Construction and Characterization of a Chloroplast Targeting Plasmid DNA Vector in *Nicotiana tabacum*

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A chimeric plasmid DNA vector designated as pMS65LBgus was constructed with the aim of targeting chloroplasts in *Nicotiana tabacum*. The Plasmid was designed to contain β-glucuronidase (*gus*A) gene under a modified chloroplast 16S rRNA gene promotor and a *psb*A gene terminator sequence. Adjacent to *gus*A gene, plasmid vector was designed to contain a selectable marker aminoglycoside 3' adynyle-transferase (*aad*A) gene under *atp*A gene promotor and *rbc*L gene terminator sequences. The entire cassette with two genes was flanked by ~1.0 kb fragment on each side, spreading through 3'*rps*12 gene and *orf*131 of the tobacco chloroplast genome. Both *gus*A and *aad*A genes orient in opposite directions and are expressed in *Escherichia coli*. The entire cassette with *aad*A and *gus*A genes along with their expression signals can be excised by *Nde* I restriction endonuclease digestion. Multiple cloning sites at both ends of the *gus*A coding sequence enable a desired passenger gene to be replaced with *gus*A. PCR amplification, Southern hybridization, restriction mapping and dot blot analysis of the vector revealed a high degree of homology of the 2 kb tobacco chloroplast targeting fragment in the vector with various plant species, suggesting that the targeting vector may also be useful in other plants.

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INTRODUCTION

Expression of prokaryotic genes in plant chloroplasts has been successfully achieved [1, 2, 3, 4] specially because of the availability of the chloroplast sequence data [5], recombination activity in plant chloroplasts [6, 7] and the development of biolistic gun [8]. Its advantages include high number of chloroplast genome copies per cell resulting in accumulation of high levels of a gene product [9], its similarity to prokaryotic regime & thereby efficient expression of natural prokaryotic genes and its maternal inheritance [10, 11]. Stable chloroplast transformation in higher plants, other than tobacco, has been demonstrated for Arabidopsis [12], Potato [13], Rice [14] and Tomato [15]. The vectors have been constructed which contain an antibiotic resistance gene for selection of transformants along with passenger genes e.g. *gus*A and *cry* IAC [2, 16, 17]. All of these vectors contain a chloroplast fragment flanking these genes, which help in targeting the integration of desired genes at a predetermined locus in a chloroplast genome. A homologous targeting sequence is a prerequirement for resident chloroplast and vector DNA to align and undergo recombination. Although chloroplast genome is highly homologous among plant species, sequence differences exist in spacer regions found between genes. These regions are usually chosen for plastid transformation, in order to avoid gene silencing. The vectors constructed with targeting sequences from particular species usually exhibit low rates of recombination in heterologous species and may not even work at all for some species. It necessitates construction of different vectors for different species, which requires skilled personnel, costly infrastructure and a significant amount of intellectual time. We have shown

earlier that chloroplast rRNA operon and its neighboring region is highly conserved in the family Asteraceae [18] and, therefore, could be the fragments of choice for construction of chloroplast transformation vectors. We show here construction and characterization of a plasmid DNA, designed to target tobacco chloroplasts and its potential for other species.

MATERIALS AND METHODS

Construction of DNA vectors: Standard genetic engineering techniques were used to construct and analyze the plasmid vectors [19]. To clone the tobacco plastid targeting fragment, a 6 kb *Hind* III-*Pst* I fragment of plasmid pTB9 [20] was ligated in pBluescript SK [+] corresponding sites. The resulting plasmid was digested with *Bst* EII, the ends were filled with klenow and ligated with a *Not* I linker (New England Biolabs). The resulting plasmid was digested with *Not* I restriction endonuclease and a 2.1 kb fragment was extracted off the gel, purified and ligated in *Not* I site of pSK [-] plasmid. The resulting 5 kb plasmid was digested with *Pst* I (to remove a duplicated 30 bp multiple cloning site) and self ligated, giving rise to plasmid pMS6.42.1 (Figure 1). Thus pMS6.42.1 contains a 2.1 kb fragment of tobacco chloroplast, flanking the parts of *orf*131 and *rps*12 gene (neighboring to rRNA operon) of the chloroplast inverted repeat region. It serves as a homologous recombination site between the exogenous vector plasmid and the resident chloroplast genome. The *Pst* I site in the 2.1 kb fragment of chloroplast refers to the sequence numbered as 142541, while *Not* I refers to the sequence numbered 140392 in tobacco chloroplast sequence [5].

To construct the *aad*A and *gus*A gene cassette, the chloroplast *psb*A gene terminator region [21] was amplified by using the oligos psbA5 and psbA3 (Table 1) and pTB28 [20] plasmid DNA as a template. The amplified 410 bp PCR product was doubly digested with *Nde* I & *Sac* I and ligated in the corresponding sites of pNEB193 vector (New England Biolabs) resulting in plasmid pMS62.22. Similarly the tobacco chloroplast 16SrRNA gene promoter was amplified by using the oligos 16SrDNA5 and 16SrDNA3 (Table 1) and pTB9 plasmid DNA as a template. This promoter was modified to create a synthetic *Shine-Dalgarno* (ribosome binding site) sequence during its PCR-amplification (Table 1, 16SrDNA3 primer). The amplified 130 bp product was digested with *Sal* I & *Xba* I and ligated in the corresponding sites of pMS62.22 resulting in plasmid pMS63.11 (Figure 1). The direction of transcription in pMS63.11, from the 16SrRNA gene promoter is oriented towards *psb*A gene terminator fragment and was confirmed by restriction mapping. The 1.89 kb expression cassette, containing bacterial amino-glycoside 3' adenyle-transferase (*aad*A) gene with flanking *Chlamydomonas reinhardtii* promoter (from *atp*A gene) and terminator (from *rbc*L gene) sequences, was excised from the plasmid pUCatpX-AAD [22] by doubly digesting with *Eco*RI & *Spe* I restriction endonucleases. The fragment was klenow filled and ligated in the *Nhe* I digested and klenow filled plasmid pMS63.11, resulting in plasmid pMS64L. The plasmid pMS64L was digested with *Nde* I. The 2.443 kb band was taken out of the gel, purified and ligated with similarly digested pMS6.42.1, resulting in plasmid pMS65LB. To clone the gusA gene, plasmid pDMC206 [23] was digested with *Eco*RI. The 1.87 kb *Eco*RI fragment containing a promoter and terminator-less *uid*A gene (also known as *gus*A gene) was excised off the gel, purified and mixed with *Pac* I digested pMS65LB plasmid. The mixture was klenow filled and ligated, resulting in the plasmid pMS65LBgus, which is about 9.3 kb in size.

***Escherichia coli* transformation, selection of clones and DNA extraction:** *E. coli* XL1Blue or DH5α competent cells were routinely transformed with various sub-clones as per standard protocols [19]. *E. coli* cells were grown in Luria Bertani (LB) media and selected on Ampicillin (50 µg/mL) and/or Spectinomycin (50 µg/mL). The plasmid DNA from *E. coli* cells was routinely isolated using Qiagen columns or by

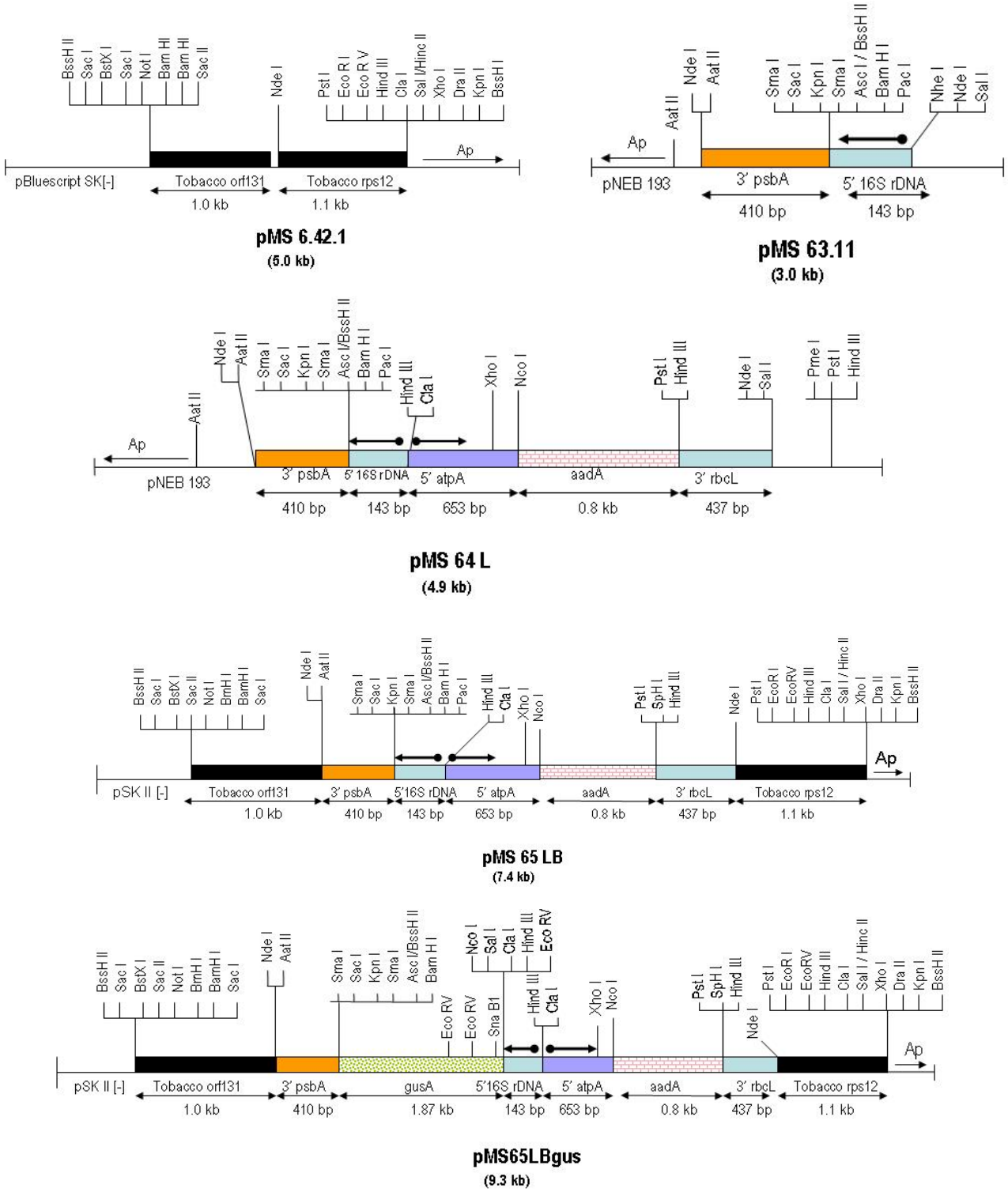

Figure 1. Diagrammatic sketch of plastid transformation vectors: 3' psbA and 5' 16SrDNA represent terminator and promoter fragments, respectively, from the corresponding genes of tobacco chloroplast genome. Similarly, 3' rbcL and 5' atpA represent terminator and promotor regions of the corresponding genes of *Clamydomonas reinhardii* chloroplast genome. Tobacco represents Tobacco chloroplast genome, flanking through *orf*131 and 3'*rps*12 gene. The *gus*A and *aad*A represent corresponding genes (ORF only) of *E. coli*. The numbers at bottom represent the size of various DNA fragments in base pair (bp) or kilo base (kb). The upper arrows show direction of transcription. The restriction sites are shown at the upper side of the map. Map is only diagrammatic and not to scale.

alkaline lyses method. The total genomic DNA from various plant species was extracted as described [18].

PCR amplification: The PCR reaction was carried out in a final volume of 50 µl containing 100 µM each of the four dNTPs, 5 Pico mol of each primer, 20 ng of genomic DNA and 0.3 units of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India). Each reaction tube was loaded with 20 µl of mineral oil. The amplification was performed in a Robocycler (Stratagene) which was programmed for 1 cycle of 5 min at 95°C followed by 37 cycles of 1 min at 94°C, 1 min at 58°C and 2 min at 72°C. The last extension cycle was programmed at 72°C for 7 mins. The amplified products were resolved in 1% agarose gel using 1X TAE buffer.

Southern Transfer: The PCR amplified products were purified using QIAquick PCR purification kit (Qiagen). The eluted DNA was quantified spectrophotometrically and 1 µg of each of the PCR product was run in 1% agarose gel and transferred onto Hybond N+ nylon membrane (Amersham) as per manufacturer's instructions in a 20X SSC transfer buffer. The DNA was fixed onto the membrane by baking for 30 min at 120°C.

Dot blot analysis: The Qiagen purified PCR products from various plant species were diluted in 1X TE buffer. PCR product was denatured for 10 min at 95°C and then snap-cooled on ice for 5 min. Various concentrations of DNA (50, 20, 5 and 2 ng) in 2 µl final volume were spotted onto nylon membrane. The membrane was baked at 120°C for 30 min and hybridized with DIG labeled 2 kb PCR fragment of tobacco chloroplast (as shown in plasmid pMS6.42.1 of Figure 1).

Probe-labeling with DIG, Southern hybridization and detection: The tobacco PCR product was resolved in a 1% agarose gel. The 2 kb band was excised, purified using a QIAquick gel extraction kit (Qiagen) and was used as a probe. The probe was labeled with digoxigenin (DIG) random primer labeling kit (Boehringer Mannheim). The prehybridization (5X SSC, 1% blocking reagent, 0.1% N-laurylsarcosine, 0.2% SDS), hybridization and washing were performed as described [24]. Hybridizations were carried out at 68°C in 20X SSC buffer (3M NaCl, 300mM sodium citrate pH 7.0). Filters were washed in 2X SSC buffer, 0.1% SDS at RT twice for 15 min, in 1X SSC, 0.1% SDS twice for 15 min and finally in 0.5X SSC buffer, 0.1% SDS twice for 15 min. The detection of bands was carried out with X-Phosphate and NBT color substrates as per manufacturer's instructions (Boehringer Mannheim).

RESULTS

Plastid targeting vector(s): A plasmid DNA vector pMS65LBgus was constructed to target tobacco chloroplasts through homologous recombination. During its construction several intermediate constructs resulted, some of which can be of importance in experiments designed to construct a plastid vector with different passenger genes or a different targeting sequence, for example, plasmid pMS65LB (Figure 1) is ideal to clone any passenger gene in a novel *Asc* I restriction site. In pMS65LBgus a promoter and terminator-less *uid*A gene was cloned under the tobacco chloroplast 16SrRNA gene promoter in which a synthetic Shine-Dalgarno (ribosome binding site) sequence was created during its PCR amplification (Table 1, 16SrDNA3 primer). To the distal 3' end of *gus*A gene, 3' untranslated region of tobacco plastid *psb*A photosynthetic gene (which is required for mRNA stability) was added. This *uid*A gene with tobacco plastid promotor and terminator signals was cloned next to a spectinomycin resistant transcriptional unit containing *Chlamydomonas* plastid *atp*A gene promotor and *rbc*L gene terminator fragments. Both of these *uid*A and *aad*A transcriptional units orient in opposite directions and are expressed efficiently in *E. coli*. The *uid*A and *aad*A genes along with their expression and stability signals are flanked by tobacco plastid DNA in both sides so as to

Table 1: Oligonucleotides used in this work

Restriction Sites	Oligo name	Sequence (5'---3')	Position
Sac I, Sma I	psbA5	gggagctcccggGATCTTGGCCTAGTCTATAGGAGGTTTTG	1063-1091
Nde I, Aat II	psbA3	cccatatgacgTCGAATATAGCTCTTCTTTCTTATTTC	1455-1429
SalI, NdeI, NheI	16SrDNA5	gggtcgacatatgctaGCCGTCGTTCAATGAGAATGGATAAG	139960-139935
Xba I	16SrDNA3	tttctagatccctccCTTGTATCCgTGCGCTTCATATTCGC	139849-139874
	TobC5	CCTCCGTGGAAAGGATGATTCATTCGG	140506-140531
	TobC3	CAGTACCTCGACGTGACATGAGCGTG	142518-140544

Note: The oligos used as primers for PCR are listed in 5'-3' orientation with the bases matching the template in upper case and the created restriction sites and *Shine-Dalgarno* sequence (RBS) in lower case. Note that the RBS is underlined. In 16SrDNA3 primer, a single base change has also been made in the primer (shown in lower case) where A has been changed to G to eradicate internal ATG in the primer. Numbering (position) is as in the references [Sugita & Sugiura, 1984] for *psb*A terminator and [Shinozaki et al., 1986] for 16SrDNA promotor sequence and the TobC chloroplast targeting sequence.

target their insertion into the plastid genome at a defined location. These homologous flanking plastid DNA sequences direct the insertion of foreign DNA between 3'*rps*12 gene and *orf*131 in the inverted repeat region of the plastid genome. This position of the chloroplast genome consists of mostly spacer region and is transcriptionally inactive.

Restriction mapping of plasmid pMS65LBgus: The various junctions of the final plasmid pMS65LBgus (Figure 1) were analyzed for correct orientation of the individual fragments. Figure 2 shows the restriction pattern of pMS65LBgus with twelve different restriction enzymes. It should be noted that *Not* I and *Asc* I enzymes exhibited single bands, while other enzymes exhibited 2 or more than 2 bands as expected, and as outlined in figure 1. *Nde* I digestion can be used to excise entire cassette with *gus*A and *aad*A genes along with their expression and stability signal sequences.

PCR primers designed from ends of 2.1 kb tobacco sequence amplify the corresponding plastid fragments in various plant species: The 2.1 kb tobacco chloroplast fragment was chosen as a target for homologous recombination

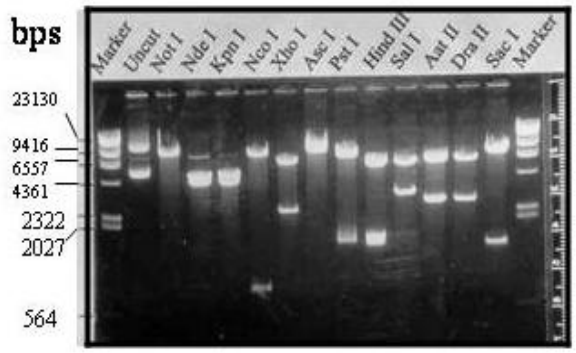

Figure 2. Agarose gel (1%) showing the restriction digestion pattern of the plasmid pMS65LBgus with respect to twelve different restriction endonuclease enzymes. Marker represents the λDNA digested with *Hind* III.

between resident chloroplasts and the chloroplast transformation vector pMS65LBgus. The forward and reverse PCR primers (TobC5 and TobC3) were designed from its flanking sequences (Table 1). The amplified fragment from tobacco genomic DNA using these primers was approximately 2 kb fragment (Figure 3, lane 1). The PCR amplification using genomic DNA isolated from twelve other plant species also exhibited a single major band of almost similar size in all the species except Rice (Figure 3, lane 10). The mobility of the PCR product for some plant species differed slightly, for example,

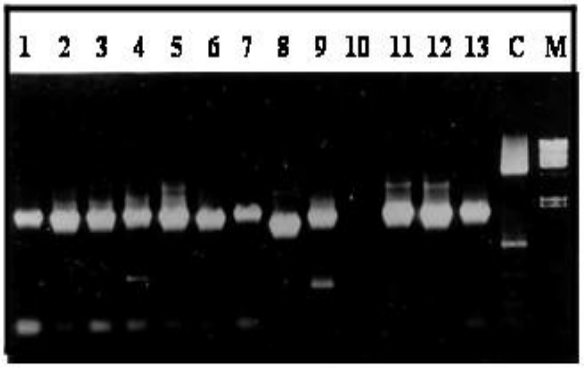

Figure 3. PCR-amplification products of thirteen different plant species using the primers TobC5 and TobC3 (Table 1). 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 represent Nicotiana tabacum, Artemisia vulgaris, Camellia assamica, Philodendron, Valeriana jatamansi, Rosa demascena, Ocimum sanctum, Olracea (Spinach), Triticum aestivum, Oriza sativa, Mentha piperata, Phaseolus vulgaris & Dendrocalamus hamiltonii, respectively. 'C' is PCR product of control plasmid pMS65LBgus. 'M' is Mol. Wt. marker λ*Hind* III digest.

mobility of the spinach PCR product was slightly higher (Figure 3, lane 8) as compared to others. Amplification of the philodendron and wheat DNA, in addition to a major band, also exhibited a band of about 300 bp (Figure 3, lanes 4 and 9). The control plasmid pMS65LBgus PCR product, in addition to a 6 kb expected major band, also exhibited a minor band of about 500 bp (Figure 3 & 4, lane C). The intensity of the amplified products was different for different species.

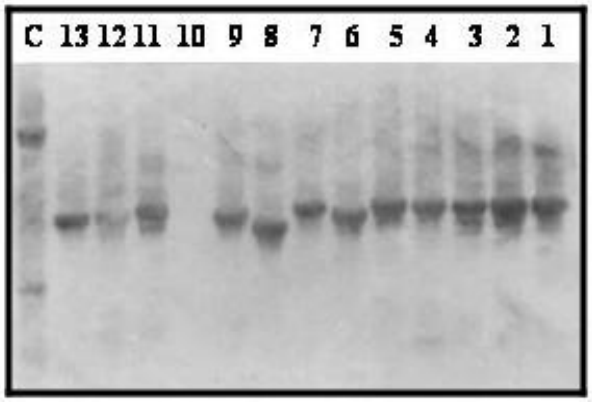

Figure 4. Southern hybridization of the PCR products of 13 different plant species (as in Figure. 3) using 2 kb PCR product of tobacco chloroplast genomic DNA as a probe. The sequence of the lanes is as shown in Figure 3.

Tobacco PCR product hybridizes with corresponding plastid fragments in various plant species: The 2 kb PCR product obtained with tobacco genomic DNA when used as a probe, hybridized with similarly obtained PCR products from twelve different species of plants (Figure 4). The intensity of hybridization signals for various plants was comparable to tobacco control (Figure 4, lane 1). Two small sized PCR products (~300 bp) in case of philodendron and wheat also hybridized but with lower intensities (Figure 4, lanes 4 and 9). The PCR products from the control vector pMS65LBgus (lane C) exhibited a 6 kb major band as expected but it also exhibited a band of about 500 bp. Dot blot hybridization also exhibited positive signals in all cases comparable to the PCR product of tobacco genomic DNA (Figure 6).

An internal *Nde* I site of tobacco PCR fragment is retained in majority of the plant species: When digested with *Nde* I, the 2 kb PCR amplified fragment of all the species except rose and spinach (Figure 5, lanes 6 & 8) exhibited two bands indicating presence of one internal restriction endonuclease site as expected. The sizes of the bands were quite similar for Tobacco, Artemisia, Camellia, Philodendron, Triticum and Mentha (Figure 5, lanes 1, 2, 3, 4, 9 and 11). The bands in lane 7 (Ocimum) and lane 12 (Phaseolus) were doublets and resolved on longer runs of the gel. It seems that in lane 5 (Valeriana) two *Nde* I sites were present in the fragment and, therefore, one band is similar to other species while other is truncated.

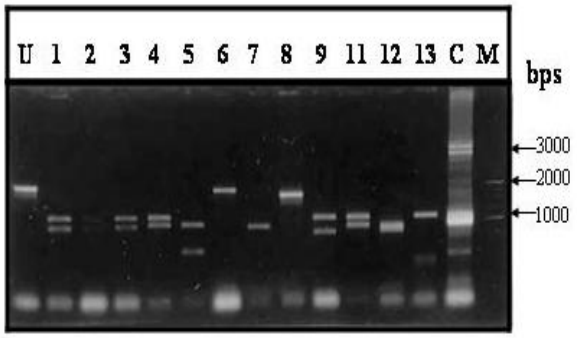

Figure 5. *Nde* I digestion patterns of the PCR-amplified products from 12 different plant species. The numbering is as listed in Figure 3. U is control uncut PCR product of *N. tabacum*. M is mol wt marker. Note that PCR product from lane 10 (Figure 3 and 4) was not included here and thus lane 10 is absent.

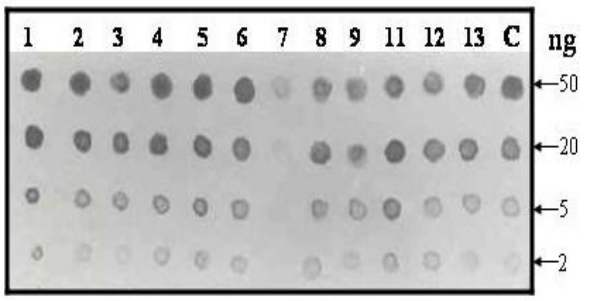

Figure 6. Dot blot analysis of the PCR products of 12 different plant species. The sequence of the lanes is as listed in Figure 3. Note that the PCR product from lane 10 (Figure 3 and 4) was not spotted here and thus lane10 is absent, similar to Figure 5.

DISCUSSION

Since the first appearance of transgenic plants in early eighties [25], much progress has been made in this area. A variety of DNA constructs are now available for nuclear transformation of plants. Majority of these vectors can be used for multiple plant species. However, low levels of expression (even with the strongest promoters available), gene silencing due to random integration of foreign genes, and the transgenic release into the environment, are some of the major concerns associated with nuclear transformation of plants. Chloroplast transformation obviates many of these problems, since gene integration is at a predetermined loci and the gene is contained within maternal compartment. The aim of this study was to design a plasmid DNA vector to transform chloroplasts of plants, and which can also serve as a basal vector, in which genes of commercial potential can be manipulated. In order to achieve these goals, we selected a region of tobacco chloroplast genome, which is known to be a spacer region flanking the 3'*rps*12 gene and *orf*131 of the tobacco chloroplast genome. Using this segment as a targeting sequence we have developed a pSK plasmid based chloroplast transformation vector. In the chimeric vector we have assembled a cassette with a selection marker *aad*A (an antibiotic resistance gene) and an expression gene (*gus*A). These genes were hooked with promotor and terminator signal sequences from chloroplastic genes, in order to express and stabilize the transcripts in the chloroplasts. The 16S rRNA promotor was modified so as to create a *Shine-Dalgarno* sequence, in order to enhance the gene expression. Similarly *psb*A terminator sequence was attached to 3' end of the gene, so as to stabilize the transcripts. We have shown earlier that both of these genes in plasmid pMS65LBgus express in *E. coli* efficiently [26]. Earlier large size of targeting sequences have been used [27] limiting the size of passenger genes that can be taken up into chloroplasts. In this vector, only about 1 kb of homologous sequence on each side of the gene cassette has been used, so as to reduce the unnecessary load of vectors. The entire cassette containing *aad*A and *gus*A gene is excisable by *Nde* I restriction endonuclease and, therefore, can be sub-cloned easily in any other plasmid vector or chloroplast backgrounds of other plant species. Both the transcriptional units of the vector have been assembled in such a way that they orient in opposite directions and, therefore, reduce the chances of polymerase crash or overlapping transcripts. We also analyzed the possibility of these vectors for targeting chloroplasts of other plant species. Three lines of evidences suggest that tobacco plastid targeting fragment in this vector is highly conserved across plant species and may be used for targeting many plant species: 1) The PCR primers designed after the tobacco plastid genome amplify the corresponding fragments in many plant species. Also most of the plant species retained *Nde* I site in their corresponding PCR fragments indicating high homology to tobacco. 2) The Southern hybridization of PCR bands from different species show comparable band intensities. 3) Dot blot analysis of total genomic DNA revealed intense signals in many cases (comparable to control).

In conclusion, we have constructed a unique plasmid DNA vector designed to target tobacco chloroplasts to express desired genes in tobacco plants, and possibly in other plant species. The vector and its intermediates can be

manipulated to develop species-specific targeting vectors with desired genes.

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