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Silenced Phytoene desaturase Gene as a Scorable Marker for Plant Genetic Transformation

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Abstract: Currently used marker systems either require cumbersome assays and specialized equipment, or have bio-safety concerns. Here we report construction of Phytoene desaturase (PDS) intron-hairpin RNAi vector for its application as a scorable marker in plant genetic transformation. The transgenic nature of the transformants can be confirmed by mere phenotypic scoring of the bleached and dwarf regenerants, as a result of PDS gene silencing. Thus, the described method will bypass the needs of time consuming, skill and labor intensive molecular and biochemical transgenic confirmation assays that are generally employed for selection and screening of the true transformants.

Key words: Noninvasive transgenic confirmation, phytoene desaturase, RNAi, transgenic confirmation, transcript expression

INTRODUCTION

Marker genes are routinely used in plant genetic transformation protocols to ensure the selection/scoring of transformed cells/tissues from that of non-transformed. Among the selectable markers, antibiotic and herbicide resistance genes have been the most widely used in plant genetic transformation (Miki and McHugh, 2004). An examination of the selectable marker genes used in commercial transgenic varieties showed that neomycin phosphotransferase-npt II was the choice of marker in genetic transformation. The product of npt II gene can detoxify Kanamycin and other aminoglycosidic antibiotics by phosphorylation, thereby imparting selective advantage to the transgenic. Popularity of these marker systems reflects the efficiency and general applicability of their use across a wide range of species and regenerable tissue culture systems. However, the toxicity of the antibiotic varies greatly with the genotype and the growth stage. Therefore, it is necessary to standardize the optimum concentration of antibiotics for selecting the transgenic plants. Further, co-transformation with these selectable marker genes has its own bio-safety concerns (Rosellini, 2012; Yau and Stewart, 2013). As against the selectable marker genes, reporter genes do not provide a transformed cell with a selective advantage, but mark it so as to enable detection of transgenic material from that of non-transformed. Green Fluorescent Protein (GFP) has been particularly important as the assay is noninvasive and simple to apply without the addition of exogenous substrates or cofactors (Stewart, 2001). However, the marker system suffers with drawbacks such as low stability of the protein and the interference from cellular auto-fluorescence (Jouzani and Goldenkova, 2005). The reporter gene β-galactosidase (GUS) was a very important tool in transgenic research though suffers with disadvantages such as similar endogenous activities by the host enzymes and requirement of destructive assays (Jefferson, 1987). As a reporter, luciferase can be monitored in living tissue but this requires cumbersome assays and specialized detection equipment (Ow *et al*., 1986).

RNA interference (RNAi), a biological process of degradation of specific mRNA molecules, has become a widespread method for gene silencing since its discovery in 1998 (Fire *et al*., 1998). In order to find alternatives to the use of antibiotics as selection agents, or cumbersome assays for the scorable markers, we followed a targeted approach utilizing a RNAi technology to silence an expression of plant gene encoding Phytoene desaturase (PDS), a key enzyme of carotenoid synthesis pathway. The PDS, a highly conserved gene (Lopez *et al*., 2008), has been cloned and characterized from a number of plant

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species (Matthews *et al*., 2003; Liu *et al*., 2013). The catalytic activity of PDS when blocked, leads to accumulation of phytoene, characterized by albino and dwarf appearance (Qin *et al*., 2007). Thus, silencing of the gene would result in dwarf albino regenerants, which can potentially be used as a strong scorable marker for a number of scientific quests.

Here we describe construction of RNAi silencing vector for PDS gene and its application as a scorable marker in *Agrobacterium* mediated genetic transformation in Tobacco (*Nicotiana tabacum* L.). The modified binary vector appears superior to other contemporary selection/scorable systems as no post-transformation assays are required to be performed and thus saves both time and cost of biochemicals.

MATERIALS AND METHOD

Arabidopsis thaliana PDS gene (NM_202816.2) fragment of 128 bp (601-728 bp) was custom synthesized (GeneScript, USA) in sense and antisense orientations, separated by a 192 bp fragment of intronic sequence derived from the second intron of *ST-LS1* gene (X04753). The recombinant polynucleotide molecule (Fig. 1) thus, derived was inserted in MCS of binary cum shuttle vector pRI 101-AN (Takara Bio Inc., Japan) at *Kpn* I and *Bam*H I sites (pRI 101-AN-PDS-Sil), which was further transferred to *E. coli* strain DH5α using heat shock transformation method. The transformation was confirmed by double digestion of isolated plasmids with *Kpn* I and *Bam*H I enzymes (Fermentas, Canada) and through real-time PCR targeting specific detection of co-transformed *npt* II gene (Fig. 1). The recombinant plasmid was further transferred into *Agrobacterium tumefaciens* strain *GV3101* using heat shock and the transformation was validated using double digestion and real time PCR, as above.

Agrobacterium tumefaciens strain *GV3101* harboring the recombinant vector were grown overnight at 28°C and cells were harvested as pellet by centrifuging (6000 g) the cultures next morning. Pellet was re-suspended in liquid plant regeneration medium (MS+1 mg L^{-1} BAP+0.1 mg L^{-1} NAA) containing acetosyringone (100 μM) to a final OD 600 nm of 0.1, for further use in infection process. The leaf explants prepared of size 1 cm^2 from *in vitro* grown tobacco plants were infected with the *Agrobacterium* suspension for 15 min in incubator shaker at 28°C and 150 rpm. The leaf explants were blot dried and transferred to co-cultivation medium (plant culture medium

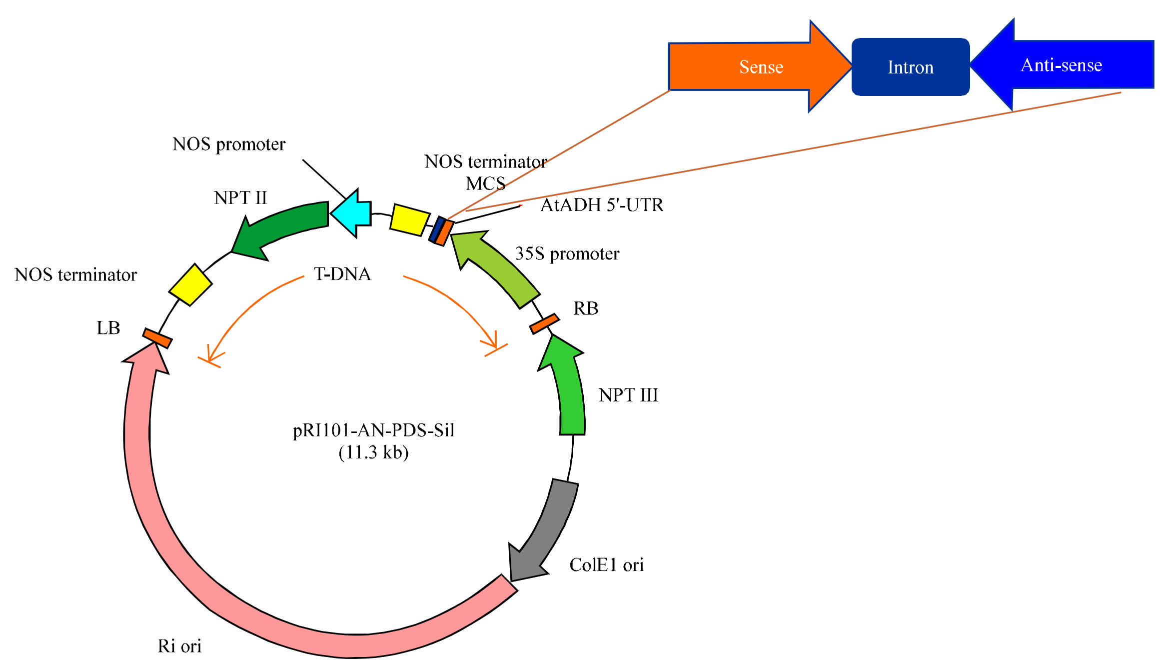

Fig. 1: Custom designed PDS silencing construct constituted of sequence of 128 bp in sense (Red color) and antisense (blue color) orientation separated by intron sequence of 192 bp

supplemented with acetosyringone) and incubated in dark for 48 h. The cultures were washed with cefotaxime (250 mg L^{-1}) solution, blot dried and transferred on plant regeneration medium supplemented with cefotaxime (250 mg L^{-1}) and incubated for 21 days in light/dark cycle of 16/8 h for shoot regeneration. The regenerants were further transferred to kanamycin (150 mg L^{-1}) selection medium to eliminate the non transformants. The surviving regenerants were grown for further cycles.

Genomic DNA isolated from the randomly selected 12 bleached plantlets was used as a template in a real-time PCR. Further, the total RNA was isolated from three of the bleached regenerants and wild type using TRIzol (TRI reagent) method to quantitate the transcript expression of the PDS gene with qPCR analysis. The normalized (using β Actin) relative transcript expression was analyzed using delta delta Ct method (Livak and Schmittgen, 2001).

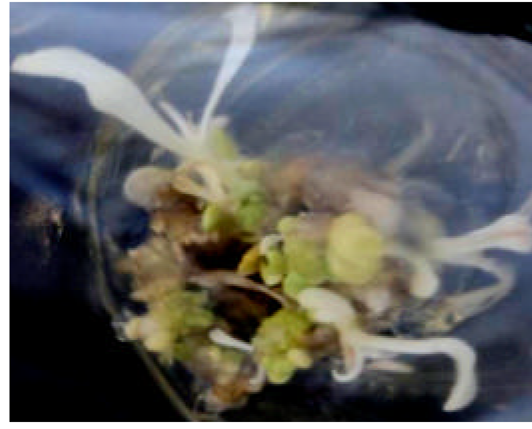

Fig. 2: Bleached phenotype regenerating on Kanamycin selection medium due to silencing of the PDS gene, wild types could not survive the selection

RESULTS AND DISCUSSION

Intron hairpin RNA (ihpRNA) constructs are efficient means of gene silencing compared to the hpRNA, co-suppression and anti-sense silencing constructs in a range of plant species (Wesley *et al*., 2001). We have used ihpRNA construct with arm sequence of 128 bp from the coding sequence of the PDS gene for development of distinct, bleached and dwarf phenotypes, a scorable trait in transgenic plants, for its potential use as a 'marker'. The designed PDS gene RNAi construct was validated in tobacco, a model system. The regenerants from the transformed and control (wild type) leaf explants were transferred to the regeneration medium supplemented with Kanamycin for selection of the putative transformants. Growth of the wild type regenerants was gradually declined on the selection medium and all the regenerants ultimately died. Putative transformants successfully survived on the selection medium. Bleaching in transformed regenerants (Fig. 2), due to silencing of the PDS gene was observed after 35 days of transformation. The bleached regenerants appeared dwarf as compared to the non-transformed counterparts without selection, in line with an earlier report (Qin *et al*., 2007), wherein albino and dwarf phenotypes were obtained in a T-DNA insertion mutant of PDS gene in *Arabidopsis*. The PDS gene silencing impairs the chlorophyll, carotenoid and gibberellin biosynthesis, leading to the bleached and dwarf phenotypes (Qin *et al*., 2007). Further, the genomic integration of the silencing cassette in all the randomly selected 12 bleached regenerants was confirmed by npt II TaqMan® probe based real time PCR analysis, using genomic DNA as a template. Cts were obtained in all the tested bleached plantlets indicating the presence of the npt II gene and the silencing construct but not in genomic DNA isolated from non transformed plantlets/regenerants (Fig. 3). Further, the quantitative transcript expression analyzed from three of the confirmed transformed plantlets, revealed the reduced expression of PDS gene by up to 45% than the wildtype control (Fig. 4).

Recently, Liu *et al.* (2013) reported engineering of *Chlamydomonas reinhardtii* PDS gene to enhance its desaturation activity and thereby resistance to herbicide norflurazon for its application as a dominant selectable marker in the green alga. However, the advantage of the silenced PDS as a reporter gene over the engineered counterpart is its potential use as a scorable marker to assess successful transformation without employing any selection. Appearance of bleached tissues is an early and powerful indicator of the successful transformation event. Noninvasive transgenic confirmation based on phenotypic scoring of the regenerents is time and cost effective, easy and does not need any specialized training, unlike skill and labor intensive molecular and biochemical assays currently popular for selection and screening of the true transformants. The polynucleotide construct described here and the methods employed may find immense uses by the community of plant molecular biologists for optimization of genetic transformation in other plant systems.

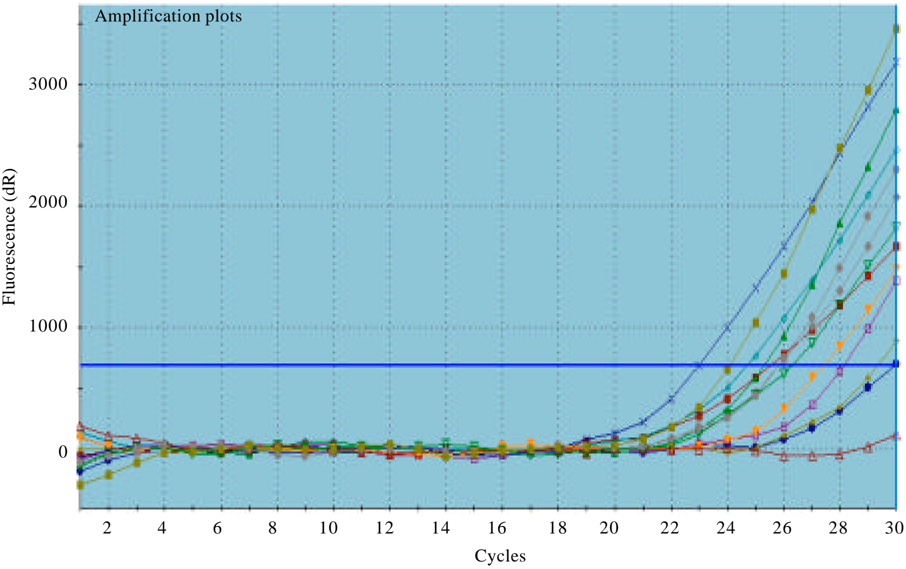

Fig. 3: Amplification plot depicting npt II TaqMan® probe real time PCR based transgenic confirmation of tobacco regenerants transformed with the PDS silencing construct, Genomic DNA isolated from the transformants and wild type was used as a template in a real time PCR. Cts were obtained only in transformants, confirming their transgenic nature

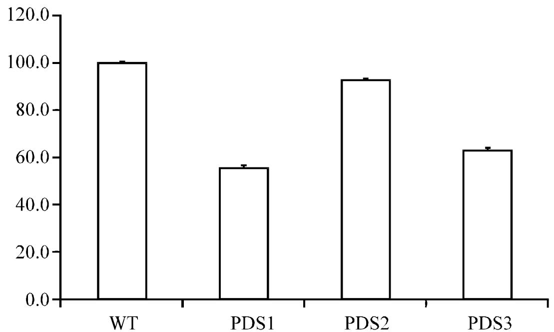

Fig. 4: Transcript expression analysis of the PDS gene in the bleached regenerants transformed with the RNAi construct. Total RNA was isolated from the regenerants (Wild type: WT; Bleached: PDS1-3) for the confirmation of the reduced transcript expression of the gene in the silenced lines. The columns indicate mean (n = 2) relative transcript expression in the bleached regenerants over the WT. Error bars indicate the SEM

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