ISSN (online): 2250-141X
Vol. 2 Issue 4, September 2012

CHLOROPLAST EXPRESSION VECTOR SYSTEM & ITS TRANSFORMATION

Navaneeth Beeramganti, #Haritha Beeramganti, Koona Subramanyam*, Pinnamanrni Rajasekhar

Department of Biotechnology, Sreenidhi Institute of Science and Technology (Autonomous), Jawaharlal Nehru Technological University, Hyderabad, Andhra Pradesh 501301, India.

#Department of Microbiology, St Pious College for women, Habsiguda,Hyderabad, Andhra Pradesh, 500039- India.

ABSTRACT

Chloroplast genetic engineering offers several advantages, including high level transgene expression, multi-gene engineering in a single transformation event. Lack of transgene silencing, pleotropic effect has made chloroplast an efficient vector for carrying foreign DNA. In current study, we discuss the methods for designing chloroplast expression vectors and their transformation into chloroplast (PEG treatment followed by biolistic method). Tobacco plastids have been transformed for production of high levels of vaccines, antigens, biopharmaceuticals, etc.

KEYWORDS: Chloroplast Expression Vector, Transformation, Transgene.

INTRODUCTION

Chloroplast is members of class of organelles known as plastids. Chloroplast contains multiple copies of circular DNA. Recent advancement in field of biotechnology has proved the use of chloroplast as excellent ideal host for expression of Gene of Interest or Transgene. Use of chloroplast is advantageous as chloroplast DNA is not transmitted through pollen, which prevents gene flow from the genetically modified plant to other plants. This makes plastid transformation a valuable tool for the creation and cultivation of genetically modified plants that are biologically contained, thus posing significantly lower environmental risks. This biological containment strategy is therefore suitable for establishing the coexistence of conventional and organic agriculture. While the reliability of this mechanism has not yet been studied for all relevant crop species, recent results in tobacco plants are promising, showing a failed containment rate of transplastomic plants at 3 in 1,000,000 [1].

To achieve efficient chloroplast transformation we use chloroplast vector, the stable integration of Transgene is by homologous recombination of flanking sequence used in vectors (chloroplast vectors), the trnA and trnI genes from the IR region of the tobacco chloroplast genome as flanking sequences for homologous recombination to transform several other plant species (of unknown genome sequence) was suggested several years ago.[2] Because of the high similarity in the transcription and translation systems between *E.coli* and

ISSN (online): 2250-141X
Vol. 2 Issue 4, September 2012

chloroplasts, the chloroplast expression vectors are tested in *E.coli* first before proceeding with plant transformation. Once expression of transgenes is confirmed in *E. coli*, the transformation vector is delivered into leaves (tobacco/lettuce) via particle bombardment. PCR analysis is used to screen the transgenic shoots and distinguish true chloroplast transgenic events from mutants or nuclear transgenic plants. Site-specific chloroplast integration of the transgene cassette is determined by using a set of primers, one of which anneals to the native chloroplast genome and the other anneals within the transgene cassette. Mutants and nuclear transgenic plants are not expected to produce a PCR product with these primers.

Chloroplasts are one of the many different types of organelles in the plant cell. They are considered to have originated from cyanobacteria through endsymbiosis. This was first suggested by Mereschkowsky in 1905[3] after an observation by Schimper in 1883 that chloroplasts closely resemble cyanobacteria.[4] All chloroplasts are thought to derive directly or indirectly from a single endosymbiotic event(in the Archaeplastida), except for *Paulinella chromatophora*, which has recently acquired a photosynthetic cyanobacterial endosymbiont which is not closely related to chloroplasts of other eukaryotes.[5] In that they derive from an endosymbiotic event, chloroplasts are similar to mitochondria, but chloroplasts are found only in plants and protista. The chloroplast has its own DNA,[6] which codes for redox proteins involved in electron transport in photosynthesis; this is termed the plastome[7].

Recently, chloroplasts have caught attention by developers of genetically modified plants. In most flowering plants, chloroplasts are not inherited from the male parent, although in plants such as pines, chloroplasts are inherited from males. [8] Where chloroplasts are inherited only from the female, transgenes in these plastids cannot be disseminated by pollen. This makes plastid transformation a valuable tool for the creation and cultivation of genetically modified plants that are biologically contained, thus posing significantly lower environmental risks. This containment strategy is therefore suitable for establishing the coexistence of conventional and organic agriculture.

Chloroplasts are ideal hosts for expression of transgenes. Transgene integration into the chloroplast genome occurs via homologous recombination of flanking sequences used in chloroplast vectors. Identification of spacer regions to integrate transgenes and endogenous regulatory sequences that support optimal expression is the first step in construction of chloroplast vectors. Thirty-five sequenced crop chloroplast genomes provide this essential information. Various steps involved in the design and construction of chloroplast vectors, DNA delivery, and multiple rounds of selection are described. Several crop species have stably integrated transgenes conferring agronomic traits, including herbicide, insect, and disease resistance, drought and salt tolerance, and phytoremediation. Several crop chloroplast genomes have been transformed via organogenesis cauliflower[*Brassica oleracea*], cabbage [*Brassica capitata*], lettuce[*Lactuca sativa*], oilseed rape [*Brassica napus*], petunia [*Petunia hybrida*], poplar [Populus spp.], potato[*Solanum tuberosum*], tobacco[*Nicotiana tabacum*],and tomato [*Solanum lycopersicum*]or embryogenesis (carrot [Daucus carota], cotton [*Gossypium hirsutum*], rice [*Oryza sativa*], and

ISSN (online): 2250-141X
Vol. 2 Issue 4, September 2012

soybean [Glycine max]), and maternal inheritance of transgenes has been observed. Chloroplast derived biopharmaceutical proteins, including insulin, interferon's (IFNs), and somatotropin (ST), have been evaluated by in vitro studies. Human INFa2b transplastomic plants have been evaluated in field studies. Chloroplast-derived vaccine antigens against bacterial (cholera, tetanus, anthrax, plague, and Lyme disease), viral (canine parvovirus [CPV] and rotavirus), and protozoan (amoeba) pathogens have been evaluated by immune responses, neutralizing antibodies, and pathogen or toxin challenge in animals. Chloroplasts have been used as bioreactors for production of bio-polymers, amino acids, and industrial enzymes. Oral delivery of plant cells expressing proinsulin (Pins) in chloroplasts offered protection against development of insulitis in diabetic mice; such delivery eliminates expensive fermentation, purification, low temperature storage, and transportation. Chloroplast vector systems used in these biotechnology applications are described.

Chloroplast Genome Organization

The chloroplast genome typically consists of basic units of double stranded DNA of 120 to 220 kb arranged in monomeric or multimeric circles as well as in linear molecules.[9] The chloroplast genome generally has a highly conserved organization, with most land plant genomes having two identical copies of a 20 to 30-kb inverted repeat region (IR_A and IR_B) separating a large single copy (LSC) region and a small single copy (SSC) region. Plastid transformation is typically based on DNA delivery by the biolistic process or occasionally by polyethylene glycol (PEG) treatment of protoplasts [10]. This is followed by transgene integration into the chloroplast genome via homologous recombination facilitated by a RecA-type [11] system between the plastid-targeting sequences of the transformation vector and the targeted region of the plastid genome. Chloroplast transformation vectors are thus designed with homologous flanking sequences on either side of the transgene cassette to facilitate double recombination. Targeting sequences have no special properties other than that they are homologous to the chosen target site and are generally about 1 kb in size. Both flanking sequences are essential for homologous recombination. Transformation is accomplished by integration of the transgene into a few genome copies, followed by 25 to 30 cell divisions under selection pressure to eliminate untransformed plastids, thereby achieving a homogeneous population of plastid genomes. If the transgene is targeted into the IR region, integration in one IR is followed by the phenomenon of copy correction that duplicates the introduced transgene into the other IR as well.

Transgenes have been stably integrated at several sites within the plastid genome. Transgenes were first integrated into transcriptionally silent spacer regions [12]. However, transcriptionally active spacer regions offer unique advantages, including insertion of transgenes without 5^1 or 3^1 untranslated regions (UTRs) or promoters. To date, the most commonly used site of integration is the transcriptionally active intergenic region between the trnI-trnA genes, within the rrn operon, located in the IR regions of the chloroplast genome. The foreign gene expression levels obtained from genes integrated at this site are among the highest ever reported. It

ISSN (online): 2250-141X
Vol. 2 Issue 4, September 2012

appears that this preferred site is unique and allows highly efficient transgene integration and expression.

Chloroplast vectors may also carry an origin of replication that facilitates replication of the plasmid inside the chloroplast, thereby increasing the template copy number for homologous recombination and consequently enhancing the probability of transgene integration. oriA is present within the trnI flanking region[13], and this might facilitate replication of foreign vectors within chloroplasts, enhance the probability of transgene integration, and achieve homoplasmy even in the first round of selection[14].This is further confirmed by the first successful Rubisco engineering obtained by integrating the rbcS gene at this site[15]. All other earlier attempts on Rubisco engineering at other integration sites within the chloroplast genome were only partially successful. Integration of transgenes between exons of trnA and trnI also facilitates correct processing of foreign transcripts because of processing of introns present within both flanking regions.

Chloroplast Expression Vector: Method for Construction of Expression Vector and transformation into Chloroplast

Plastid gene expression is regulated both at the transcriptional and posttranscriptional levels. Protein levels in chloroplasts depend on mRNA abundance, which is determined by promoter strength and mRNA stability. However, high mRNA levels do not result in high-level protein accumulation as posttranscriptional processes ultimately determine obtainable protein levels. Therefore, we have designed expression cassettes for transgene assembly to achieve optimal levels of protein accumulation in leaves (Fig. 1).The basic plastid transformation vector is comprised of flanking sequences and chloroplast-specific expression cassettes (Fig. 1).Species-specific chloroplast flanking sequence (e.g. trnI/trnA) is obtained by PCR using the primers designed from the available chloroplast genomes. The chloroplast expression cassette is composed of a promoter, selectable marker, and $5^1/3^1$ regulatory sequences to enhance the efficiency of transcription and Translation of the gene. The chloroplast specific promoters and regulatory elements are amplified from the total cellular DNA using primers designed on the basis of the sequence information available for the chloroplast genome. Suitable restriction sites are introduced to facilitate gene assembly.

Because of the high similarity in the transcription and translation systems between *E. coli* and chloroplasts, the chloroplast expression vectors are tested in E.coli first before proceeding with plant transformation. The growth of *E.coli* harboring the plastid transformation vector with the aadA gene in the presence of spectinomycin confirms expression of the aadA gene. Western blot with extracts from E.coli confirms expression of the gene of interest.

ISSN (online): 2250-141X
Vol. 2 Issue 4, September 2012

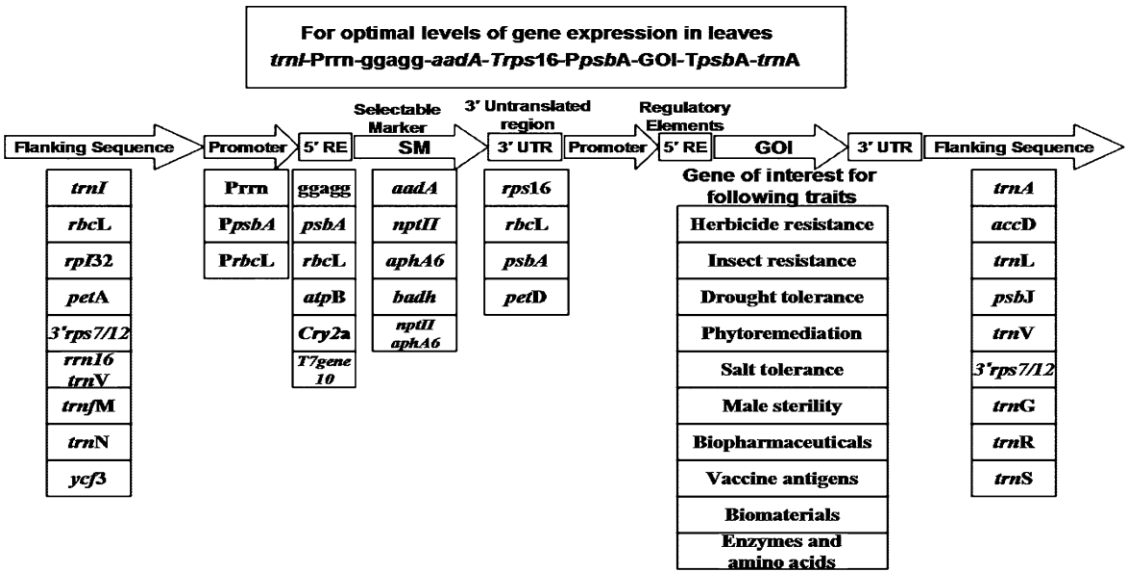

Figure 1. Schematic representation of the chloroplast-specific expression cassette.

Map of the chloroplast expression vector shows the integration sites, promoters, selectable marker genes, regulatory elements, and genes of interest. For a list of regulatory elements and genes of interest used for chloroplast transformation.

Once expression of transgenes is confirmed in *E.coli*, the transformation vector is delivered into leaves (tobacco/lettuce) via particle bombardment. The leaves used for bombardment should be young, green, and healthy. The bombarded leaves are placed on selection medium with an appropriate concentration of antibiotics (RMOP in tobacco). Normally, in 3to 10weeks, putative transgenic shoots appear (Fig. 2, A and D). PCR analysis is used to screen the transgenic shoots and distinguish true chloroplast transgenic events from mutants or nuclear transgenic plants. Site-specific chloroplast integration of the transgene cassette is determined by using a set of

primers, one of which anneals to the native chloroplast genome and the other anneals within the transgene cassette. Mutants and nuclear transgenic plants are not expected to produce a PCR product with these primers. The leaf pieces from PCR-positive shoots are further selected for a second round to achieve homoplasmy (Fig. 2, B and E).The regenerated shoots are rooted with the same level of selection (Fig. 2, C and F) and checked for homoplasmy by Southern-blot analysis . The Southern blot is probed with radio labeled flanking sequences used for homologous recombination. Transplastomic genome contains a larger size hybridizing fragment than the untransformed genome because of the presence of transgenes. If the transgenic plants are heteroplasmic, a native fragment is visible along with the larger transgenic fragment. Absence of the native fragment confirms the establishment of homoplasmy. Transgene expression is confirmed by western-blot analysis, and the effectiveness or properties or functionality of the introduced transgene is assessed. Seeds from the transgenic plants and untransformed plants are grown on spectinomycin containing medium to check for maternal inheritance.

ISSN (online): 2250-141X
Vol. 2 Issue 4, September 2012

Transgenic seeds germinate and grow into uniformly green plants. The absence of Mendelian segregation of transgenes confirms that they are maternally inherited to progeny.

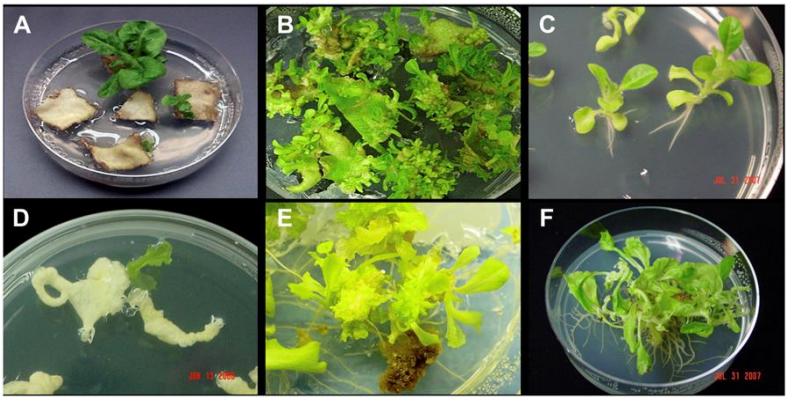

Figure 2. Selection of transplastomic plants. Shown are representative photographs of transplastomic tobacco and lettuce shoot undergoing first (A and D), second (B and E), and third (C and F, rooting) rounds of selection, respectively.

Screening of transformants: Selectable markers

The gene coding for spectinomycin resistance (encoded in the mutant 16S ribosomal RNA (rRNA) gene) was used as selectable marker for identification of transformants.[29][30]. Stable integration and expression of the aadA gene was first reported in the chloroplast genome of *Chlamydomonas* [31]. The aadA gene encodes the enzyme aminoglycoside 3^1 adenylyltransferase that inactivates spectinomycin and streptomycin by adenylation and prevents binding to chloroplast ribosome's. The aadA gene was later used as a selectable marker in tobacco, and the frequency of transformation events increased to 100-fold more than the mutant 16S rRNA genes. Due to the recessive nature of the mutant 16S rRNA marker gene, the phenotypic resistance was not expressed until sorting out of the transgenomes was essentially completed. Lack of phenotypic resistance permitted the loss of the resistant rRNA gene in 99 out of 100 potential transformation events. Although it was first explained that spectinomycin offers nonlethal selection [12] by not inhibiting cell growth at high concentrations. It was observed lethalin other crop plants.

A different kanamycin resistance gene (aphA6) with relatively high transformation efficiency was reported later [32]. Another selection strategy utilizing a ''double barrel'' vector was used for cotton transformation where explants for transformation was nongreen cells [17]. The cotton plastid transformation vector contained two different genes (aphA6 and nptII) coding for two different enzymes. The aphA6 gene was regulated by the 16S rRNA promoter and gene 10 UTR capable of expression in the dark and in nongreen tissues. The nptII gene was regulated by the psbA promoter and UTR capable of expression in the light. Both genes with different regulatory sequences facilitated detoxification of the same selection agent (kanamycin) during day and night as well as in developing plastids and mature chloroplasts. The double barrel transformation vector was reported to be at least 8-fold more

ISSN (online): 2250-141X
Vol. 2 Issue 4, September 2012

efficient than single gene (aphA6)-based chloroplast vectors.

To avoid potential disadvantages of antibiotic resistance genes, several studies have explored strategies for engineering chloroplasts that are free of antibiotic resistance markers. The spinach (*Spinacia oleracea*) betaine aldehyde dehydrogenase (badh) gene has been developed as a plant-derived selectable marker gene to transform chloroplast genomes [33]. The selection process involved conversion of the toxic compound betaine aldehyde to beneficial Gly betaine by the chloroplast localized gene-encoding enzyme BADH. Because the BADH enzyme is present only in chloroplasts of a few plant species adapted to dry and saline environments, it is considered as a suitable selectable marker in many crop plants. The transformation study showed rapid regeneration of transgenic shoots within 2 weeks in tobacco, and betaine aldehyde selection was 25-fold more efficient than spectinomycin. In addition, the Badh enzyme conferred salt tolerance in carrot [16].

Reporter genes

GUS, chloramphenicol acetyl transferase, and GFP have been used as plastid reporters [34]. The enzymatic activity of GUS can be visualized by histochemical staining [35], whereas GFP is a visual marker that allows direct imaging of the fluorescent gene product in living cells. The GFP chromophore forms autocatalytically in the presence of oxygen and fluoresces green when absorbing blue or UV light [36]. GFP has been used to detect transient gene expression and stable transformation events [20] [37] in chloroplasts.

GFP has also been fused with AadA and used as a bifunctional visual and selectable marker [38]. Further, GFP has been used to test the concept of receptor mediated oral delivery of foreign proteins. Cholera toxin B-subunit (CTB)-GFP fusion protein with a furin cleavage site in between CTB and GFP has been used to elucidate the path of CTB and GFP in the circulatory system [37]. Mice were fed with CTB-GFP expressing plant leaf material. GFP was detected in the intestinal mucosa and sub mucosa, the hepatocytes of the liver, as well as various cells of spleen utilizing fluorescence microscopy and anti-GFP antibodies. In mice fed with untransformed leaf material or IFN-GFP fusion protein expressing plant leaf material, no GFP fluorescence was observed. This confirmed the receptor mediated oral delivery of a foreign protein (GFP) across the intestinal lumen into the systemic circulation. Moreover, GFP was not detected in any substantial amount in the liver or spleen of mice fed with IFN-GFP expressing plants, suggesting that a transmucosal carrier such as CTB is required for delivery of an adequate amount of a foreign protein across the intestinal lumen into the systemic circulation. Thus, GFP has been used as a reporter gene in chloroplast expression and in animal studies.

Examples: Tobacco Chloroplast Transformation

Tobacco has been the most widely exploited plastid transformation system because of its ease in genetic manipulations. A single tobacco plant is capable of generating a million seeds and 1 acre of tobacco can produce more than 40 metric tons of leaves per year [39]. Harvesting leaves before flowering can offer nearly complete transgene containment in addition to protection offered by maternal inheritance. Recent studies have reported that escape of transgenes

ISSN (online): 2250-141X
Vol. 2 Issue 4, September 2012

in tobacco is 0.0087% to 0.00024% [12][25], making this an ideal system for use of chloroplasts as bioreactors. In addition, CMS has been engineered via the tobacco chloroplast genome as a failsafe method [40]. As a bioreactor, tobacco has been estimated to be more than 50 times less expensive than the frequently used Escherichia coli fermentation systems. Additionally, tobacco eliminates contamination of food because it is a non-food and non-feed crop. Plastid transformation in higher plants was first successfully carried out in tobacco and is now a routine procedure because many foreign genes have been expressed to engineer agronomic traits, biopharmaceuticals, vaccines, or biomaterials. However, presence of nicotine or other alkaloids has been a disadvantage for pharmaceutical production, but the chloroplast genome of low nicotine varieties like LAMD has been used to engineer therapeutic proteins [41]. For oral delivery studies, there is a need to move beyond tobacco.

Expression vector for Tobacco:-

Expression of many vaccine antigens and biopharmaceuticals proteins has been seen like human somatotropin, cholera toxin, interferon α, monoclonal antibodies, tetanus toxins etc, have been successfully expressed in Tobacco plant.

For example if we choose production of Interferon α then expression system would be as follows:

1. **Flanking sites** :- trnI/trnA
2. **Promoter**:- PpsbA
3. **5′/3′ UTRs**: - PpsbA/TpsbA.
4. **Gene of interest** :- INF-α

When the expression vector with desired promoter, flanking sites, with gene of interest is designed. The vector so designed is transferred in to chloroplast (leaf). Then we go for screening of transformants as mentioned above

Expression vector for Agronomic Trait (Salt Tolerance)

Several useful transgenes have conferred valuable agronomic traits, including insect and pathogen resistance, drought tolerance, phytoremediation, salt tolerance, and CMS through chloroplast genetic engineering.

Salt stress is a major abiotic stress in plant agriculture. Carrot (*Daucus carota* L) is any important vegetable crop as it is an excellent source for sugars, vitamin A & C, and fiber diet. Carrot is salt-sensitive plant, its growth decreases by 7% for every 10mM increase in salt concentration. Transgenic plants accumulating Glycine betaine exhibits moderate level of salt tolerance. This resistance was due to expression badh-gene which codes for BADH enzyme.

Expression Vector Salt Tolerance:-

- **Flanking sequences** :- trnI/trnA
- **Promoter** :- Prrn-F
- **5′/3 ′ UTRs** :-ggagg/rps16
- **Gene of interest** :- badh

This vector was developed at Daniell laboratory.

Advantages

- Use of chloroplast genetic engineering offers several advantages over nuclear transformation.
- Small genome size
- Chloroplast a viable alternative to conventional production system.
- Its ability to accumulate large amounts of foreign protein.
- Lack of transgene silencing.
- Site-specific integration of GOI by homologous recombination.

ISSN (online): 2250-141X
Vol. 2 Issue 4, September 2012

- Expression of multiple genes.
- No pleotropic effect.

Uses & Limitations of chloroplast transformation: Uses

- **Plastid as a biopharmaceutical bioreactor**:-

Several chloroplast derived biopharmaceutical have been reported. Many medical and therapeutic proteins like Human ST (HST), HSA, and IFN have been developed.

- **Plastid as vaccine bioreactor**:-

Anthrax vaccine developed from Bacillus anthracis contain a protective antigen which may be lethal, hence its use is limited. Anthrax vaccines produced by chloroplast transformation are clean and safe for use. Other vaccines like plague, tetanus, and amebiasis have been expressed by chloroplast.

- **Plastid as biomaterial bioreactors**:-

Besides vaccine antigens, biomaterial and amino acids have also been expressed in chloroplast. p-Hydroxybenzoic acid is produced in small quantities.

Limitations

- Delivery of foreign gene into chloroplast and it stable integration.
- Introducing of foreign DNA into non green tissues.
- Identification of appropriate regulatory sequences whose function is important to achieve foreign gene expression.
- Low transformation efficiency.

REFERENCES

1. Ruf S, Karcher D, Bock R (April 2007). "Determining the transgene containment level provided by chloroplast transformation". *Proc. Natl. Acad. Sci. U.S.A.* **104** (17): 6998–7002.doi:10.1073/pnas.0700008104. PMC 1849964. PMID 17420459. Archived from the original on 2010-11-18.
2. Daniell H, Datta R, Varma S, Gray S, Lee SB (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nat Biotechnol 16: 345–348
3. Mereschkowsky C (1905). "Über Natur und Ursprung der Chromatophoren im Pflanzenreiche".*Biol Centralbl* 25: 593–604
4. Schimper AFW (1883). "Über die Entwicklung der Chlorophyllkörner und Farbkörper". *Bot. Zeitung* **41**: 105–14, 121–31, 137–46, 153–62.
5. Patrick J. Keeling (2004). "Diversity and evolutionary history of plastids and their hosts". *American Journal of Botany* **91** (10): 1481–1493.
6. Patrick J. Keeling (2004). "Diversity and evolutionary history of plastids and their hosts". *American Journal of Botany* **91** (10): 1481–1493.
7. Krause K (September 2008). "From chloroplasts to "cryptic" plastids: evolution of plastid genomes in parasitic plants". *Curr. Genet.* **54** (3): 111–21.
8. Powell W, Morgante M, McDevitt R, Vendramin GG, Rafalski JA (August 1995). "Polymorphic simple

ISSN (online): 2250-141X
Vol. 2 Issue 4, September 2012

sequence repeats regions in chloroplast genomes: applications to the population genetics of pines". *Proc. Natl. Acad. Sci. U.S.A.* **92** (17): 7759–63. Doi:10.1073/pnas.92.17.7759.PMC 41225. PMID 7644491. "In the pines, the chloroplast genome is transmitted through pollen.

9. Palmer JD (1985) Comparative organization of chloroplast genomes. Annu RevGenet 19: 325–354.
10. 10. Golds T, Maliga P, Koop HU (1993) Stable plastid transformation in PEG-treated protoplasts of Nicotiana tabacum. Nat Biotechnol 11: 95–9.
11. Cerutti H, Osman M, Grandoni P, Jagendorf AT (1992) A homolog of
12. Escherichia coli RecA protein in plastids of higher plants. Proc Natl Acad Sci USA 89: 8068–8072
13. Svab Z, Maliga P (1993) High-frequency plastid transformation in tobacco by selection for a chimeric aadA gene. Proc Natl Acad Sci USA 90: 913–917
14. Kunnimalaiyaan M, Nielsen BL (1997) Fine mapping of replication origins (ori A and ori B) in Nicotiana tabacum chloroplast DNA. Nucleic Acids Res 25: 3681–3686
15. Guda C, Lee SB, Daniell H (2000) Stable expression of a biodegradable protein-based polymer in tobacco chloroplasts. Plant Cell Rep 19: 257–262
16. Dhingra A, Portis AR Jr, Daniell H Enhanced translation of a chloroplast-expressed RbcS gene restores small subunit levels and photosynthesis in nuclear RbcS antisense plants. Proc Natl Acad Sci USA 101: 6315–6320
17. Kumar S,Dhingra A, Daniell H Plastid-expressed betainealdehyde dehydrogenase gene in carrot cultured cells, roots, and leaves confers enhanced salt tolerance. Plant Physiol 136: 2843–2854
18. Kumar S, Dhingra A, Daniell H Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. Plant Mol Biol 56: 203–216
19. Lee SM, Kang K, Chung H, Yoo SH, Xu XM, Lee SB, Cheong JJ, Daniell H,Kim M Plastid transformation in the monocotyledonous cereal crop, rice (Oryza sativa) and transmission of transgenes to their progeny. Mol Cells 21: 401–410
20. Nugent GD, Coyne S, Nguyen TT, Kavanagh TT, Dix PJ (2006) Nuclear and plastid transformation of Brassica oleracea var. botrytis (cauliflower) using PEG-mediated uptake of DNA into protoplasts. Plant Sci 170:135–142
21. Lelivelt C, McCabe M, Newell C, deSnoo C, Dun K, Birch-Machin I, Gray J, Mills K, Nugent J (2005) Stable plastid transformation in lettuce (Lactuca sativa L.). Plant Mol Biol 58: 763–774
22. Liu CW, Lin CC, Chen J, Tseng MJ (2007) Stable chloroplast transformation in cabbage (Brassica oleracea var. capitata L.) by particle bombardment. Plant Cell Rep 26: 1733–1744

ISSN (online): 2250-141X
Vol. 2 Issue 4, September 2012

23. Kanamoto H, Yamashita A, Asao H, Okumura S, Takase H, Hattori M, Yokota A, Tomizawa K (2006) Efficient and stable transformation of Lactuca sativa L. cv. Cisco (lettuce) plastids. Transgenic Res 15: 205–217
24. Hou BK, Zhou YH, Wan LH, Zhang ZL, Shen GF, Chen ZH, Hu ZM (2003) Chloroplast transformation in oilseed rape. Transgenic Res 12:111–114
25. Zubko M, Zubko E, Zuilen K, Meyer P, Day A (2004) Stable transformation of petunia plastids. Transgenic Res 13: 523–530
26. Ruf S, Hermann M, Berger IJ, Carrer H, Bock R (2001) Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. Nat Biotechnol 19: 870–875
27. Nguyen TT, Nugent G, Cardi T, Dix PJ (2005) Generation of homoplasmic plastid transformants of a commercial cultivar of potato (Solanum tuberosum L.). Plant Sci 168: 1495–1500
28. Okumura S, Sawada M, Park YW, Hayashi T, Shimamura M, Takase H, Tomizawa K (2006) Transformation of poplar (Populus alba) plastids and expression of foreign proteins in tree chloroplasts. Transgenic Res 15:637–646
29. Sidorov VA, Kasten D, Pang SZ, Hajdukiewicz PTJ, Staub JM, Nehra NS (1999) Stable chloroplast transformation in potato: use of green fluores- cent protein as a plastid marker. Plant J 19: 209–216
30. Harris EH, Burkhart BD, Gillham NW, Boynton JE (1989) Antibiotic resistance mutations in the chloroplast 16S and 23S rRNA genes of Chlamydomonas reinhardtii: correlation of genetic and physical maps of the chloroplast genome. Genetics 123: 281–292
31. Svab Z, Hajdukiewicz P, Maliga P (1990) Stable transformation of plastids in higher plants. Proc Natl Acad Sci USA 87: 8526–8530
32. Goldschmidt-Clermont M (1991) Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker of site-directed transformation of chlamydomonas. Nucleic Acids Res 19: 4083–4089
33. Huang FC, Klaus S, Herz S, Zou Z, Koop HU, Golds T (2002) Efficient plastid transformation in tobacco using the aphA-6 gene and kanamycin selection. Mol Genet Genomics 268: 19–27
34. Daniell H, Muthukumar B, Lee SB (2001b) Marker free transgenic plants: engineering the chloroplast genome without the use of antibiotic selec- tion. Curr Genet 39: 109–116
35. Daniell H, Krishnan M, McFadden BA (1991) Transient expression of b-glucuronidase in different cellular compartments following biolistic delivery of foreign DNA into wheat leaves and calli. Plant Cell Rep 9:615–619
36. Ye GN, Daniell H, Sanford JC (1990) Optimization of delivery of foreign DNA into higher-plant chloroplasts. Plant Mol Biol 15: 809–819

ISSN (online): 2250-141X
Vol. 2 Issue 4, September 2012

37. Hanson MR, Kohler RH (2001) GFP imaging: methodology and application to investigate cellular compartmentation in plants. J Exp Bot 52:529–539
38. Limaye A, Koya V, Samsam M, Daniell H (2006) Receptor-mediated oral delivery of a bioencapsulated green fluorescent protein expressed in transgenic chloroplasts into the mouse circulatory system. FASEB J 20:959–961
39. Khan MS, Maliga P (1999) Fluorescent antibiotic resistance marker for tracking plastid transformation in higher plants. Nat Biotechnol 17:910–915
40. Cramer CL, Boothe JG, Oishi KK (1999) Transgenic plants for therapeutic proteins: linking upstream and downstream strategies. Curr Top Micro- biol Immunol 240: 95–118
41. Ruiz ON, Daniell H (2005) Engineering cytoplasmic male sterility via the chloroplast genome by expression of b-ketothiolase. Plant Physiol 138:1232–1246
42. Arlen PA, Falconer R, Cherukumilli S, Cole A, Cole AM, Oishi KK, Daniell H (2007) Field production and functional evaluation of chloroplast- derived interferon-a2b. Plant Biotechnol J 5: 511–525.