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Epigenetic regulation of dihydroflavonol reductase in petunia and sensitivity of chalcone synthase promoter to RNA dependent DNA methylation

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Abstract

Petal colour is an excellent marker to study regulation of dihydroflavonol 4-reductase (DfrA) and chalcone synthase (Chs) involved in plant pigment biosynthesis. Still, alternative regulation of genes without altering their primary DNA sequences is still developing. In this study, fragments of DfrA promoter and coding region were transformed and tested for activity in petunia *(Petunia hybrida*) severely methylated in the promoter. On the other hand, we examined if inverted repeat construct of ChsA promoter is sufficient to methylate homologous sequences to induce silencing in petunia. We report that W80 petunia lines having W4 constructs produced flowers with pink petals. Stunted growth, delayed maturation and patched yellow coloration of leaves were evident in V26 lines transformed with V2, though the plants did not yet flower by the end of the study period. However, all V26 lines with V4 produced flowers with purple petals. It was unexpected to note that W80 plant lines with V4 produced white flowers. The fact that W80 plant lines with W4 produced purple flowers suggests that both the transgene and DfrA promoter may have been active in a white petal plant. The results suggest that the promoter was active to drive the expression of pigmentation genes.

Keywords: petunia, DNA methylation, *Agrobacterium tumefaciens*, chalcone synthase, dihydroflavonol 4-reductase

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Introduction

Mendel's demonstration of colour variation in pea has remained one of the most studied metabolic processes in plants (Winkel-Shirly, 2001). Over time, other important pathways were unraveled including those involved in plant secondary metabolism and biosynthesis of flavonoids (Quattrocchio et al., 1993). Apart from other crucial physiological functions, floral pigments play a role in plant signaling, UV protection, defense, and recruitment of pollinators and seed dispersers (Winkel-Shirly, 2001, Pervaiz et al., 2017). As precursors of flavonoids, several enzymes are critical in converting phenylalanine to 4-coumaroyl CoA and subsequently to chalcones, flavanones, flavanols, flavandiols, anthocyanins, and condensed tannins (Mol et al., 1989, Napoli et al., 1990, Winkel-Shirly, 2001). However, chalcone synthase (Chs) catalyzes initial enzymatic reaction that yields various classes of flavonoids (Quattrocchio et al., 1993; Kanazawa et al., 2007). Other members of Chs gene family exist, nonetheless, only ChsA and ChsJ are expressed in floral tissues during normal development and synthesis of secondary metabolites (Koes et al., 1989). Previous studies

have shown that genes encoding Chs and dihydroflavonol 4-reductase (Dfr) are jointly activated via similar transcriptional mechanisms to regulate anthocyanin biosynthesis (Napoli et al., 1990, Dooner et al., 1991). In petunia, however, the Dfr gene family consists of DfrA, DfrB, and DfrC located on chromosomes IV, II and VI, respectively (Beld et al., 1989; Huits et al., 1994). As the most active, DfrA is expressed predominantly in plant petals (Beld et al., 1989). It contains six exons that are separated by five introns (Figure 1) (Huits et al., 1994). Additionally, the DfrA promoter contains regulatory motifs (-CACGTG-) that facilitate transcriptional activation (Figure 1).

Plants evolved methylation-induced silencing of repetitive sequences and transposon immobility to regulate large genome sizes as well as maintain variety across species (Kumar & Bennetzen, 1999; Buckler & Thornsberry, 2002). Briefly, DNA methylation is the transfer of methyl group to cytosine residue in a DNA methyltransferase-catalyzed reaction (Rabinowicz et al., 2005). Patterns of DNA methylation have been shown to correlate with gene expression considering that epigenetic features affect binding and interaction of regulatory molecules (Zhang et al., 2016). For instance, methylation is associated with sequence repeats and transposon silencing and varies in range from 6% in Arabidopsis to 25% in maize (Papa et al., 2001; Wada et al., 2003). However, mammalian DNA methylation is mostly restricted to cytosine-guanine (CG) even though studies have shown that other sequences are methylated in mouse embryonic stem cells (Bird, 2002; Ramsahoye et al., 2000). Originally discovered in tobacco plants, RNA directed DNA methylation (RdDM) is the first known RNA-guided epigenetic modification of the genome (Matzke & Birchler, 2005). It involves small RNAs of 21-24 nucleotides processed from double stranded RNAs and serve as signaling molecules to direct methylation of homologous target sequences (Mathieu & Bender, 2004). Such coupling of transgenes encoded RNA hairpins into a genome has been shown to induce modification of chromatin and several endogenous repetitive sequences (Gorisch et al., 2005). Still, transgene integration in inverted repeat fashion is critical for methylation-induced silencing to occur (Kanazawa et al., 2007). This mechanism requires annealing of small RNAs to homologous endogenous sequences to form dsRNA cruciform structures that are prone to cleavage.

To successfully achieve silencing, it is crucial to consider the exact underlying mechanism, which may include virus-induced silencing, histone modification, RNA interference or DNA methylation (Fire et al., 1998, Outchourov et al., 2018). According to an earlier screening in our laboratory, the transcription factor binding domain in Dfr promoter (5'-CACGTG-3') is severely methylated in wildtype petunia line W80. This impairment may distort binding of transcription factor bHLH to hamper pigment biosynthesis (Outchourov et al. 2018). If severe methylation of Dfr promoter can lead to inactivation, amplifying regions of the promoter in vitro and subsequently introducing them into plants will possibly restore promoter activity. This is plausible given that a transcriptionally-active promoter is required to express DfrA gene and ultimately lead to pigment biosynthesis visualized as floral colour. Therefore, introducing in vitro-generated unmethylated fragments into W80 petunia lines is expected to restore a transcriptionally inactive promoter. On the other hand, if pigmentation genes usually not expressed because of methylation-induced promoter inactivation can regain activity, then methylating a previously unmethylated promoter via RdDM will likely result in silencing. To investigate these, we first examined whether in vitro-generated fragments of DfrA promoter can become active when transformed into a white-petal W80 petunia line. Secondly, we examined if inverted repeat constructs of ChsA promoter can direct sufficient methylation of homologous sequences to induce ChsA silencing in a V26 petunia line.

Materials and Methods

DfrA constructs and vectors

Three constructs V2, V4 and W4 (Figure 2) were obtained from a previous complementary study

(Reinen, 2006, MSc work at Vrije University Amsterdam). Construct V2 has Dfr promoter (pDfrA) from a V26 petunia line cloned in front of a reporter green fluorescent protein gene (EGFP) in a destination vector pKGWFS7.0. Constructs V4 and W4 contain genomic DfrA gene (pDfr with the coding region) from V26 and W80 petunia lines, respectively.

For DfrA promoter and gene alike, fragments were amplified from genomic DNA of V26 and W80 with DNA polymerase (Thermo Fischer Scientific). The PCR products generated were resolved, excised from agarose gel and subsequently purified prior to ligation into pGem-T-easy vector (Promega). The ligation mix was used to transform *E. coli* cells DH10B and the transformation product spread to grow on LB/amplicillin/isopropyl-b-D-1-thiogalactoside (IPTG)/X-gal Agar plates. The resulting recombinant clones were screened for inserts by PCR with forward and reverse M13 Gateway primers (Table 1). After harvest of DNA from colony positive for inserts, a restriction digestion was performed with *Nco*I - *BbrP*I. Further digestion was performed with *Nco*I - *Sal*I to release fragment subsequently ligated into a pre-digested pEntry-Gm vector. Again, the ligation mix was used to transform *E. coli* cells and later selected on gentamycin selective LB plates. Colonies were screened for inserts in order to determine insert orientations. Samples of DNA harvested from colony positive for insert were recombined into destination vector pKGWFS7.0. The resulting constructs were transformed into *A. tumefaciens* via electroporation and subsequently introduced into petunia W80 and V26 lines for infiltration and co-cultivation with explants.

Inverted repeat constructs of chalcone synthase promoter

Fragments of various sizes were generated from petunia genomic DNA and amplified by PCR using primers specific for ChsA and ChsJ as shown in table 1. Primers 2904 and 3410 were used for forward and reverse reactions, respectively, to generate short ChsA fragments of 349 base pairs. Similarly, 2910 and 3410, 2904 and 2909, and 2910 and 2911, respectively, were used to generate short fragments of ChsJ, long fragments of ChsA, long fragments of ChsJ corresponding to sizes of 245bp, 610bp and 378bp (Table 1). The fragments were resolved on a 1% agarose gel. Bands excised from gel were recovered using a commercially available Quiagen® Spin Miniprep Kit. Column-purified Chs inserts were cloned into an expression vector pGem T easy and later was amplified in *E. coli*. Expression vector that contained inserts was harvested and column-purified for a later PCR reaction using M13 primers. This step establishes the intact state of plasmid and generates fragments from one end of the forward M13 primer to the corresponding reverse sequence. PCR fragments contain *Nco*I and *Sal*I restriction sites and upon digestion ensure release of Chs fragments including some nucleotides from pGem T easy. PCR products of Chs were digested with *Nco*I and *Sal*I (Figure 3). Inserts of Chs released were later ligated into pENTRY Gm and amplified in *E. coli* cells.

To determine size and orientation of inserts in the entry vector, M13 primers were used in combination with Chs specific primers to generate PCR fragments (Figure 4).

Culture and harvest of cells

A single colony each of *Agrobacterium tumefaciens* AgL0 and E. coil DH10B was used to inoculate 10 ml of YT medium. An overnight incubation of *A. tumefaciens* and *E. col*i was allowed at 30°C and 37°C, respectively, to saturate the culture. Fresh precultures were diluted 100 times with YT and shaken at 200 rpm during a 5 hr incubation to achieve an optical density of 0.6 - 0.8. Cultures were transferred into centrifuge bottles and spun down at 5000 rpm for 10 mins in 4°C pre-cooled rotors. After

supernatants were removed, cell pellets were resuspended in 200 ml of ice cold 10% glycerol prior to a further 10 mins spin at 5000 rpm. Again, supernatants were removed and cells resuspended in 4 ml of ice cold 10% glycerol before 50 µl aliquots were taken, snap-frozen in liquid nitrogen and stored at – 80°C until needed during electroporation.

Bacterial transformation

Ice cold 0.1cm cuvettes were used for bacterial transformation. 500 ng of each construct each of the DfrA T-DNA constructs was added into 50 µl of competent *E. coli* cells and subsequently the mixture was subjected to electrical pulse at 1.5V and a resistance of 200? for 2 secs of electroporation. One milliliter of pre-heated SOC medium was added immediately and mixed before cells were transferred to 1.5 ml centrifuge tubes and incubated at 37°C for 30 mins to allow cell recovery. Cells were centrifuged, resuspended in 50 µl SOC medium prior to overnight selection on *rifampicine*. The resulting colonies were screened for inserts prior to an overnight culture to amplify copies of the plasmid. Using a Quiagen® Spin Miniprep Kit, plasmid DNA were harvested and later used to transform *A. tumefaciens* via electroporation. Cells were incubated at 30°C and other subsequent steps were similar to the above stipulated protocol for *E. coli.*

Transformation of Petunia

Handling of petunia leaves

Overnight cultures of *A. tumefaciens* were prepared having DfrA constructs V2, V4 or W4. Cultures of A. tumefaciens having ChsA and ChsJ constructs were used to transform V26 petunia line. For transformation of W80 and V26 petunia lines with DfrA constructs, young leaves were harvested from greenhouse of the Vrije University Amsterdam. For optimum transformability, young leaves not fully expanded were harvested and subjected to routine sterilization. Leaves were dipped in 70% ethanol followed by complete immersions in 0.5% hypochlorite for 10 mins and repeated rinse steps in sterile water.

Co-cultivation and generation of explants

Young leaves were cut with renewed sterile scalpel into small sections (0.5cm x 0.5cm) in Petri dishes. Explants were submersed in a 1:10 dilute culture of transformed *A. tumefaciens* in a 20 ml Petri dish. Co-cultivation was performed for 15 mins to facilitate bacterial attachments to explants and thereafter transferred to a non-selective Murashige (MS) medium (Murashige and Skoog, Duchefa, Haarlem, The Netherlands) at 25°C. Medium consisted of 4.4 g of MS salts and vitamins, 20 g saccharose, 7 g microagar, 1 mg folic acid, 100 mg/l inositol, 2 mg/l naphthalene acetic acid (NAA), and 1 mg/l benzyl aminopurine (BAP).
After 3 days of co-cultivation, explants were transferred to MS plates supplemented with carbenicillin and kanamycin to inhibit bacterial growth and select for transformed calli, respectively. Cultures were watched closely for possible rescue upon fungal growth and routinely transferred to fresh plates. Except in cases of infection, all explants were routinely brought on fresh plates without NAA and BAP every four days. Tissues that were generated from plasmid-treated explants were intensely green and in morphology resembled the crown-gall tissues, while media were renewed till shoots emerged from calli. Shoots were allowed to attain a reasonable height before they were excised and transferred on plates with fresh medium.

Restriction cleavage analysis

For both fragments of ChsA promoter that showed consistent band pattern on agarose gel for every single orientation in pENTRY Gm vector, an LR recombination reaction was performed with the destination vector pK7GWIWG2(I). Prior to recombination, however, DNA was recovered from the entry clone using a Quiagen® Spin Miniprep kit. Thereafter, 80 ng and 100 ng of short and long fragments of ChsA promoter region, respectively, were recombined with approximately 100 ng of destination vector. Reaction volume was brought to 8 µl with TE buffer, which was further catalyzed by ice-cold clonase II. Further incubation (25°C) of reaction mixture was allowed but was quenched with proteinase k after 1hr. Four micro liter of each

reaction mixture was transformed via electroporation into competent *E. coli* cells as described earlier. Transformants were selected on spectromycin plates after an overnight incubation at 37°C. Further screening of colonies using gateway primers (2285, 2286) in combination with ChsA specific primers (Table 1) confirmed the presence of inserts. Overnight cultivation of each transformant ensured a substantial DNA yield, which was harvested using a Quiagen® Spin Miniprep kit. The DNA was used to transform competent A. tumefaciens and later selected on spectinomycin plates at 30°C. After the presence of inserts was confirmed via PCR, a single colony for each ChsA construct was used for a pre-culture. One milliliter volume of each pre-culture was used to inoculate an overnight cultivation at 30°C with continuous agitation at 200 rpm. Each of ChsA inverted repeat constructs of short and long fragments was used to transform V26 petunia line by using fresh culture of each *A. tumefaciens* transformant. Apart from the difference in DNA constructs, transformation procedures were similar to the Dfr protocol described earlier.

DNA sequencing

To monitor transgenes in DfrA transformants, determine insert copy number and affirm transgenicity of plants, the neomycin phosphotransferase (*NPTII*) gene is a good tracer. Hence, it is important to distinguish transcripts of endogenous genes from those derived from transgenes. For this reason DfrA gene was sequenced to facilitate design of unique primers, which were used to quantify transgene transcript levels in background of the endogenous gene.

Nucleotide sequence of DfrA gene in V30 petunia line is known and partly determined in V26 (Reinen, 2006, MSc work at Vrije University Amsterdam). Prior to this study, DfrA promoter sequence of W80 was partly determined and revealed a few base differences. To follow mRNA from transgenes, we distinguished DfrA in the transgenics from those of endogenous gene by designing primers that took advantage of possible nucleotide differences necessary to mine W80 DfrA mRNA in a V26 background. Therefore, DNA fragments were generated to cover the entire W80 DfrA gene by using the following set of primers 0097/0098, 1243/0098, 0097/1244, 1243/1244, and 1113/0098 (Table 1). The resulting DNA fragments were excised from agarose gel and column-purified using Quiagen® Spin Miniprep kit. Pure fragments were subjected to 'BigDye' sequencing reaction.

PCR amplification conditions – sequencing reactions

PCR amplification of cloned ChsJ in entry vector yielded irregularly unexpected bands for both possible orientations. To ascertain the exact orientation and insert copy number per location, we sequenced fragments of ChsJ within the vector. Therefore, PCR amplification was performed using the following vector specific as well as insert specific primers: 3118/3119, 1777/1778, 2910/3410 (Table 1). Amplification conditions were 96°C for 30 sec, 55°C for 30 sec, and 60°C for 1.5 min for 25 cycles.

Results and Discussion

I*nsert orientation of inverted repeat constructs*

Insert orientations in clones containing long and short fragments of ChsA were determined as aligned in a tail-to-tail position (Figure 5). Each clone was subjected to a recombination reaction described earlier to determine the orientation of each ChsJ insert in entry vector. This proved challenging because yields of sizeable PCR fragments for both possible insert orientations of ChsJ were not directly helpful in determining ChsJ insert number per location. For this reason, overnight restriction cleavages at 37°C with *Sal*I - *Nco*I in a single and double digest fashion were necessary as shown in figure 5. The purpose of the step was to determine by size any possible insertion of multiple fragments that would become apparent upon resolution on agarose gel. For each single digestion, a 30 µl cleavage reaction volume was subjected to 1 hr incubation. Each reaction comprised of 2 µl plasmid clone, 1 µl each of *Nco*I and *Sal*I, 3 µl reaction buffer H and water for a reaction volume of 30 µl incubated for 1 hr. Each digest was resolved on agarose gel to visualize cleaved fragments. However, analysis of pChsJ revealed the presence of a single insert per locus.

Orientations of pChsA fragments in pENTRY Gm vector were successfully

determined. This provides adequate prediction of insertion patterns during recombination with gateway destination vector, pK7GWIWG2(I). Although these vectors recombine only in one possible pattern, it was necessary to confirm the presence of pChsA inserts in the vector. Figure 6 shows that PCR products resolved on agarose gel correspond with expected length of fragments.

Transformants of V26 line having inverted repeat pChsA transgenes in early growth stage had developed clear shoots by the end of this research stay. Plant specimens intended for use in a confirmatory analysis of transgenic plants with inverted repeat constructs were lost and thus recommended in a future study.

Plants transformed with DfrA constructs showed a wide range of phenotypic variations that were apparent in petal colour, leaf texture, leaf colour and plant growth rate. A visual presentation of characteristics during two growth stages of the transgenics is shown in figure 7. We observed stunted growth, delayed maturation and a later appearance of patched yellow coloration of leaves in several V26 lines transformed with V2 constructs [L7059] (Figure 7A). A few of these transformants had leaves that were particularly rough in texture with irregular white patches (Figure 7B). However, for all constructs having the entire DfrA gene, tranformants showed visibly healthy phenotypes (Figure 7C).

All flowering V26 lines having V4 constructs [L7059] showed purple floral characteristics (Figure 7D). We obtained similar characteristics in all V26 transgenic plants containing W4 and thus suggest DfrA promoter activity in both transformants (L7059 and L7060). Considering that V4 was derived from active DfrA gene, it was rather exciting to note that all eleven W80 transformants having V4 constructs [L7061] produced white flowers (Figure 7E). These plants later were confirmed in PCR reactions to be transgenics and contained NPTII gene in the construct. A plausible reason is that the promoter region of V4 DfrA gene is methylated and hence is not able to drive gene expression. However, we do not rule out the possibility of other mechanisms besides methylation. We think that further analysis may reveal the methylation status as well as the correctness of the construct. On the other hand, all W80 transgenic plants transformed with W4 constructs produced flowers with light pink petals and may suggest a moderately active DfrA promoter (Figure 7F). These observed phenotypes suggest that unmethylated DfrA gene can maintain active status when transformed into a normally white flowering plant. This is in keeping with data from Zhang and colleagues (2016), which suggest that higher DNA methylation of gene body regions correlate with lower expression levels. It implies that unmethylated DfrA gene can drive transcriptional activity and ultimately restore petal colour in a white flowering plant. Our results suggest other possible substitute transcriptional role of transgenes in the background of endogenous methylated promoter.

While it is important to establish the status of DfrA in regulating pigment synthesis in transgenic plants, we think that further analysis of relative mRNA levels is necessary to support other evidence of transcriptional activity of DfrA promoter. What is not clear is whether an unknown mechanism was responsible for demethylation of endogenous DfrA promoter or transgene expression can account for the observed phenotypes. It is possible to verify methylation status of DfrA promoter via bisulphate sequencing or by the use of methylation-sensitive restriction enzymes for cleavage analyses of transgenes as well as endogenous DfrA fragments.

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FIGURES AND TABLE

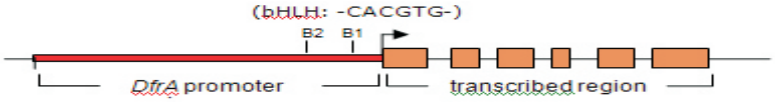

Figure 1. The DfrA gene showing regulatory sequence motifs of promoter at B1 and B2 sites that is necessary for transcriptional activation of coding region that consist of six exons.

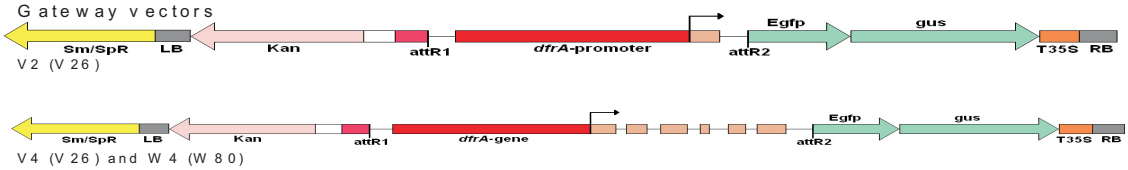

Figure 2. Map of V2 constructs showing DfrA promoter cloned into the destination vector pKGWFS7.0. (A). (B) depicts DfrA gene coupled with a corresponding DfrA promoter shown as V4 and W4 (Adapted from Reinen, 2006).

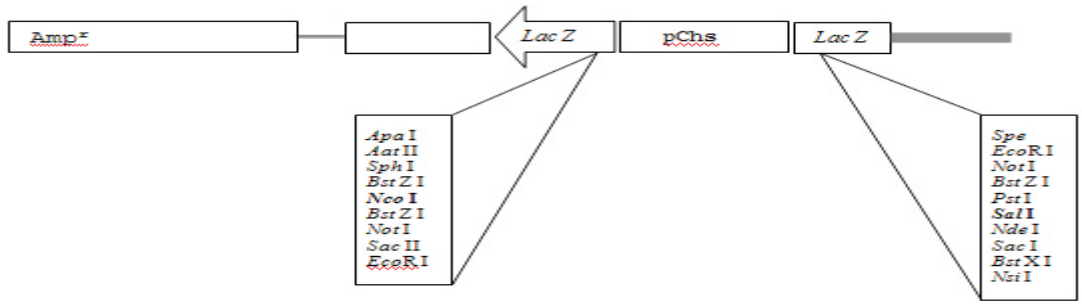

Figure 3. Restriction cleavage assay with *Nco*I and *Sal*I to release cloned fragments of chalcone synthase promoter from expression vector pGem T-easy.

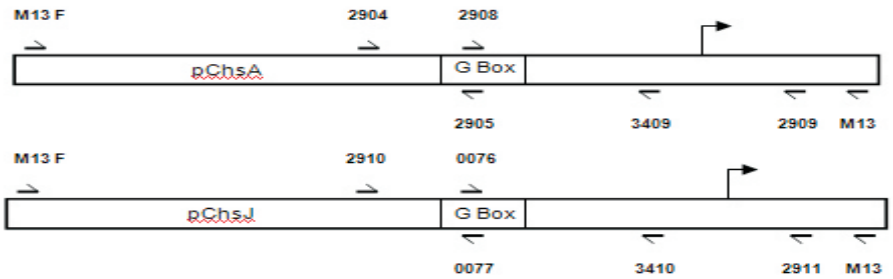

Figure 4. Map of primers to generate chalcone synthase promoter fragments cloned into a vector

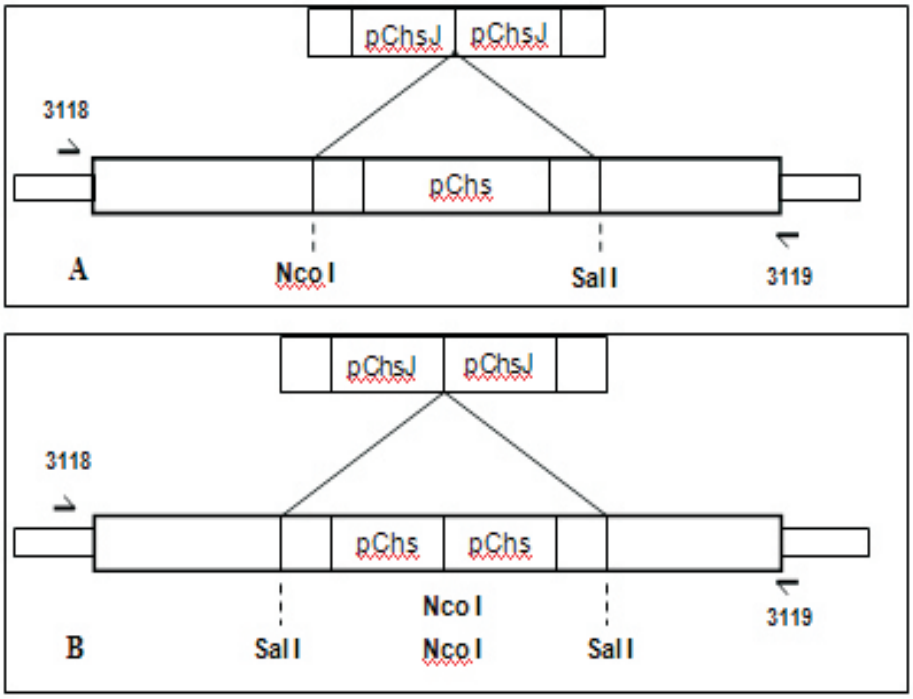

Figure 5. Restriction enzyme digestion to ascertain orientations of ChsJ insert in entry vector. A single copy of insert was cloned into *Sal*I /*Nco*I -cleaved site, implying that digests with the same enzymes will release only a single insert copy of ChsJ corresponding to a presumed length [A]. For multi-copy insertions having either *Sal*I or *Nco*I at both ChsJ insert-vector boundaries, cleavage with either of both enzymes would yield fragments corresponding to lengths of the supposed multi-copy insertions [B]. Similarly, a single-enzyme would cleave in a single digest fashion to yield large-sized fragments determined on gel by size and migration pattern.

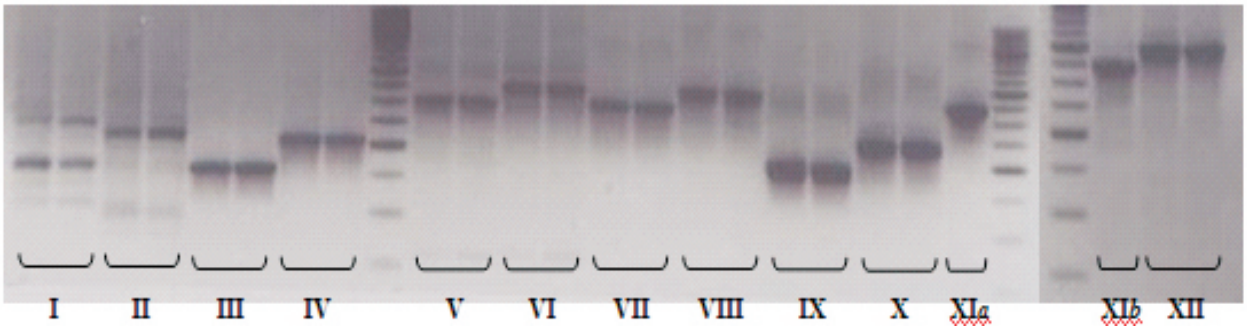

Figure 6. Confirmation of p*ChsA* insertion in vector pK7GWIWG2(I) showing fragment sizes of approximately 757bp:I:AS; 1018bp:II:AL; 757bp:III:AS; 1018bp:IV:AL; 1463bp:V:AS; 1724bp:VI:AL; 1463bp:VII:AS; 1724bp:VIII:AL; 931bp:IX:AS; 1192bp:X:AL; 1553bp:XIa/b:AS; 1814bp:XII:AL. *AL depicts long fragment of ChsA promoter region while AS is fragment of short Chs, bp is the number of base pairs.*

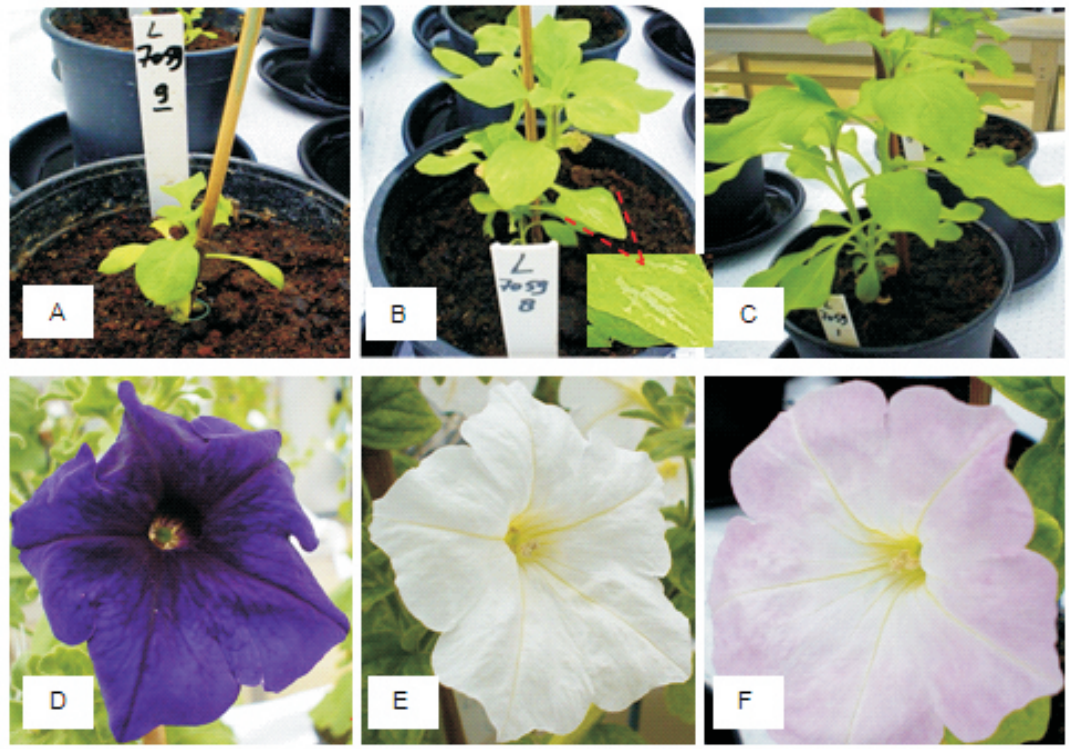

Figure 7. Phenotypic characteristics of DfrA transformants showing different traits in early and maturing stages of growth.

Table 1. List of some primers used for PCR amplification and sequencing

Primer code	sequence [5'…3']	Tm (°C)	F/R
0001 [M13]	**GTAAAACGACGGCCAGTGA**	**57**	**F**
0002 [M13]	**GGAAACAGCTATGACCATGA**	**56**	**R**
0027[35Sprom]	**AGAAGACGTTCCAACCACGTCT**	**60.5**	**F**
0076 [ChsJ]	**CCCCTCAAACACGTGAACACTAGCTACCAGTT**	**70**	**F**
0077 [ChsJ]	**GGGGAACTGGTAGCTAGTGTTCACGTGTTTGA**	**70**	**R**
0097 [Dfr]	**ACAATGTTCACGCTACTGTTC**	**56**	**F**
0098 [Dfr]	**GTAGGAACATAGTACTCTGG**	**55**	**R**
0623 [Dfr]	**CGAACAGTAGCGTGAACATTG**	**56**	**R**
0629[35S term]	**TGCTCAACACATAGCGAAAC**	**58**	**R**
1113 [ART'S]	**TGAACCCTCTTCACCGAAAATTTGTACTG**	**56**	**F**
1243 [Dfr]	**TTCTTCCTCTCACCAAACAC**	**55.5**	**F**
1244 [Dfr]	**AGTTTTGAGRAGAAATGGRAATGG**	**58**	**R**
1777 [Gateway]	**GAAACCTTACCTCATCATTTCC**	**56**	**F**
1778 [Gateway]	**GCAGGTCAGCTTGACACTGAAC**	**56**	**R**
2285 [NPTII]	**GCGGTTCTGTCAGTTCCAAACG**	**56**	**F**
2286 [RB.R]	**CCCGCCAATATATCCTGTCAAAC**	**56**	**R**
2904[Chsprom]	**AACTCGCTGTTGTGCACTGTCAA**	**56**	**F**
2905[Chsprom]	**GTGTAGCTATAACTTGATGGCAC**	**56**	**R**
2908 [ChsA]	**GTGCCATCAAGTTATAGCTACAC**	**56**	**F**
2909 [ChsA]	**TAAGATCAGTCTTGTGCTCACTG**	**56**	**R**
2910 [ChsJ]	**TTTGTGAAGTAGGTAGCCCAAGA**	**56**	**F**
2911 [ChsJ]	**ATAGCCATGATTGTGGCTGGCCC**	**56**	**R**
3118[pEntrGm]	**GATGCCTGGCAGTTCCCTACTC**	**60**	**F**
3119[pEntrGm]	**GTAACATCAGAGATTTTGAGACAC**	**60**	**R**
3409 [ChsA]	**TTTATACGAGAACCCTAAAGGAG**	**56**	**R**
3410 [ChsJ]	**ATAAGTGATCTTTGTATGCATGAG**	**56**	**R**