic pathway to produce a higher quantity of artemisinin (Malhotra *et al.*, 2016) to meet its global demand for ACTs.

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Conflict of interest

The authors declare that they have no competing or conflicting interests.

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Authors' contributions

CK carried out the transformation optimization experiments and molecular analysis on transplastomic plants. MZA assisted in interpreting the results, reviewed the study. SK conceptualized the study and supervised the whole experimental research, data and analysis of results. CK and SK interpreted results and wrote the final manuscript.

Ethical approval

Not applicable.

Consent to publish

All authors read the manuscript and approved to publish.

Data Availability Statement

All data generated or analysed during this study are included in this article and whole vector description, and DNA sequence information is uploaded to public domain with GenBank Accession # MT096403.

References

Alam, P. and Abdin, M.Z. (2011) Over-expression of HMG-CoA reductase and amorpha-4,11-diene synthase genes in *Artemisia annua* L. and its influence on artemisinin content. *Plant Cell Rep.* **30**, 1919–1928.

Bock, R. (2015) Engineering plastid genomes: methods, tools, and applications in basic research and biotechnology. *Annu. Rev. Plant Biol.* **66**, 211–241.

Ikram, N. and Simonsen, H.T. (2017) A review of biotechnological artemisinin production in plants. *Front. Plant Sci.* **8**, 1966.

Jin, S. and Daniell, H. (2015) The engineered chloroplast genome just got smarter. *Trends Plant. Sci.* **20**, 622–640.

Kumar, S. and Daniell, H. (2004) Engineering the chloroplast genome for hyperexpression of human therapeutic proteins and vaccine antigens. *Meth. Mol. Biol.* **267**, 365–383.

Malhotra, K., Subramaniyan, M., Rawat, K., Kalamuddin, M., Qureshi, M.I., Malhotra, P., Mohmmed, A. *et al.* (2016) Compartmentalized metabolic engineering for artemisinin biosynthesis and effective malaria treatment by oral delivery of plant cells. *Mol. Plant.* **9**, 1464–1477.

Olins, P.O., Devine, C.S., Rangwala, S.H. and Kavka, K.S. (1988) The T7 phage gene 10 leader RNA, a ribosome-binding site that dramatically enhances the expression of foreign genes in *Escherichia coli*. *Gene,* **73**, 227–235.

Wetzstein, H.Y., Porter, J.A., Janick, J., Ferreira, J.F.S. and Mutui, T.M. (2018) Selection and clonal propagation of high artemisinin genotypes of *Artemisia annua*. *Front. Plant Sci.* **9**, 358–369.

Zhang, G., Gurtu, V. and Kain, S.R. (1996) An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells. *Biochem. Biophys. Res. Commun.* **227**, 707–711.

