

## INTEGRATION OF NOVEL CHLOROPHYLL GENES FROM BLACK PINE INTO THE CHLOROPLAST GENOME OF TOBACCO

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### Abstract

Gymnosperms owing the presence of dark-operative protochlorophyllide oxidoreductase pathway are able to reduce protochlorophyllide to chlorophyllide leading to the formation of chlorophyll in dark, whereas angiosperms are unable to do this and are photosynthetically less efficient. Plastid encoded genes *chlL* and *chlN* are reported to be involved in the functioning of the enzyme Dark-operative Protochlorophyllide Oxidoreductase (DPOR). The genes were isolated from black pine and cloned into the species-specific chloroplast targeting vector; harboring FLARE-S, a fluorescent selection marker. The final transformation vector was used to introduce genes into the tobacco chloroplast genome using biolistic approach. Here, we report stable integration of both genes along with translationally fused marker genes (*gfp* and *aadA*) into the inverted repeat region of plastome which was confirmed by Polymerase Chain Reaction and Southern blot analysis. Morphological and physiological analyses of the transgenic plants compared with non-transformed wild type tobacco plants revealed that the activation of dark-operative pathway requires additional factors/genes to *chlL* and *chlN* genes to develop chlorophyll, and consequently photosynthetically competent chloroplasts.

### Introduction

Chlorophyll is the most plentiful natural pigment in a mature plant cell whether in the leaf of a plant or in the algal species. Chlorophyll is synthesized in the chloroplasts from glutamate, a precursor amino acid (Taiz & Zeiger, 2006). A number of enzymes are involved in the biosynthetic pathway and each of these has its own importance. Protochlorophyllide (Pchl) is a main metabolite for the biosynthesis of chlorophyll (*chl*) and bacteriochlorophylls (*bchl*). In photosynthetic organisms, there are 2 entirely different enzymes that catalyze the stereo-specific reduction of D-ring of Pchl to form chlorophyllide (*chl*). The first enzyme the light dependent Pchl oxidoreductase (LPOR) is ubiquitous among eukaryotic phototrophs and is consisted of nucleus-encoded subunits that are post-translationally targeted to the chloroplasts (Aronsson *et al.*, 2003). LPOR is present in almost all chlorophyll manufacturing organisms but missing in photosynthetic bacteria (Adamson *et al.*, 1997). In contrast, dark-operative Pchl oxidoreductase (DPOR) can reduce Pchl to chl and synthesize chlorophyll in the absence of light. DPOR is a plastid-encoded and multi-subunit enzyme and is found in photosynthetic bacteria, cyanobacteria, algae and gymnosperms but angiosperms lack DPOR (Armstrong, 1998). Organisms containing DPOR can have functional chloroplasts in the dark and start photosynthesis upon exposure to light (Kusumi *et al.*, 2006). Photosynthetic organisms that contain both LPOR and DPOR pathways of chlorophyll formation, for example, black pine are photosynthetically more competent as compared to angiosperms that contain only LPOR enzyme. Plastid encoded chlorophyll genes *chlL* and *chlN* are encoding key subunits of the DPOR enzyme. To-date, studies have shown that all organisms capable of synthesizing the chlorophyll in the dark are carrying these genes (Fujita, 1996) in the inverted repeat region, creating an operon in the plastid genome of black pine (Wakasugi

*et al.*, 1994). Introduction of foreign genes into chloroplast offers unique features when we compare it with nuclear transformation. These includes (i) high level of foreign gene expression due to its polyploidy nature (ii) introduction of more than one genes in a single transformation event in operon fashion as is prokaryotic in nature (iii) gene containment due to its maternal inheritance (iv) absence of pleiotropic/toxic effects due its compartmentalization status (v) targeted insertion of foreign genes also eliminates the position effect (vi) gene silencing has also not been observed in chloroplast transformation (Khan *et al.*, 2001; Daniell *et al.*, 2002; Bock & Khan, 2004). The aim of the present work is isolation and integration of plastid encoding DPOR genes (*chlL* and *chlN*) from black pine into the chloroplast genome of tobacco which is lacking the pathway.

### Materials and Methods

**Plant material and growth conditions:** Seeds of *Nicotiana tabacum* L. var. Petite Havana, were sterilized with 70% ethanol (in 50ml Falcon tube) for 3min to either loose gelatinous seed coat or to remove fungicide. Ethanol was removed and seeds were thoroughly rinsed with sterile water. A 10% Domestose (w/v) (house hold bleach containing 3-5.7% active hypochlorite) was added and shaken for 3min. Approximately 150 seeds were sown on seed germination medium containing plates (Murashige & Skoog, 1962).

**Species-specific vector construction for chloroplast transformation:** Transformation vector was constructed to integrate the chlorophyll genes into the chloroplast genome of tobacco. A PCR amplified fragment of ~2.2kb encoding flanking sequences was used for site specific integration of transgenes into the inverted repeat regions of chloroplast genome of tobacco. To make easy the cloning steps, an oligo containing MCS (multiple cloning sites) was introduced in-between the flanking sequences which divides it into left targeting region (LTR) and right

targeting region (RTR) as described by Khan *et al.*, (2007). For selection and screening of transformation events, FLARE-S (Fluorescent Antibiotic Resistance Enzyme, Spectinomycin and Streptomycin) marker gene was cloned in the MCS. This marker was comprised of two genes i.e. *aadA* a gene for spectinomycin resistance and a reporter *gfp* gene which facilitate visual identification of transformed cells. These two genes were translational fused to facilitate selection as described by Khan & Maliga, (1999). The expression of marker gene was driven by *Prm* promoter which provides the signals for binding of RNA polymerase to initiate transcription while *TpsbA* was used as terminator sequence for the stability of transcribed RNA.

**Tobacco chloroplast transformation and regeneration of transformants:** Integration of final tobacco chloroplast vector into the plastome was achieved by the biolistic DNA delivery method, using PDS 1000/He gene gun (Bio-Rad, Germany) after coating plasmid DNA on 1µm size gold particles. For transformation, fully expanded dark green leaves of 4-6 weeks old were used. Leaves were cut under aseptic conditions and placed abaxial side up on Whatman No.1 filter paper on MS medium plates. After 48 hours of transformation tobacco leaves were chopped into 5x5mm small pieces and placed on RMOP regeneration medium, supplemented with 500 mg/L spectinomycin dihydrochloride (Svab & Maliga, 1993). Regenerated shoots on selective medium were shifted into magenta boxes, containing MS rooting medium for the development of roots and proliferation of shoots. Rooted plants with expanded leaves were used for further analysis.

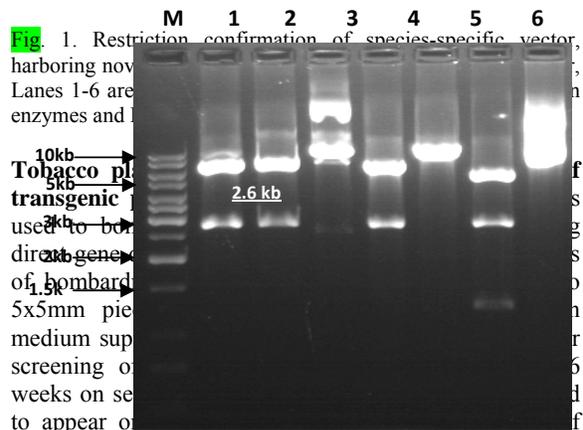
**Genetic analysis of antibiotic resistant tobacco plants:** Genetic analysis of antibiotic resistant as well as wild type tobacco plants was carried out by isolation of total cellular DNA by CTAB (hexadecyltrimethyl ammonium bromide) method with some modifications (Rogers & Bendich, 1985). This DNA was used as template in PCR and Southern blot analysis using biotin labeling kit (MBI Fermentas, Italy). Integration of transgenes into tobacco plastome was confirmed by using different set of primers. The PCR programme consisted of 30 cycles of 95°C for 2 min. 56°C for 2 min. and 72°C for 3 min. with a step of 72°C for 10 min. The amplified product was visualized under UV gel documentation system (VilberLourMat, France). Integration of expression cassette was also measured with the help of Southern blotting by using selection marker (*aadA*) as a probe.

**Inspection of transgenic plants for FLARE-S:** Transgenic plant leaves were inspected for the expression of FLARE-S with the help of an Olympus SZX (Olympus SZX9, Japan) stereomicroscope equipped for *gfp* detection with a CCD camera. The images generated by *gfp* fluorescence were recorded using computer (Khan & Maliga, 1999). The size of the fluorescent sectors was different in different leaves of transgenic plants

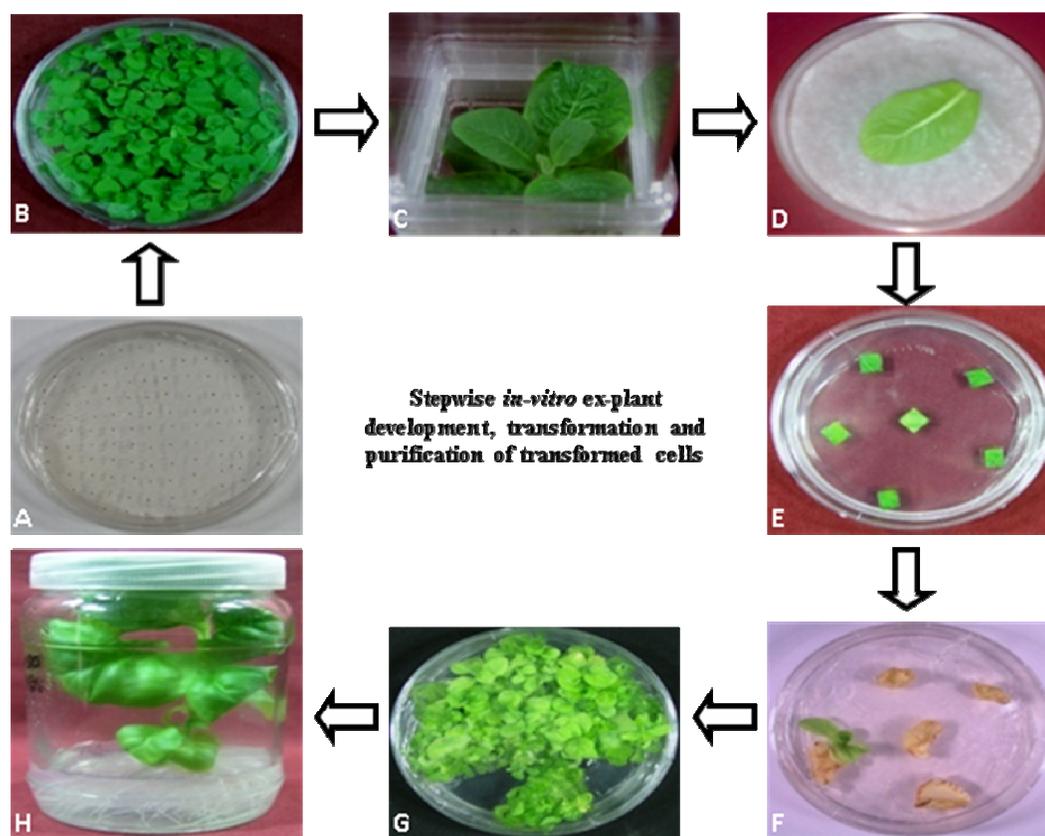
depending upon the segregation of the transformed cells from wild type cells.

## Results

**Development of species-specific vector for integration of chlorophyll genes:** Plastid transformation vector was developed to integrate the novel chlorophyll genes into the inverted repeat regions of tobacco through homologous recombination technique due to the targeted sequences that were cloned in the transformation vector along with FLARE-S marker. An operon of ~2.2 kb encoding chlorophyll genes (*chlL* and *chlN*) was PCR amplified from the total genomic DNA of black pine and cloned in PCR cloning vector pTZ57R (MBI Fermentas, Italy). The genes were regulated by a strong ribosomal RNA promoter (*Prm*) which was ligated by using *NheI/KpnI* restriction enzymes. This chlorophyll gene expression cassette was introduced at the upstream of the marker gene by using *PstI* restriction site in the final transformation vector. The orientation of genes in the final transformation vector was confirmed by using a series of restriction enzymes (Fig. 1) as well as with the help of polymerase chain reaction using gene specific primers.



harboring novel chlorophyll genes. Lanes 1-6 are digested with different restriction enzymes and the resulting fragments were visualized under UV light. The 2.6 kb band in lane 1 indicates the presence of the chlorophyll gene expression cassette. The 1.5 kb band in lane 2 indicates the presence of the marker gene. The 5x5mm pieces of tobacco leaves were cultured on MS medium supplemented with 500 mg/L spectinomycin for 4-6 weeks on selective medium to appear of antibiotic resistant shoots. The selection marker gene *aadA* in the transformation vector. The gene confers resistance to antibiotics, spectinomycin and streptomycin which inhibit growth of untransformed cells (Fig. 2F) on the medium. The antibiotic resistant shoots formed on the regeneration medium were transferred onto MS medium, containing spectinomycin to the level of 500mg/L (the same concentration as used in regeneration medium) for the development of roots and proliferation of shoots (Fig. 2H). Antibiotic resistant plants exhibiting the same phenotype as that of wild type plants were maintained on MS medium without antibiotic. The transplastomic shoots were screened from the escapes and nuclear transformants (due to illegitimate recombination events) through repeated rounds of selection and regeneration on antibiotic containing media. Regenerated resistant plants were then analyzed through Polymerase Chain Reaction (PCR) after every round of regeneration and screening to detect the plasmid level (Khan *et al.*, 2007).

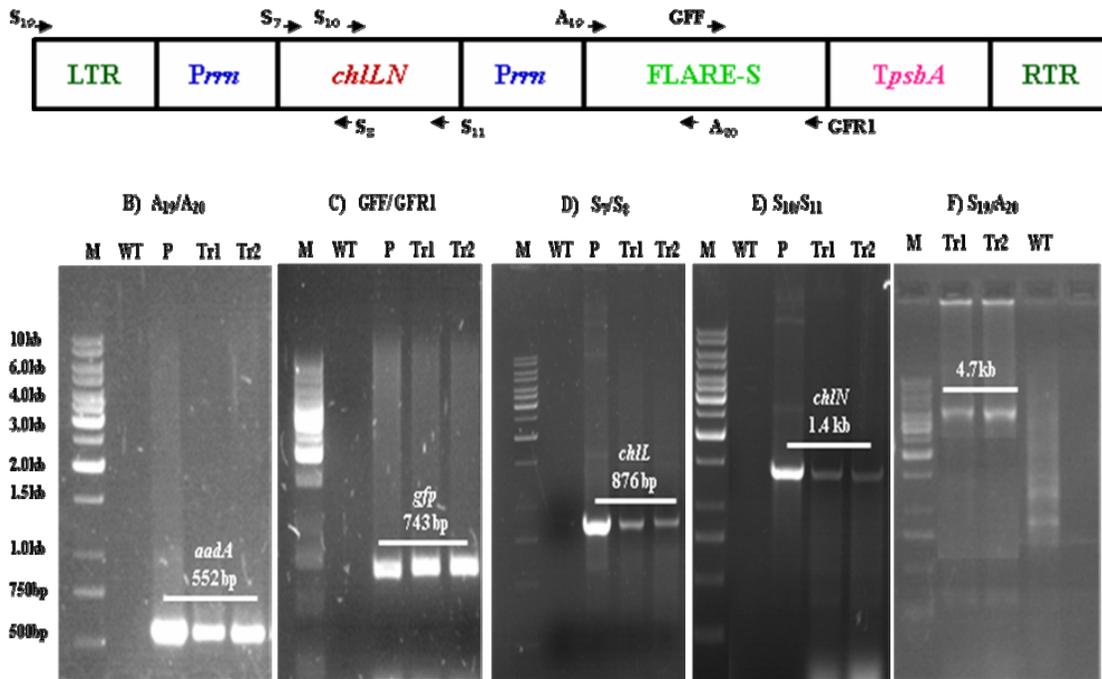


**Fig. 2.** Stepwise *in-vitro* ex-plant development, transformation, selection and regeneration of tobacco plants under aseptic conditions. (A) sterilized tobacco seeds on MS medium (B) Seedlings on MS medium (C) Plants with proliferated leaves in the magenta box (D) Fully expanded leaves used for biolistic transformation (E) Chopped bombarded leaves on antibiotic containing regeneration medium (F) Regenerated shoots on selection medium (G) Second round of regeneration (H) Transgenic tobacco plant with proliferated leaves and roots on selection media.

#### PCR based screening of antibiotic resistant clones:

Early stage purification of putative transgenic plants is very important for eliminating the undesired transformation events, for example, events due to illegitimate recombination of transformation vector with nuclear genome or escapes that manage to grow on antibiotic containing medium. The transformation vector was composed of FLARE-S marker gene hence two approaches were employed for the screening of transformation events including Polymerase Chain Reaction (PCR) and by fluorescence. Total cellular DNA was extracted from both putative transgenic and wild type tobacco plants using CTAB method (Rogers & Bendich, 1985) for template in PCR reactions. Fig. 3B shows the amplification of 552bp of selection marker gene (*aadA*) by using primer A<sub>19</sub>/A<sub>20</sub>. A 743bp fragment of visual marker gene (*gfp*) was also amplified with GFF/GFR1 primer set (Fig. 3C). The wild type tobacco DNA was used as a negative control and plasmid DNA as positive control. For the amplification of novel chlorophyll genes from putative transgenic plants two primer sets S<sub>7</sub>/S<sub>8</sub> and S<sub>10</sub>/S<sub>11</sub> were used for *chlL* and *chlN* genes, respectively. The amplification of 876bp of *chlL* and 1.4kb of *chlN* shows that the integration of transformation vector into tobacco plants with novel chlorophyll genes has been successfully achieved (Fig.

3D, E). The transformation vector was planned to incorporate the foreign genes into the inverted repeats of plastome. For the confirmation of site specific integration another primer pair was employed. In this experiment, one primer anneals with the native plastid genome sequence while second primer anneals within the transforming cassette. This primer set eliminates the amplification of any fragment from nuclear transformants and escapes. The amplification of 4.7kb fragment using primers S<sub>19</sub>/A<sub>20</sub> confirms the successful integration of transformation vector into the inverted repeats of tobacco plastome (Fig. 3F).

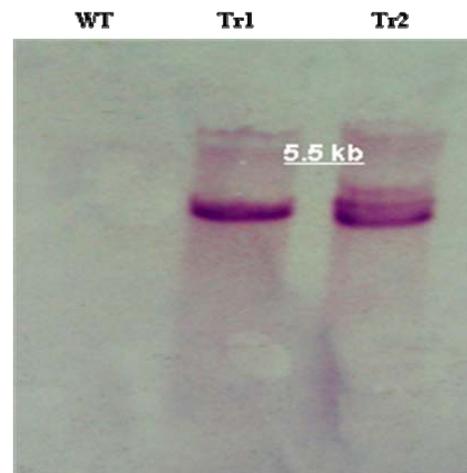


**Fig. 3.** PCR based genomic analysis to confirm transgene integration into the plastome of tobacco (A) Physical map of chloroplast transformation vector with primer positions (B,C) amplification of marker genes (*aadA*, *gfp*) used for selection of transformation events (D,E) amplification of genes of interest (*chlL*, *chlN*) (F) confirmation of chloroplast integration of transgenic cassette. Lane M is 1kb DNA ladder, WT is wild type plant DNA, P is the plasmid DNA, Tr1+Tr2 represent DNA from transgenic plants.

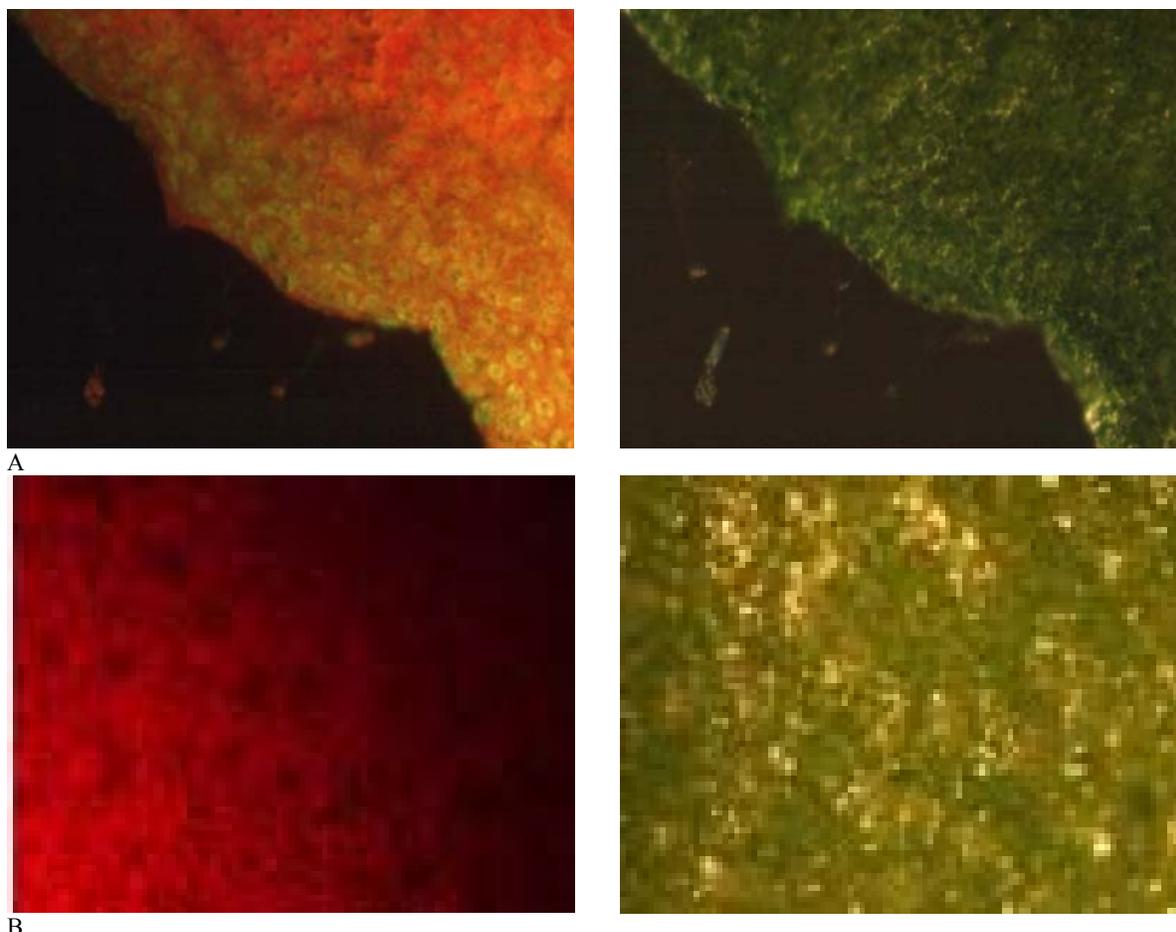
**FLARE-S tracking by stereomicroscopy:** As the transformation vector also carries FLARE-S marker gene, therefore visual screening of transformed shoots on medium was carried out. Basically it was to identify and differentiate transformed shoots from heterozygous population as described elsewhere (Khan & Maliga, 1999). When exposed to blue light, *gfp* fluoresced green, the intensity of fluorescence was directly proportional to the amount of protein present (Fig. 4). The size of the fluorescent sectors was different in different leaves in heterozygous transgenic clones depending upon the segregation of the transformed cells from wild type cells. Thus regeneration of transgenic plants was significantly helped by visual identification of the fluorescence at different stages of development.

**Confirmation of tobacco transplastomes by Southern blot analysis:** Transgenic plants were also analyzed for the integration of transgenes by using Southern blotting technique. Genomic DNA of 15µg from transgenic as well as wild type plants was digested with *Apal* restriction enzyme and electrophoresed on 1% agarose gel at low voltage. Upon restriction with *Apal* enzyme the *chlLN* transplastomic plants produced two fragments; one of 5.5kb carrying the marker gene (*aadA*) and other of 3.3kb having only expression cassette, whereas untransformed tobacco plants produced only one fragment of 4.2kb, containing only inverted repeat regions. When *aadA* was used a probe, only 5.5kb fragment from

transformed plants which carry the marker gene cassette was hybridized with the probe however, no DNA fragment was hybridized with the probe from DNA extracted from wild type tobacco plants (Fig. 5), confirming the successful integration of transformation cassette into tobacco plastome.



**Fig. 5.** Southern-blot analysis to determine integration of transformed cassette into tobacco plants using marker gene (*aadA*) as a probe. WT represents wild type plant with no hybridized fragment whereas Tr1 and Tr2 represent transgenic plants, carrying the marker gene containing transformation vector.



**Fig. 4.** Identification of transplastomic leaf sectors for FLARE-S. Left panel represents leaf sectors, transgenic (A) and wild type (B) under fluorescent illumination whereas, right panel under bright field illumination.

## Discussion

During the process of evolution, photosynthetic bacteria, cyanobacteria and gymnosperms maintain the ability of chlorophyll synthesis in the dark while angiosperms including rice, wheat, tobacco etc. have lost this capability. Organisms that contain the dark chlorophyll synthesis pathway can develop functional chloroplasts in the dark and start photosynthesis upon exposure to light. These plants are also photosynthetically more competent than plants that are lacking the pathway (Kusumi *et al.*, 2006). Gymnosperms, carrying dark operative chlorophyll formation pathway contains DPOR in addition to LPOR. DPOR is the sole operative reductase in the dark, LPOR is necessary in the high light whereas in low light both function (Fujita *et al.*, 1998). In our studies, plastid encoded DPOR genes (*chlL* and *chlN*) were introduced into the tobacco chloroplasts that lacks these genes. For selection and screening of transformation events FLARE-S marker was used. This marker was composed of two genes *aadA* for selection against spectinomycin and *gfp* to facilitate visual identification. These were translationally fused to generate FLARE-S as described by Khan & Maliga, (1999). PCR, being the most sensitive technique to evaluate the transformation events, was used to confirm transgenic tobacco plants developed using biolistic DNA

delivery approach. In PCR reactions, gene specific primers in various combinations were used (Fig. 3B, C, D and E). Transformation event was also verified by Southern hybridization using selection marker gene (*aadA*) as a probe. It confirmed that the final transformation vector have been successfully integrated into tobacco plants (Fig. 5). However these experiments only confirmed the development of transgenic tobacco plants with chlorophyll genes but not the integration of transgenes into the plastome. By extending the screening period on selection medium, regeneration of screened transplastomic clones was carried out. For confirmation of site specific integration into the plastome, another primer set ( $S_{19}/A_{20}$ ) was used that resulted in the amplification of desired fragments, eliminating the possibility of nuclear transformants and mutants (Fig. 3F). Recently, *gfp* has appeared as a versatile marker for the analysis of biological processes in living organisms (Hibberd, *et al.*, 1998). When exposed to blue light, *gfp* emits bright green fluorescence, comparable to the amounts of expressed transprotein. Unlike luciferase and  $\beta$ -glucuronidase, GFP requires no substrate and have no harmful effects on phenotype (Shen, *et al.*, 2012). Leaves of transgenic plants, showing variable green sectors under blue light confirmed the presence of *gfp* gene and the selection cassette (Fig. 4). Photosynthetic parameters like stomatal conductance, water use efficiency,

transpiration rate etc were also estimated through infra red gas analyzer (IRGA) (Kanwal, *et al.*, 2011), but no significant differences were observed between the transgenic and wild type plants, suggesting the activation of dark-operative pathway requires additional factors/genes to *chlL* and *chlN*. Further, transplastomic plants were phenotypically indistinguishable when compared to wild type plants, indicating the absence of peliotropic effects.

#### Acknowledgement

This research work was funded by the Higher Education Commission (HEC) Islamabad, Pakistan. Authors thank Ministry of Science and Technology (MoST) and Pakistan Science Foundation (PSF) for awarding projects to MSK.

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(Received for publication 1 September 2012)