

SCIENTIFIC REPORTS

OPEN

A polymorphic (GA/CT)*n*- SSR influences promoter activity of *Tryptophan decarboxylase* gene in *Catharanthus roseus* L. Don

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Received: 24 May 2016

Accepted: 17 August 2016

Published: 13 September 2016

Simple Sequence Repeats (SSRs) of polypurine-polypyrimidine type motifs occur very frequently in the 5' flanks of genes in plants and have recently been implicated to have a role in regulation of gene expression. In this study, 2 accessions of *Catharanthus roseus* having (CT)₈ and (CT)₂₁ varying motifs in the 5'UTR of *Tryptophan decarboxylase* (*Tdc*) gene, were investigated for its role in regulation of gene expression. Extensive *Tdc* gene expression analysis in the 2 accessions was carried out both at the level of transcription and translation. Transcript abundance was estimated using Northern analysis and qRT-PCR, whereas the rate of *Tdc* gene transcription was assessed using *in-situ* nuclear run-on transcription assay. Translation status of *Tdc* gene was monitored by quantification of polysome associated *Tdc* mRNA using qRT-PCR. These observations were validated through transient expression analysis using the fusion construct [CaM35S:(CT)₈₋₂₁:GUS]. Our study demonstrated that not only does the length of (CT)*n*-SSRs influences the promoter activity, but the presence of SSRs *per se* in the 5'-UTR significantly enhances the level of gene expression. We termed this phenomenon as "microsatellite mediated enhancement" (MME) of gene expression. Results presented here will provide leads for engineering plants with enhanced amounts of medicinally important alkaloids.

Simple sequence repeats (SSRs) or microsatellites occur ubiquitously in eukaryotic genomes as tandem reiterations of short sequence motifs. They exhibit extensive length polymorphisms due to variation in the copy number of repeat motifs and are considered as genetic markers used in DNA fingerprinting, analysis of genetic diversity and linkage mapping. Numerous lines of evidence now suggest that SSRs are non-randomly distributed across transcribed regions of plant genomes^{1,2} wherein, UTRs harbor more SSRs than the coding regions¹⁻⁶. Moreover, the 5'-UTRs in particular, contain a majority of di- and tri-nucleotides that exhibit a strong bias towards polypurine-polypyrimidine sequences such as GA/CT and CTT/GAA repeats⁴⁻⁷. Such DNA elements, which till some time ago were termed as "junk DNA," can have multiple roles in the genomes of higher eukaryotes and have often been found to be associated with gene regulation based on their location in the genome^{8,9}.

Several studies in the animal kingdom have indicated the functional role of polypurine-polypyrimidine sequences in gene expression through transcription factor binding, methylation of CpG and/or DNA structure modification². In particular, the 'GAGA' elements comprising of the dinucleotide repeat sequence (GA)*n*/(CT)*n* have been found in the promoters of numerous genes¹⁰⁻¹⁶.

GAGA elements have been most thoroughly examined in *Drosophila*, where they have been shown to be present in the promoter regions and are involved in the regulation of developmental genes by binding to a protein called the GAGA factor, which results in local nucleosome disruption thereby allowing gene expression¹⁷. A recently described GAGA like element (GLE), homologous to GAGA factor binding sequences, regulate core promoter activity and preferentially affect the transcription start site selection clustered around the 5' end of core promoter¹⁸.

However, in plants, such studies are limited. One of the earliest known example in plants is the soybean *Gsa1* that encodes the chlorophyll heme synthesis enzyme Glu2-semialdehyde aminotransferase and has a (GA)₉/(CT)₉ element in its promoter that has been implicated in regulating expression of that gene in a tissue specific manner^{19,20}. In another study, a polymorphic (CT)*n* microsatellite identified in the 5'UTR region of the *wx*

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gene of rice²¹ was correlated with amylose content and microsatellite length polymorphism was thought to affect the expression of the related genes of amylose synthesis²². Recently, Joshi-Saha and Reddy²³ have suggested that (CT)*n* repeat length variation in 5'-UTR of the chickpea *myo-inositol monophosphatase gene* (*CaIMP*), might regulate phytic acid levels to confer drought tolerance in natural populations of chickpea. Additionally, at the whole genome level it has been shown that ~10% of 5' non-coding CT/GA and CTT/GAA repeats are conserved in *Arabidopsis* and are thought to be involved in regulation of gene expression in plant-specific pathways⁶.

More recently, while analyzing the *Catharanthus roseus* transcriptome we have demonstrated that GA/CT and GAA/CTT repeats were most frequent in 5'-flanks of genes that are known to be involved in enzymatic, regulatory and housekeeping functions⁷. Such preferential distribution and conservation of SSRs in the 5'-UTRs^{1,7} strongly suggests that they may be strong contenders for being characterized as a regulatory element. However, more comprehensive analysis needs to be undertaken in order to assess their role in regulating gene expression especially in plant species.

Catharanthus roseus is a model medicinal plant species that produces a wide array of pharmaceutically important alkaloids, including anticancer drugs such as vincristine and vinblastine^{24,25}. These alkaloids are produced in low quantities, making extraction and purification difficult which leads to high market price and poor availability. The terpenoid indole alkaloids originate from tryptophan via the TIA biosynthetic pathway which has been thoroughly investigated^{26–28}, but most of it still remains largely unresolved. Tryptophan decarboxylase (TDC) catalyses the first committed step of indole alkaloid synthesis by decarboxylation of tryptophan to form tryptamine²⁹ and is a key enzyme in the biosynthetic pathway by virtue of its position at the interface of primary and secondary metabolism. It has been characterized in *C. roseus*^{28–30} but its complex, coordinated regulation needs to be investigated in order to exploit it for genetically engineering plants with enhanced levels of useful indole alkaloids.

Therefore, our aim in the present study was to investigate the effect of (CT)*n* microsatellite length variation on *Tdc* gene expression since the *Tdc* genes have been shown to possess variable number of CT motifs in their 5'-UTRs³¹, but the functional role of these SSRs has not been elucidated. It is expected that a thorough investigation of the variations in the number of microsatellite repeat motifs near the TSS within individual accessions of *C. roseus* would provide new information with regard to the putative function of these microsatellites. To the best of our knowledge, this is the first time that the functional role of microsatellites (especially CT repeats), present in the 5'UTR of *Tdc* gene of the medicinally important plant *C. roseus* is being described. In this study, through parallel measurement of transcript abundance vs rate of transcription and translation, we demonstrate that the plant (CT)*n* repeats indeed modulate the promoter activity of *Tdc* gene in a length dependent manner.

Results

Analysis of sequence variation in the 5'-UTR of *Tdc* gene in *C. roseus*. The *Tdc* gene, which catalyses the conversion of tryptophan to tryptamine in the TIA pathway, had been cloned and characterized from *C. roseus*³² and shown to possess (CT)*n* repeats in the 5'-UTR region^{32,33}. Moreover, an earlier study in our lab had shown (CT)*n* polymorphism in the 5' UTR region of *Tdc* gene³¹. Therefore in the present study, primers P1 and P3 were designed from the regions flanking the (CT)*n* motifs in the 5'UTR of *Tdc* gene (Fig. 1). PCR amplification of genomic DNA from 15 *C. roseus* accessions collected from diverse geographical locations was performed using the primers P1 and P3 (Fig. 1). The amplified products revealed bands of various lengths ranging in size from 200 bp to 350 bp in the different accessions (Fig. 2A). The *C. roseus* accessions Kew1 and Prabal showing significant length variation were chosen for further studies.

To begin with, the minimal promoter sequences of the *Tdc* genes³³ of the two *C. roseus* accessions, Kew1 and Prabal, were compared. For this, genomic DNA of *C. roseus* accessions Kew1 and Prabal was isolated and the *Tdc* 5'UTR as well as the basal promoters were amplified and sequenced using gene specific primers (P1 and P4). Sequence alignment revealed that significant variation existed in the number of (CT)*n* microsatellite motifs in the basal promoter regions of the *Tdc* genes of the 2 *C. roseus* accessions, wherein Kew1 had (CT)₈ motifs, Prabal had (CT)₂₁ motifs (Fig. 2C). Sequence comparison also revealed that no other nucleotide variation was present within the basal promoter (~300 bp) region except the variation of the (CT)*n* motif number (Fig. 2C).

Further, to confirm the copy number of *Tdc* gene in the accession Kew1 and Prabal, qPCR was performed. The real time PCR analysis showed that both the accessions of *C. roseus* had the same copy number of *Tdc* gene as no significant variation in levels of amplified product (*Tdc* gene) from the genomic DNA was observed [Fig. 2(D)]. Our results demonstrated that the 5'UTRs of the *Tdc* genes of the two accessions Kew1 and Prabal differed only in number of (CT)*n* motifs. No other sequence variation was found in the (CT)*n* flanking regions in the *Tdc* 5'UTR and also the copy number of *Tdc* gene was found to be same in both the accessions, Kew1 and Prabal.

Estimating the effect of (CT)*n* motif number variation on transcript abundance. Gene expression analysis is of utmost importance and its measurement may be done efficiently by estimating the mRNA abundance. Therefore, gene expression analysis at the mRNA level was carried out by (1) hybridization based Northern analysis (2) mRNA copy number based semi-Q PCR and (3) real time PCR. Total RNA was isolated from the *C. roseus* accessions, Kew1 and Prabal and Northern hybridization analysis was performed using radio labeled *Tdc* cDNA probe. This revealed differential expression of the *Tdc* gene between the two accessions. In Prabal, which had (CT)₂₁ di-nucleotide repeat motifs, the *Tdc* gene transcript was more abundant in comparison to Kew1 which had only (CT)₈ di-nucleotide repeat motifs [Fig. 3I(a)]. Furthermore, quantification of expression was done by semi-Q PCR and real time PCR experiments using cDNA template made from same sample of RNA that was used for Northern analysis. The semi-Q PCR [Fig. 3I(b)] as well as real time PCR [Fig. 3I(c)] also revealed higher levels of expression of *Tdc* gene in Prabal [(CT)₂₁] in comparison to Kew1 [(CT)₈]. Real time data suggested that the *Tdc* transcript abundance in Prabal was approximately 8 times more in comparison to Kew1 [Fig. 3I(c)], thereby validating the results of Northern analysis.

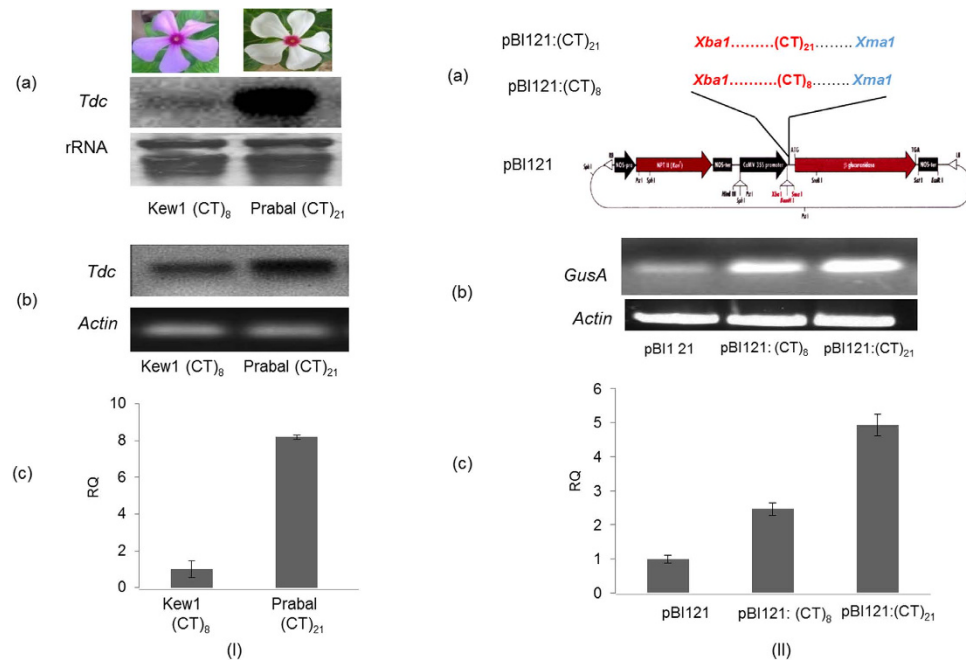


Figure 3. (I) Expression analysis of *Tdc* gene in the two *C. roseus* accessions, Kew1 and Prabal having (CT)₈ and (CT)₂₁ motifs respectively in the 5'-UTR of *Tdc* gene (a) Northern blot analysis. 10 μg of total RNA was probed with cDNA. Ribosomal RNA was used as loading control (b) Semi quantitative PCR analysis: endogenous actin gene was used as positive control and (c) Real Time quantification of *Tdc* mRNA. It revealed approximately 8 times higher expression in Prabal (CT)₂₁ than Kew1 (CT)₈. (II). Schematic representation of cloning of 5'-UTRs of *Tdc* harboring (CT)_n repeat motifs into pBI121 expression vector using *Xba*I and *Xma*I restriction enzymes. The sequences of recombinant constructs were confirmed by sequencing (a) Sequence of 5'-UTR (129 bp from Kew1 accession) harboring (CT)₈ motifs in pBI121:(CT)₈ construct and 5'-UTR (155 bp from Prabal accession) harboring (CT)₂₁ motifs in pBI121:(CT)₂₁ construct. Analysis of transiently expressed *GusA* mRNA, driven by respective recombinant pBI121 vectors, by (b) semi-quantitative PCR. Tobacco actin gene was used as a control and (c) Real time PCR expression analysis of transiently expressed *GusA* gene transcript, wherein pBI121 empty vector was used as a control. Relative expression profile of recombinant vectors pBI121:(CT)₈ and pBI121:(CT)₂₁. (The original figure from which Fig. 3.I (a) was cropped and derived is present as Supplementary Figure 1).

Further, transient expression analysis in *Nicotiana benthamiana* was also carried out. For this, the 5'-UTRs of *Tdc* genes harboring the microsatellite motifs (CT)₈ and (CT)₂₁ were cloned into the pBI121 vector upstream of the reporter gene *gusA* [Fig. 3II(a)] and transformed into *agrobacterium* as described in Methods. The pBI121 empty vector was also transformed into *agrobacterium* and used as a control. The positive recombinant clones were used for agro-injection into tobacco (*N. benthamiana*) plants. Leaf discs were harvested from agro-injection sites after three days and total RNA was isolated and subjected to the transient expression analysis of *gusA* gene by using (A) semi-quantitative PCR and (B) real time PCR, using *gusA* specific primers. This revealed differential expression of *gusA* transcripts in tobacco leaves transformed with construct pBI121:(CT)₈ and pBI121:(CT)₂₁ [Fig. 3II(b,c)]. The maximum expression of *gusA* gene was seen in the plant transformed with recombinant pBI121:(CT)₂₁, whereas *gusA* expression in plant agro-injected with recombinant pBI121:(CT)₈ was two-fold less as compared to recombinant pBI121:(CT)₂₁ but two-fold excess as compared to control (Fig. 3IIc). The above expression analysis revealed that the (CT)_n copy number dependent mRNA abundance of *Tdc* gene in *C. roseus* was observed both *in situ* and in heterologous reporter gene (*gusA*) expression, thereby establishing that microsatellite length variation in the 5'-UTR of a gene may directly affect transcript abundance.

Assessing the effect of (CT)_n length variation on rate of gene transcription and translation. Flow of genetic information follows the path of transcription and translation. However, sometimes mRNA abundance may not be directly indicative of the rate of transcription or translation of a given gene. Therefore to investigate the role of SSRs in transcription and/or translation the following two experiments were performed (A) Nuclear run-on transcription assay to assess the effect of (CT)_n length variation on rate of transcription and (B) Quantification of polysome associated *Tdc* mRNA to assess the effects of SSRs variation on translation. In nuclear run-on transcription assay the intact nuclei of *C. roseus* accessions Kew1 and Prabal was used to synthesize the radio labeled transcripts. These were hybridized with cold probe made of PCR amplified cDNA of *Rps9*, SAND (positive control), *Tdc* (target gene) and MATE (negative control) on membrane. The indicated that the rate of *Tdc* gene transcription was significantly higher in Prabal accession which contained 21 (CT)_n motifs, as compared to Kew1 having only 8 (CT)_n motifs [Fig. 4(a)]. This clearly suggested that (CT)_n length variation contributed in promoter activity of *Tdc* gene.

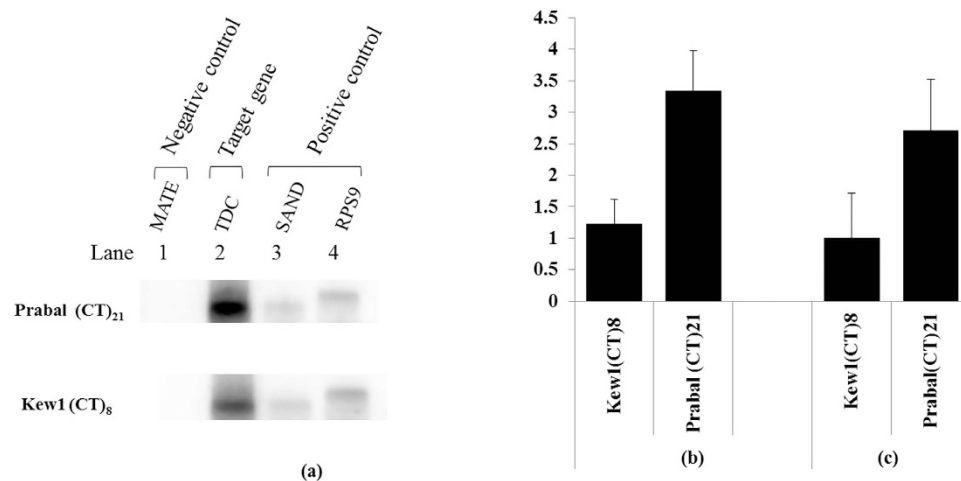


Figure 4. Resolution of flow of information both at the level of transcription and translation. (a) Nuclear run-on transcription to measure rate of *Tdc* gene transcription. PCR amplified DNA of *Tdc* (target gene), *Rps9* and SAND (positive control) and MATE (negative control) was blotted on to the nitrocellulose membrane and hybridized with the nascent UTP labeled run-on transcripts from Kew1 and Prabal. (b) Quantitative RT-PCR for quantification of *Tdc* mRNA cell extract of Kew1 and Prabal. (c) Quantitative RT-PCR for quantification of polysome associated *Tdc* mRNA in Kew1 and Prabal. (The original figure from which Fig. 4.(a) was cropped and derived is present as Supplementary Figure 2).

Site Name	Organism	Position	Strand	Matrix score.	Sequence	Function	Reference
5UTR Py-rich stretch	<i>Lycopersicon esculentum</i>	154	+	14	TTTCTCTCTCTCTC	cis-acting element conferring high transcription levels	34

Table 1. In-silico identification of putative protein factors binding to the promoter of *Tdc* by PlantCARE.

Further, to assess the role of (CT)*n* motifs in translation, *Tdc* mRNA from cell extract and polysomal fractions from both the *C. roseus* accessions, were parallelly quantified by quantitative real-time PCR. This revealed no significant variation in expression pattern between steady-state mRNA and transcriptionally engaged *Tdc* mRNA in accessions Kew1 and Prabal [Fig. 4(b,c)]. The coefficient of correlation between them was found to be significantly high ($r=0.99$). This suggested the putative involvement of the (CT)*n* motif number variation in the process of transcription rather than translation.

In-vitro protein-microsatellite interaction. To investigate the role of the microsatellite sequence as a putative *cis*-acting regulatory element, *in-silico* analysis was performed using the plant *cis*-acting responsive elements (PlantCARE) database³⁴. The 5'-UTR of *Tdc* gene containing the microsatellite motif (CT)*n* was used as the query. The output result revealed that a protein factor from *Lycopersicon esculentum* recognized the (CT)*n* motif and had a putative role in transcription (Table 1). Therefore, in order to establish the protein-microsatellite interaction, EMSA was performed using the *C. roseus* cell extract and three oligonucleotide probes [single stranded (SS) (CT)*n*, (SS) (GA)*n* and double stranded DS (CT/GA)*n*]. Band shifting was observed preferentially with single stranded (CT)*n* [Fig. 5(a)]. However, with other conformations such as SS (GA)*n* and DS (CT/GA)*n*, no protein-DNA interaction was observed. Further, competition assay was performed for confirmation of affinity of (CT)*n*-protein interaction, which showed a gradual fading of band intensity from 5× to 10×, which finally disappeared at 25×, 50×, and 100× of cold probe [Fig. 5(b)]. The present experiment demonstrated that a protein factor, which probably could be a transcription factor, interacted specifically with the single stranded (CT)*n* element. Further, it had been earlier reported that members of a plant specific transcription factors family known as the basic pentacysteine proteins (BPC) or barley B recombinants proteins (BBR), specifically interact with the CT/GA motifs²⁰. Therefore, *in-silico* analysis of the available *C. roseus* transcriptome⁷ was carried out in order to identify the members of the BPCs/BBR TFs. This resulted in the identification of four members of BPCs/BBR TF family in *C. roseus*. These members are Cr_TC33596, a BPC_4 like protein, Cr_TC33775, a BPC_6 like protein, Cr_TC06660, a BPC_7 like protein and TC_57534, a member of BPC_2/1 like protein. CLUSTAL W alignment of these proteins showed that all the members of the *C. roseus* BPCs/BBR contained the characteristic conserved pentacysteine C-terminal DNA binding domain (Fig. 6).

Discussion

This study examines the putative role of (CT)*n*- 5'UTR SSR, as well as its repeat length variation, in transcriptional regulation of *Tdc* gene of model medicinal plant *C. roseus*, *in-vivo*. Microsatellite repeats, especially the (CT)*n* motifs, are known to exist in abundance in the 5'UTRs of several eukaryotic genes including plants^{5-7,35}.

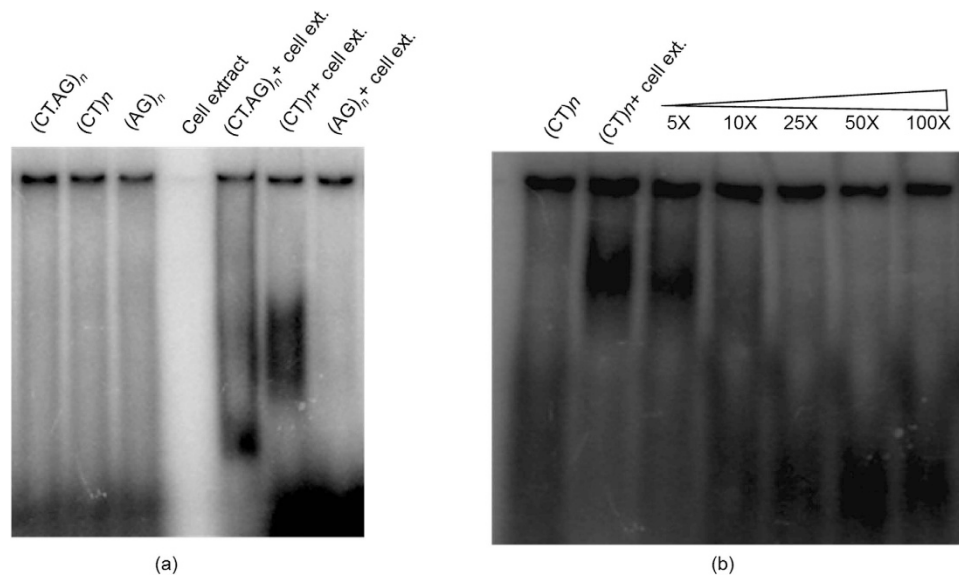


Figure 5. Interaction of protein from cell extract with dinucleotide repeat motifs: EMSA was carried out with three oligonucleotide probes (i) single stranded $(CT)_n$ (ii) single stranded $(GA)_n$ and (iii) double-stranded $(CT/GA)_n$ [$n = 21$]. (a) Band-shifting of band was observed in case of single stranded $(CT)_{21}$ with respect to free probe and (b) The protein- microsatellite binding affinity was confirmed by using SS $(CT)_n$ as cold probe (non radioactive), wherein 5X, 10X, 25X, 50X, 100X refer to molar excess of cold probe included in the reaction.

However, the involvement of the microsatellite motif *per-se* or based on their length variation, in transcriptional regulation is only beginning to be investigated in plants²³.

Hence, to elucidate the role of $(CT)_n$ variation in the 5'UTR of *Tdc* gene of *C. roseus*, a systematic approach utilizing $(CT)_n$ variant alleles from 2 accessions was followed. While selecting the microsatellite for analysis in this study, the following criteria were considered (i) the position/location of microsatellite since regional preference is important as those present in the vicinity of TSS are more likely to be involved in transcription/translation (ii) the variation in the microsatellite repeat motif number - longer repeats were preferred since they have greater probability of being polymorphic and (iii) the nature of repeat motif- the polypurine-polypyrimidine were preferred since they have a higher probability of conformational polymorphism. These criteria were especially important since they increased the likelihood of the chosen microsatellite being a part of the gene regulatory circuits.

Analysis of the sequence variation in the 5'UTR and upstream region of *Tdc* gene from the 2 *C. roseus* accessions clearly revealed that no nucleotide variation existed in the regulatory regions of *Tdc* genes of both the accessions, except the variation in the number of polypurine-polypyrimidine $(CT)_n$ motifs wherein Kew1 possessed $(CT)_8$ and Prabal had $(CT)_{21}$ motifs. Further, qRT-PCR was adopted to estimate the relative copy number of *Tdc* gene in the *C. roseus* accessions. The copy number of a gene in general is determined by Southern blotting³⁶. However, recently quantitative RT-PCR assay has been successfully used to determine gene copy number^{37,38}. In this study, quantitative real-time PCR analysis revealed no relative difference in the *Tdc* gene copy number in the 2 *C. roseus* accessions, Kew1 and Prabal since no significant variation in the level of *Tdc* amplification was observed. Since the findings of an earlier report³² had suggested that in *C. roseus*, TDC was encoded by an intron less single copy gene therefore taken together it may be suggested that both the *C. roseus* accessions had single copy of *Tdc* gene.

The untranslated mRNA leader sequences (UTRs) or downstream promoter sequences (DPE) are thought to play a pivotal role in the regulation of eukaryotic gene expression at various levels: transcriptional, post-transcriptional and translational^{23,39-44}. Moreover, presence of SSRs in the leader sequence or within promoter regions are being implicated in regulation of gene expression. For example, the $(CTC)_n$ microsatellites located in the promoter of the paired box gene 7 (*PAX7*) was found to have three different alleles, with 8, 10, or 11 repeats and it was found that the alleles with 11 repeats gave a significantly higher transcriptional activity than alleles with 8 or 10 repeats⁴⁵. In rice tungro bacilliform virus (RTBV), the DNA sequence from +50 to +90 stimulates RTBV promoter activity in a copy number dependent manner⁴⁶.

In soybean *Gsa1* that encodes the chlorophyll heme synthesis enzyme Glu2-semialdehyde aminotransferase and has a $(GA)_9/(CT)_9$ element in its promoter that has been implicated in regulating expression of that gene in a tissue specific manner^{19,20}. In another study, a polymorphic $(CT)_n$ microsatellite identified in the 5'UTR region of the *wx* gene of rice²¹ was correlated with amylose content and microsatellite length polymorphism was thought to affect the expression of the genes related to amylose synthesis²². More recently it was shown that the length variation of $(CT)_n$ repeat motifs present in the 5'UTR of *CaIMP* leads to differential transcription in chickpea drought tolerant and susceptible accessions. The *CaIMP* allele with the smaller number of $(CT)_9$ repeat motifs in drought tolerant accession was almost 2-fold up regulated as compared to a susceptible accession having the longer $(CT)_{25}$ repeat²³.

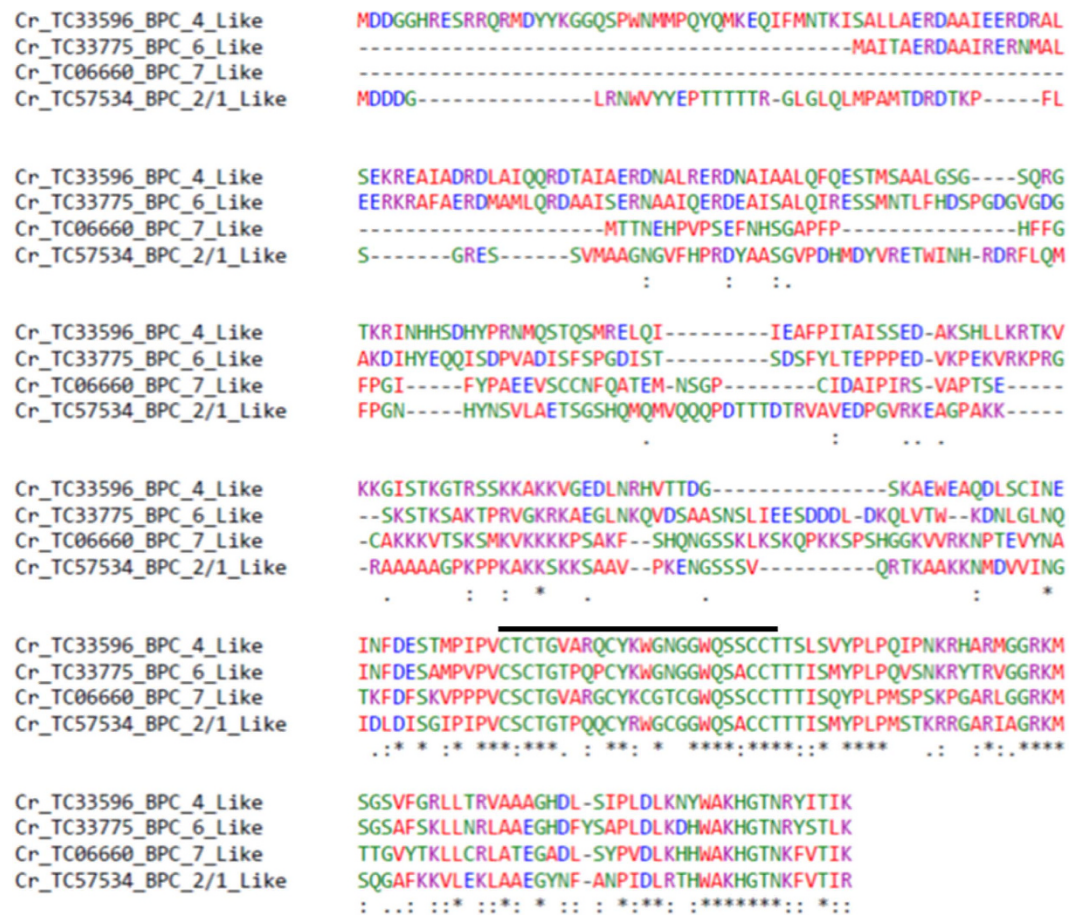


Figure 6. CLUSTAL W alignment of putative BPC/BBR protein from *C. roseus*. Putative protein sequences of Cr_TC33596, a BPC_4 like protein, Cr_TC33775, a BPC_6 Like protein, Cr_TC06660, a BPC_7 like protein and TC_57534, a member of BPC_2/1 like proteins were subjected to CLUSTAL W alignment. This revealed all the members of *C. roseus* BPCs/BBR have the characteristic conserved pentacysteine C-terminal DNA binding domain.

Similarly in our study, comparison of *Tdc* gene expression in the 2 *C. roseus* accessions varying in the (CT) n motif numbers [(CT) $_8$ and (CT) $_{21}$] revealed that motif number variation in the regulatory non-coding element (5'UTR) of *Tdc* gene may be responsible for significant quantitative and temporal difference in gene expression between the *C. roseus* accessions. This was clearly established by measuring *Tdc* mRNA abundance through Northern analysis and qRT-PCR analysis, where the accession with longer repeats (CT) $_{21}$ showed higher abundance of *Tdc* mRNA transcript than accession with shorter repeats (CT) $_8$.

Our results also demonstrated that the (CT) n repeat motifs from +24 to +66 stimulate *Tdc* promoter activity in a copy number dependent manner. Transient expression analysis of plants transformed with 5'-UTR of *Tdc* harboring two different lengths of repeat motifs [(CT) $_8$ and (CT) $_{21}$] cloned upstream of *gus A* reporter gene clearly indicated that the level of expression was dependent on copy number of repeat motif: longer the repeat motif, higher the expression level of reporter gene. This was in agreement with the report of De Amicis *et al.*⁴⁷ where *gusA* leader when replaced with (CT) n repeat sequence led to enhanced expression of reporter gene. Hence, the (CT) n microsatellite motifs *per se* as well as their copy number variation govern mRNA transcript abundance *in situ* of *Tdc* gene in *C. roseus*.

It is well established that transcript abundance has no direct quantitative relationship to its rate of synthesis^{43,44}. In many cases, certain transcripts maybe are rarely transcribed but may exhibit a prolonged half-life in response to external or internal cues^{48,49}. In either case, the transcript levels are not true indicators of active transcription and therefore an accurate assessment of transcription rate is often desired to describe the important metrics of promoter activity and relative transcript stability.

Therefore, in this study nuclear run on transcription assay for measurement of the relative rate of *Tdc* gene transcription in *C. roseus* was successfully performed. This indicated that the differential mRNA abundance as observed in previous experiment was due to differential rate of transcription of *Tdc* gene in the two *C. roseus* accessions. Microsatellite motif number variation mainly affected the synthesis of nascent transcripts rather than transcript stabilization or degradation thereby clearly indicating that the motif number dependent *Tdc* expression was solely responsible for increased promoter activity.

In eukaryotes, the translation efficiency is known to be affected by both leader sequence and structure^{40,47,50,51}. In this study, through parallel measurement of transcript abundance and protein synthesis, it was demonstrated that 5'UTR microsatellite length polymorphism [(CT)*n*] was not involved in the process of translation of *Tdc* gene. This could be suggested since the pattern of translationally engaged *Tdc* transcripts profile in the 2 *C. roseus* accessions were found to be similar to that in transcription, thereby indicating its involvement in regulating transcription rather than translation.

Many recent studies have demonstrated that repeats motifs bind to specific protein factors, which in many cases have been characterized to be TFs. In plants, GAGA binding proteins (GBPs) have been identified and characterized to be plant specific transcription factors, the BASIC PENTACYSTEINE/BARLEY B RECOMBINANT (BPC/BBR). Members of this family are characterized by the ability to bind the DNA at GA- rich sequences. These were originally detected in soybean where GBP binds to the promoter of the heme and chlorophyll synthesis gene *Gsa1*, which contains a GAGA element²⁰, and subsequently have been identified in various plant species, such as barley⁵² and Arabidopsis⁵³. Interaction of BPC/GBP with CT/GA repeat motifs leads to transcriptional activation or repression of genes^{20,53–57} involved in a diverse range of developmental processes. For example in barley, the BBR factor directly regulates transcription of the HOMEBOX transcription factor BKN3⁵². In seed development, BPC acts as a regulator of the B3-domain LEAFY COTYLEDON 2 (LEC2) gene⁵⁴. BPCs are also known to regulate the expression of INNER NO OUTER (INO), a gene involved in ovule development⁵³. BPCs also regulate the expression of the ovule identity determining SEEDSTICK (STK) gene by looping its regulatory region and through interaction with a MADS domain transcription factor containing repressor complex⁵⁶.

The EMSA analysis carried out in our study also revealed that a protein factor exclusively interacts with the single strand conformation of (CT) motifs but not with SS (GA) motif or double stranded CT/GA motifs. Further, through *in-silico* analysis of the *C. roseus* transcriptome^{7,58}, four members of BPC/BBR TF were identified that had conserved pentacysteine C-terminal DNA binding domains (Fig. 6). Taken together, our results suggested that the CT repeat motifs might be a specific *cis* element with which a protein, most likely a member of BPC/BBR transcription factor family, may interact for regulating *Tdc* gene transcription *in-vivo*.

Earlier studies have suggested that the SSRs may act as “tuning knobs”^{59,60} for modulation of gene expression, wherein the larger the number of the repeats in tandem, finer the tuning. The changes in the “tuners” of relevant genes in desirable directions and respective changes of the gene activities towards mitigation of the stress therefore get selected⁶⁰. *Tdc* is an important gene of the alkaloid producing pathway (TIA) in *C. roseus* and is known to be induced under several biotic and abiotic stresses. In this context our findings therefore valuable for genetic engineering of TIA pathway in *C. roseus* for enhanced production of alkaloid. In summary, this study presents an exhaustive investigation of the putative role of 5'UTR-SSRs in regulation of gene expression. Our study suggests that the (CT)*n* microsatellite sequence flanking the transcription start site might be a new *cis*-acting, down-stream promoter element (DPE) or enhancer element. Binding of proteins at tandemly repeated (CT)*n* sequences may influence *Tdc* gene transcription in a length dependent fashion. We termed this phenomenon as “**microsatellite mediated enhancement**” (MME) of gene expression. The results presented here will serve as a foundation for further elucidating the mechanism of 5'UTR SSRs mediated gene expression and also provide leads for engineering plants with enhanced amounts of medicinally important indole alkaloids.

Materials and Methods

Biological materials. Fifteen *C. roseus* accessions (Listed in Fig. 2A), were grown in the field and under controlled conditions in the phytotron facility (at NIPGR) as per requirement of experiments. *C. roseus* accessions (Kew1 and Prabal) were also grown in greenhouses with a 16-h (natural daylight) photoperiod and a 25/22 °C day-night temperature regime. Tissue samples were harvested in triplicates from the 2 accessions at same stage of development i.e. four week old seedlings. *Nicotiana benthamiana* plants were grown in green house at 22 °C for 6 weeks. For transformation, we used *agrobacterium tumefaciens* strain GV3101 and plant expression vector pBI121. Sample infiltrated leaf were harvested in triplicate with each leaf having 8–16 infected spots with sizes of typically 3–4 cm² in infiltrated area separated by veins on a single leaf.

Analysis of allelic polymorphism, promoter sequence and gene copy number. Genomic DNA was isolated from leaf samples of *C. roseus* accessions using GenElute™ Plant Genomic DNA Miniprep Kit (SIGMA). Quantity and quality was checked on the Nano-drop instrument (Thermo Scientific). PCR amplification of genomic DNA was performed with gene specific primers [P1: 5'-TGAATCAATGCTGCCCATAC and P3: CTGTGTTGCTGCTGCTTTTC (Fig. 1)]. The PCR reaction mixture contained 50 ng DNA, 1.0 μl of 10 μM of each primer, 1.0 μl of 10 mM dNTPs, 2.0 ul of 10X buffer and 1.0 μl of Titanium Taq DNA polymerase. Reaction conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 20 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 1 min with final extension at 72 °C for 10 min. The PCR products were electrophoresed on 3% metaphor agarose gel at 6 V/cm for 2 h in 1XTBE. The polymorphic bands were eluted using HiYield™ Gel/PCR Mini Kit (RBC) followed by cloning into pGEM-T easy vector (Promega). The putative clones were confirmed by sequencing using Big Dye Terminator kit version 3.0 (ABI, USA) and analyzed with the 3700 ABI Prism 96 capillary sequence analyzer followed by sequence comparison using CLUSTAL-W program of NCBI. For promoter sequence analysis of *Tdc* genes from 2 *C. roseus* accessions, genomic DNA was amplified using primers pair [P1: TGAATCAATGCTGCCCATAC and P4 CATTATTTTCATAATGTGTCGA (Fig. 1)] flanking the minimal *Tdc* promoter sequence. The PCR amplified product were then cloned, sequenced and pair wise alignment was made.

To assess the gene copy number, Quantitative RT PCR⁶¹ was performed on an Applied Biosystem Step One™ Real Time PCR system using 10 ng genomic DNA as template, which was added to 18 μl of reaction mixture containing 6.2 μl of sterile MQ-water, 10 μl of Power SYBR green PCR master mix (Applied Biosystems), 0.9 μl (20 pico moles) of each of forward and reverse primers. PCR amplification was carried out for 50 cycles of 94 °C for

10 sec, 76 °C for 15 sec and 72 °C for 55 sec. The temperature range for the analysis of melting curve was 60 °C to 90 °C over 30 sec. All reactions were performed in triplicate.

Northern analysis, semi-quantitative and quantitative RT-PCR analysis. For Northern analysis, total RNA was isolated using LiCl method and transferred on to nylon membrane according to Sambrook *et al.*⁶². Probe sample was prepared by random labeling using NEBlot[®] kit (Amersham Biosciences) and purified by gel filtration on Sephadex G-50 (GE Healthcare) spin column. The purified probe was denatured and used for hybridization for 14–16 hr at 60 °C. Membrane was washed twice with 2X SSC and 0.1% SDS (w/v) followed by autoradiography at –80 °C for 24–48 hrs depending upon the signal intensity.

For the quantification of gene expression, first-strand cDNA synthesis was carried out by using Clontech kit as per manufacturer instructions. The first strand cDNA sample was used as template for semi qRT-PCR analysis, which was carried out in 50 µl of PCR reaction mix having 5 µl of 10X PCR buffer, 4 µl of 2.5 µM dNTP (New England Biolab), 0.2 µl of Titanium Taq polymerase (Clontech) and 1 µl of each gene specific primer. All PCR amplifications (in triplicate) were carried out using the following program: denaturation at 95 °C for 2 min, 21/23/25 cycles at 95 °C for 15 s, 60 °C for 15 s, 68 °C for 1 min and final extension at 72 °C for 7 min. Aliquots were collected after 21 cycles, 23 cycles and 25 cycles of PCR run followed by electrophoresis on 1.2% agarose/EtBr gels.

For real time PCR analysis, the following primers were used to amplify the *Tdc* gene transcript [sense 5'-AGCGAAGTCGAACCTGGATATC and antisense 5'-GGGAGGTAAGGAGCGGTTTC] and the *C. roseus* actin gene was amplified using [sense 5'-CTATGTTCCAGGTATTGCAGATA and antisense 5'-GCTGCTTGGAGCCAAAGC]. Reaction mix was prepared as described above for quantitative RT-PCR. The PCR amplifications consisted of an initial incubation of 2 min at 50 °C followed by denaturation at 95 °C for 10 min, 40 cycles of amplification of 15 seconds at 95 °C and 1 min at 60 °C. Finally, a melt curve analysis was performed from 60 °C to 95 °C in increments of 0.5 °C to confirm the presence of a single product and absence of primer-dimers. Each sample was assayed in triplicates, and each experiment was repeated at least twice. Expression levels were calculated relative to the constitutively expressed gene (actin). Normalization was carried out using the $\Delta\Delta$ Ct method (Applied Biosystems), where Δ Ct was calculated for each sample as the difference between Ct of the target gene and Ct of actin, and final relative expression level was determined as inverse of \log_2 of Ct (target gene) – Ct (actin).

Transient expression analysis. Plasmid construction. For the heterologous gene expression analysis, the 5' UTR regions of *Tdc* gene from the two *C. roseus* accessions Kew1 and Prabal were amplified with UTR specific primer pairs which were designed in such a manner that 5' end of forward primer had *Xba*I restriction site whereas the 5' end of reverse primer had *Xma*I restriction site (F: 5'-GCTCTAGAACAGCACAGCAACTTCCTCA and R: 5'-CCCCCGGTGTGAATCAATGCTGCCCATTA). The PCR amplicon and the pBI121 plant expression vector were double digested with respective enzymes (NEB) followed by purification and ligation using T4 DNA ligase (Clontech). Recombinant and control pBI121 vector was transformed into competent *E. coli* cells (NEB-10 beta) and confirmed by sequencing using CaM35S forward primer and *GusA* reverse primers. Further, plasmid DNA was transformed into the competent GV3101 *agrobacterium* strain and selected on media containing 50 µg/ml kanamycin and 30 µg/ml rifampicin.

Agro infiltration. The colonies harboring individual constructs [pBI121, pBI121:(CT)₈ and pBI121:(CT)₂₁] were streaked on YEP solid medium (10 g⁻¹ bacto-peptone, 10 g⁻¹ yeast extract, 5 g⁻¹ NaCl, 15 g⁻¹ agar) supplemented with rifampicin (60 µg ml⁻¹) and kanamycin (50 µg ml⁻¹) and grown at 28 °C for 2 days. *Agrobacterium* colonies were then inoculated in 20 ml induction medium containing AB salts (1 g⁻¹ NH₄Cl, 0.3 g⁻¹ MgSO₄·7H₂O, 0.15 g⁻¹ KCl, 0.01 g⁻¹ CaCl₂, 0.0025 g⁻¹ FeSO₄·7H₂O), 2 mM phosphate, 1% glucose, 20 mM 2-(*N*-morpholino) ethanesulfonic acid (MES, pH 5.5), 100 µM acetosyringone as well as rifampicin and kanamycin. After overnight culture at 28 °C, *agrobacterium* cells were pelleted by centrifugation for 15 min at 3000 × g and resuspended in 10 mM MES (pH 5.5) plus 10 mM MgSO₄ solution plus MS basal medium. After supplementing with 100 µM acetosyringone, bacterial suspension was adjusted to a final OD₆₀₀ of 0.8 for agroinfiltration.

Transient transformation was conducted on fully expanded tobacco leaves that were still attached to the intact plant. Bacterial suspension was infiltrated into intercellular spaces of intact leaves using a plastic syringe of 1 ml. 100 µl of bacterial suspension was infiltrated into 8–16 spots (typically 3–4 cm² in infiltrated area) separated by veins on a single leaf. Leaf discs from each infiltration site were collected and total RNA was isolated for first strand cDNA synthesis as described earlier. Next, the transient expression analysis was performed by semi-quantitative and quantitative PCR as described earlier, wherein, reporter gene *gusA* transcript was amplified by gene specific primer pairs [sense 5'-TGCATCAGCCGATTATCATCA, antisense 5'-GTGCAGCCCGCTAACG] and control gene *actin* (tobacco) transcripts was amplified by gene specific primer pairs [sense 5'-TGGCATCATACTTTCTACAATGA, antisense 5'-CCCCTATAAATTGGGACAG].

Nuclear run-on transcription assay. Nuclei isolation and in-vitro transcription. Intact nuclei isolation and run-on transcription assay were carried out using the procedure of Kumar and Bhatia⁶³. In brief, 5 g of *C. roseus* leaf tissue was homogenized and then suspended in 1X extraction buffer (2 M hexylene glycol (2-methyl-2, 4-pentandiol), 20 mM PIPES-KOH (pH 7.0), 10 mM MgCl₂ and 5 mM 2-mercaptoethanol). The homogenate was filtered and 10% triton-X-100 was added to the solution until the final concentration was 1%. The extract was gently layered onto the top of the Percoll gradient of 30% and 80% assembled in a 50 ml culture tube followed by centrifugation at 2,000 × g for 30 min at 4 °C. The nuclei at the 30–80% interface were collected and washed followed by centrifugation at 900 × g for 5 min. The nuclei pellet was then dissolved in 500 µl of nuclei storage buffer (50 mM Tris-HCl (pH 7.8), 10 mM 2-mercaptoethanol, 20% glycerol, 5 mM MgCl₂ and

0.44 M sucrose). The run-on reaction (100 µl) was set up using 10 µl of 10X transcription assay buffer [250 mM Tris-HCl (pH 7.8), 375 mM NH₄Cl, 50 mM MgCl₂ and 50% (v/v) glycerol], 5 µl of each 100 mM CTP, GTP and ATP, 10 µl of ³²P-UTP, 50 µl nuclei in storage buffer and 15 µl of water. Reaction was allowed to proceed at 30 °C for 20 min followed by addition of 10 U of DNase I to terminate the reaction. Transcripts were purified and precipitated with 1/10 volume of 7.5 M ammonium acetate (pH 7.0), 100 µg of yeast tRNA along with ice-cold ethanol. Precipitated sample was dissolved in hybridization buffer.

Hybridization and autoradiography. The radio labelled run-on transcripts in hybridization buffer (Miracle-Hyb buffer, Stratagene USA) were denatured and hybridized with blots (harboring target (*Tdc*), reference gene (*Rps9*, SAND) and negative control (MATE) for 16 h at 60 °C. The filters were washed three times in 2×SSC, 0.1% SDS at 65 °C, twice in 0.2×SSC, 0.1% SDS at 65 °C and once in 0.1% SSC, 0.1% SDS at 65 °C. The semi-dried membrane was exposed to hyper screen (Amersham) in a cassette and was kept at room temperature for 1–12 h. Autoradiography was carried out using Molecular Dynamics Phosphor Imager and filter was scanned with high resolution scanner (Typhoon, Amersham USA). The band intensities were quantified with Alpha Imager software (Innotech Corporation, San Leandro, CA, USA).

Polysome isolation and quantification of translating *Tdc* mRNA. For polysome isolation from leaf tissue, the modified protocols of Juntawong *et al.*⁶⁴ were used. *C. roseus* leaves (0.4 g fresh weight) were homogenized in liquid nitrogen with mortar and pestle and suspended in 2 mL of ribosome extraction buffer (50 mM KCl, 200 mM Tris-acetate, pH 8.5, 25 mM MgCl₂, 5 mM EGTA, 10 mM 2-mercaptoethanol, 2% polyoxyethylene [10] tridecyl ether, 1% Triton X-100, 100 µg/ml Heparin, 0.5% [v/v] Nonidet P-40, 100 µg/ml chloramphenicol, and 100 µg/ml cycloheximide). The suspension was centrifuged for 10 min at 15,000 g and 4 °C to remove cell debris. A 500 µL aliquot was removed and frozen for subsequent total RNA isolation, cDNA preparation and quantification by qRT-PCR. Aliquots of the resultant supernatant were loaded on 10 to 50% (w/w) continuous sucrose gradients and centrifuged at 38,000 rpm for 3 h in a Beckman 90Ti rotor at 4 °C. After centrifugation, fractions were collected from the bottom to the top of the gradient with continuous monitoring of 254-nm absorbance and the fraction representing polysomes were further subjected to RNA extraction, cDNA preparation and quantification by qRT-PCR.

Electrophoretic mobility shift assay (EMSA). The cell extract was prepared using the modified protocol described in CellLytic™ PN plant nuclei isolation/extraction Kit (Sigma) protocol. For preparing the whole cell extract, leaf sample was homogenized and suspended in extraction buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 1:100(v/v) protease inhibitors cocktail), and kept on ice for 30 min followed by centrifugation for 3 min at 200×g. The resulting supernatant was desalted over PD-10 column (Sigma-Aldrich) against gravity. Three sets of oligonucleotide probes namely single stranded (CT)₂₁, single stranded (GA)₂₁, and double stranded (CT/GA)₂₁ oligo (annealed) were generated by labelling 100 pmol of oligonucleotide with 5 µl of radio-labeled γ³²p-ATP (6,000 ci/mmol, 50 µCi) and 1 µl of T4 polynucleotide kinase (10 unit) for 30 min at 37 °C. Reaction was terminated by heating at 85 °C for 5 min followed by purification by gel filtration on Sephadex G-25 spin column. Labeled DNA was denatured by heating in boiling water bath at 95–100 °C for 5 min. Sample was quickly placed in ice bath for 5 min. The protein-DNA binding reaction was performed in 50 µl reaction volume that consisted of 30 µg cell extract, 1X Binding buffer (20 mM HEPES (pH 7.5), 0.5 mM EDTA, 1 mM MgCl₂, 20 mM KCl, 6% glycerol, 1 µg poly (dI-dC) and 0.5 mM DTT) and 50–100 pmol of labeled oligo-nucleotide probe, at room temperature for 20–30 min. The bound products were separated on 6% native polyacrylamide (acrylamide: bisacrylamide, 19:1) gel in 1× TBE at room temperature for 1 h and exposed to autoradiography.

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Acknowledgements

We acknowledge the National Institute of Plant Genome Research (NIPGR), New Delhi, India for the funding support and the Council of Scientific and Industrial Research (CSIR) for fellowship grant to SK.

Author Contributions

S.K. and S.B. designed the research, S.K. performed the experiments, and S.K. and S.B. wrote the manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Kumar, S. and Bhatia, S. A polymorphic (GA/CT)*n*- SSR influences promoter activity of *Tryptophan decarboxylase* gene in *Catharanthus roseus* L. Don. *Sci. Rep.* **6**, 33280; doi: 10.1038/srep33280 (2016).



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