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RESEARCH ARTICLE

Cytosine hypomethylation at CHG and CHH sites in the pleiotropic mutants of Mendelian inheritance in *Catharanthus roseus*

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Abstract

The 5S and 18S rDNA sequences of *Catharanthus roseus* cv 'Nirmal' (wild type) and its leafless inflorescence (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*) single mutants and *lli egd*, *lli ill* and *egd ill* double mutants were characterized. The *lli*, *egd* and *ill* mutants of Mendelian inheritance bore the names after their most conspicuous morphological feature(s). They had been chemically induced and isolated for their salt tolerance. The double mutants were isolated as morphological segregants from crosses between single mutants. The morphological features of the two parents accompanied salt tolerance in the double mutants. All the six mutants were hypomethylated at repeat sequences, upregulated and downregulated for many genes and carried pleiotropic alterations for several traits. Here the 5S and 18S rDNAs of *C. roseus* were found to be relatively low in cytosine content. Cytosines were preponderantly in CG context (53%) and almost all of them were methylated (97%). The cytosines in CHH and CHG (where H = A, T or C) contexts were largely demethylated (92%) in mutants. The demethylation was attributable to reduced expression of *RDR2* and *DRM2* led RNA dependant DNA methylation and *CMT3* led maintenance methylation pathways. Mutants had gained some cytosines by substitution of C at T sites. These perhaps arose on account of errors in DNA replication, mediated by widespread cytosine demethylation at CHG and CHH sites. It was concluded that the regulation of cytosine methylation mechanisms was disturbed in the mutants. *ILL*, *EGD* and *LLI* genes were identified as the positive regulators of other genes mediating the RdDM and CMT3 pathways, for establishment and maintenance of cytosine methylation in *C. roseus*.

[Kumari R., Yadav G., Sharma V., Sharma V. and Kumar S. 2013 Cytosine hypomethylation at CHG and CHH sites in the pleiotropic mutants of Mendelian inheritance in *Catharanthus roseus*. *J. Genet.* **92**, 499–511]

Introduction

Cytosine methylation marks on DNA arise by enzymatic addition of a methyl group at the fifth position of cytosine (5-mC). The 5-mC marks carried genomewide allows cell, tissues and organs, and selfed progenies of homozygous plants to be genetically homogenous but phenotypically variable. Their presence on protein and RNA coding gene promoters and gene bodies affects gene expression negatively. The 5-mC marks are gained and lost at genes and other genetic elements in genome during organ development and in response to environmental stimuli. Not all of the methylation sensitive cytosine sites in the plant genomes are methylated simultaneously. For these reasons the 5-mC caused variation in plant populations is much more than genetic variation (Johannes *et al*. 2009; Reinders *et al*. 2009; Teixeira *et al*. 2009; Sasaki *et al*. 2012; Kumar *et al*. 2013).

Cytosine methylation in plant DNA sequences occurs at CG, CHG and CHH elements (where H = A, T or C) (Bennetzen and Zhu 2011). The cytosine methylation related mechanisms have been analysed in some detail by the use of forward and reverse genetics approaches in the model plant species, *Arabidopsis thaliana* (Bennetzen and Zhu 2011; He *et al*. 2011). It is now known that cytosine methylation at CG, CHG and CHH elements is established by the DOMAIN REARRANGED METHYLTRANSFERASE 2

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Keywords. bisulphite sequencing; CMT3; DNA demethylases; DNA methyl transferases; demethylation; excision repair; mutagenic hot spots; phylogenetic relationships; 5S rDNA; 18S rDNA; RdDM; spontaneous mutagenesis.

(DRM2) in a RNA directed DNA methylation (RdDM) pathway (Cao and Jacobsen 2002). This pathway involves synthesis of short interfering RNA (siRNAs) and deploys a protein complex for targeting those DNA sequences for methylation that are homologous to siRNAs. This process is also used for the maintenance of methylation at CHH elements (Saze *et al*. 2012). *METHYLTRANSFERASE 1* (*MET1*) and *CHROMOMETHYLTRANSFERASE 3* (*CMT3*) gene products maintain cytosine methylation, respectively, at the symmetric elements CG and CHG, during DNA replication in cell divisions (Lindroth *et al*. 2001; Kankel *et al*. 2003). There is redundancy in the demethylation enzymes [DEMETER (DME), REPRESSOR OF SILENCING 1 (ROS1), DEMETER-LIKE (DML2) and DML3] that remove 5-mC from DNA irrespective of the nature of C containing elements (Choi *et al*. 2002; Gong *et al*. 2002; Ortega-Galisteo *et al*. 2008; Zhu 2009). The net cytosine methylation status in plant genomes is determined by the antagonistic activities of methyltransferases and demethylases.

The 5-mC sites are known to be mutational hot spots. Each of the 5-mC is prone to spontaneous deamination leading to its replacement by thymine (T). Also, 5-mC can be removed from its location by the glycosylase and lyase activities of any of the demethylases (Pfeifer 2006; Walsh and Xu 2006). The abasic gap left behind by the demethylase action is filled by any of the DNA repair process involving a nuclease, DNA polymerase and DNA ligase (Agius *et al*. 2006; Bhutani *et al*. 2011). Erroneous repairs are mutagenic (Jackson and Bartek 2009).

The methyltransferase mutants such as *met1* and *drm1 drm2 cmt3* are known to demonstrate pleiotropic phenotypes which include extensive DNA demethylation, gene expression changes, transposon activation, stress tolerance, partial sterility and a spectrum of morphological alterations (Finnegan 1996; Chan *et al*. 2006; Ream *et al*. 2009; Dowen *et al*. 2012; Luna *et al*. 2012). Such pleiotropies have also been observed in plants exposed to a variety of abiotic or biotic stresses (Wada *et al*. 2004; Choi *et al*. 2007; Lira-Medeiros *et al*. 2010; Karan *et al*. 2012; Slaughter *et al*. 2012; Song *et al*. 2012; Kumar *et al*. 2013).

Nuclear ribosomal RNA genes (rDNAs) of plants occur in tandemly arranged arrays of 5S rDNA, 35S rDNA (18S + 5.8S + 26S rDNAs) and 5S and 35S linked rDNAs (Mathieu *et al*. 2003; Garcia *et al*. 2012). The rDNAs are serving as important tools in revealing the role of cytosine methylation and various types of mutations in the evolution of nuclear DNA sequences (Fulnecek *et al*. 2002; Vaillant *et al*. 2008; Blevins *et al*. 2009; Garcia-Aguilar *et al*. 2010; Wicke *et al*. 2011).

In *C. roseus*, stable Mendelian mutants of unique morphological phenotypes, such as *leafless inflorescence* (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*), have been observed to be deficient in cytosine methylation at repeat DNA sequences such as in rDNA and centromeric DNA (Kumari *et al*. 2013). They demonstrate manifold pleiotropic phenotypes and overexpress or underexpress many genes (Rai *et al*. 2003; Chaudhary *et al*. 2011; Kumar *et al*. 2012; Kumari *et al*. 2010, 2013). The experiments described here

Table 1. Salient properties of the wild type and salt tolerant-cum-morphological-cum-cytosine hypomethylated mutants of constant genetic background of *C. roseus* cv Nirmal. The mutants had been experimentally induced by the use of chemical mutagens such as ethyl methanesulphonate (Kulkarni *et al*. 1999; Rai *et al*. 2003) and were identified as *glycophytic salinity response* (*gsr*) salt tolerant mutants. They were named after their most conspicuous morphological feature(s).

Genotype	Properties	Reference(s)
Wild type	Floricultural-cum-medicinal cultivar Nirmal; white flowered, salt sensitive; tall habit; smooth leaves; secondary inflorescence arises from the axil of one of the two leaves at a node	Mishra *et al*. (2001); Kulkarni *et al*. (1999, 2003); Rai *et al*. (2003)
leafless inflorescence (*lli*)[a]	Root, stem and leaves of large biomass; large inflorescence; hyper-flowering	Rai *et al*. (2003); Kumar *et al*. (2007, 2012); Kumari *et al*. (2010, 2013); Sharma *et al*. (2012); Chaudhary *et al*. (2011, 2013)
evergreen dwarf (*egd*)[a]	High content of chlorophyll in leaves, high rate of photosynthesis, pavement cells and spongy mesophyll parenchyma cells of large size; high water content in leaves and slow rate of water loss from leaves	Rai *et al*. (2003); Kumari *et al*. (2010, 2013)
irregular leaf lamina (*ill*)[a]	Accumulation of pharmaceutical terpenoid indole alkaloids at high levels in roots and leaves; biomass of small size in root, stem and leaves; low chlorophyll content in leaves and photosynthesis at low level	Kulkarni *et al*. (1999, 2003); Kumari *et al*. (2013)
lli egd[a]	Stem/leaf ratio high; higher photosynthetic rate and water content in leaves	Kumari *et al*. (2013)
lli ill[a]	Spongy parenchyma cells of small size; high K^+ content	As above
egd ill[a]	Least plant biomass; root shoot ratio high; spongy mesophyll parenchyma cells of largest size	As above

[a]As compared to wild type, many genes were similarly upregulated and downregulated in single and double mutants (see tables 1 and 2 in electronic supplementary material at http://www.ias.ac.in/jgenet/; figure 1).

were taken up to reveal the nature of genetic deficiency responsible for cytosine hypomethylation in the mutants. The 5S and 18S rDNA sequences of the wild type and mutants were compared with each other and with those of heterologous plant species. It was observed that the 5S and 18S rDNAs of *C. roseus lli*, *egd* and *ill* mutants were on the one hand hypomethylated, especially at the CHG and CHH elements, and on the other hand had added 5-mC at new sites, mostly creating CHH elements. The mutants were found to be defective in the expression of RdDM and CMT3 pathways for cytosine methylation establishment and maintenance.

Materials and methods

Catharanthus roseus plant material

Seven true breeding genotypes of *C. roseus* maintained by selfing comprised the plant genetic material used (table 1).

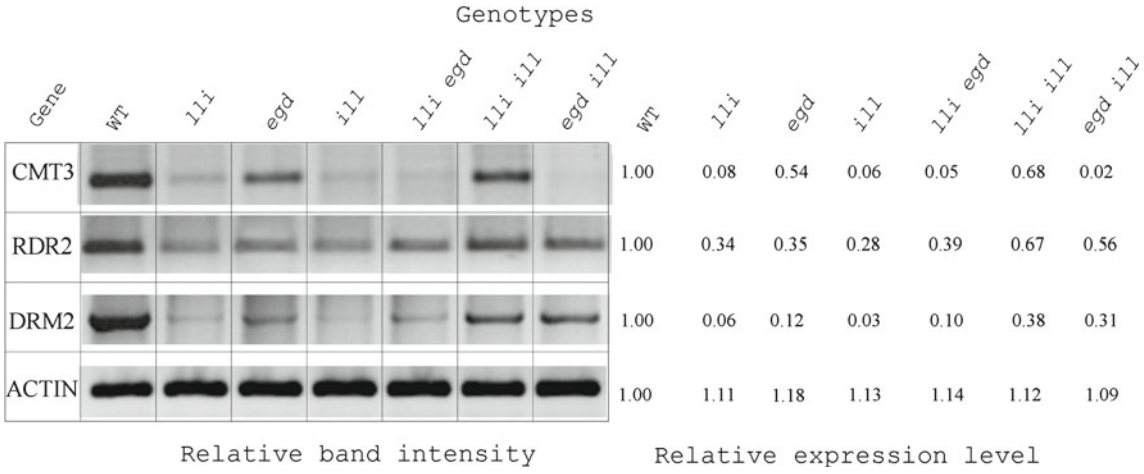

Figure 1. Transcript levels of *CMT3* (*Chromomethylase 3*), *DRM2* (*Domain rearranged methyltransferase 2*) and *RDR2* (*RNA-DEPENDENT RNA POLYMERASE 2*) by semiquantitative RT-PCR in which *ACTIN* served as control gene. The primer sequences and the procedures used are described in Kumari *et al*. (2013).

Pattern of C-methylation in a 120 bp region of 5S rRNA gene in wild type and *gsr* mutants of *Catharanthus roseus*

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WT          ---CGAAGAATTTGAGATATTAA-AGAATTTTAGAAGATGAGAAATTAATAAAAGGTAAG
lliegd      ---CGAAGAATTTGAGATACTAA-AGAATTTTAGAAGATGAGAAATTAATAAAAGGTAAG
ill         ---CGAAGAATTTGAGATATTAA-AGAATTTTAGAAGATGAGAAATTAATAAAAGGTAAG
egdill      ---CGAAGAATTTGAGATATTAA-AGAATTTTAGAAGATGAGAAATTAATAAAAGGTAAG
egd         ---CGAAGAATTTGAGATATTAA-AGAATTTTAGAAGATGAGAAATTAATAAAAGGTAAG
lli         ---TATTTAGTGGTTGATGGTGTTGGTGGAATTGTAG--GAGAAATTAATAAAAGGCAAG
lliill      ---TATTTAGTGGTTGATGGTGTTAGTGGAATTGTAG--GAGAAATTAATAAAAGGCAAG
                  * *    ***  *          * * **  ***************** ***
WT          AGGGTGAGTTTATCTTATGAAAAGCCGAAAAGAGTGTTAAATACGAGTGTTAAGTGATTA
lliegd      AGGGTGAGTTTATTTTATGAAAAGCCGAAAAGAGTGTTAAATACGAGTGTTAAGTGATTA
ill         AGGGTGAGTTTATTTTATGAAAAGCCGAAAAGAGTGCTAAATACGAGTGTTAAGTGATTA
egdill      AGGGTGAGTTTATTTTATGAAAAGCCGAAAAGAGTGTTAAATACGAGTGTTAAGTGATTA
egd         AGGGTGAGTTTATTTTATGAAAAGCCGAAAAGAGTGTTAAATACGAGTGTTAAGTGATTA
lli         AGGGTGAGTTTATTTTATGAAAAGTCGAAAAGAGTGTTAAATACGAGTGTTAAGTGATTA
lliill      AGGGTGAGTTTATTTTATGAAAAGTCGAAAAGAGTGTTAAATACGAGTGTTAAGTGATTA
            ************ ********** *********** ************************
WT          TCTTCA
lliegd      TCTTTA
ill         TTTTTA
egdill      TTTTTA
egd         TTTTTA
lli         TTTTTA
lliill      TTTTTA
            * ** *
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Figure 2. Clustal W alignment of bisulphite-treated DNA sequence of bases in a 120 nucleotide mRNA coding region of 5S rDNA in *C. roseus* cv Nirmal wild type and its salt tolerant-cum-morphological single mutants, *leafless inflorescence* (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*) and *lli egd*, *lli ill* and *egd ill* double mutants. Methylated cytosines are shown in red Cs, unmethylated cytosines are shown in green Ts and gained methylated cytosines are shown in blue Cs.

Leaves borne at stem tips in adult plants were used for extracting DNA. The plants sampled for DNA had been grown in the field, by use of already described cultivation procedures (Mishra *et al*. 2001; Rai *et al*. 2003; Sharma *et al*. 2012; Chaudhary *et al*. 2013).

Bisulphite sequencing of 5S and 18S rDNAs of C. roseus variants

Five micrograms DNA of each genotype extracted from the leaves (Saghai-Maroof *et al*. 1984; Kumari *et al*. 2013) was digested with *Hin*dIII and then the bisulphite modification was done with EZ DNA methylation-Gold™ Kit (Zymo Research, Orange, USA) (Choi *et al*. 2007). This purified bisulphite treated DNA was used for the amplification with primers specific for bisulphite-modified DNA (designed by Meth primer; http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) (Li and Dahiya 2002) for 5S rRNA and 18S rRNA regions under PCR condition of 94°C 3 min, 35 cycles of 94°C 30 s, 58°C 30 s, 72°C 30 s and a cycle of 72°C 10 min (Yang *et al*. 2004; Carr *et al*. 2007). The primer sequences for 5S rRNA were: F = 5′-GGTGATTTTTTGGGAAGTTTT-3′ (21 nucleotides; nt), R = 5′-AAATCAAACAAATAATATATCCAC-3′ (24 nt), and for 18S rRNA, the primer sequences were: F = 5′-TAATTTGATTTGAAAGATGAATAATT-3′ (26 nt) and R = 5′-CCCATATTAAATCAAATTAAAC-3′ (22 nt). The PCR product in each case was gel purified (QIAEX II Agarose Gel Extraction method, Qiagen, Hilden, Germany) and used as insert. The products were cloned into the pGEMT-Easy Vector System (Promega, Madison, USA). The clones so obtained (figure 1 in electronic supplementary material) were sequenced using ABI Prism 3700 Sequencer (figures 2 and 3).

Intraspecies and interspecies analyses of 5S and 18S rDNA sequences

The base sequences obtained after sequencing also included the vector sequences. These were removed with the help

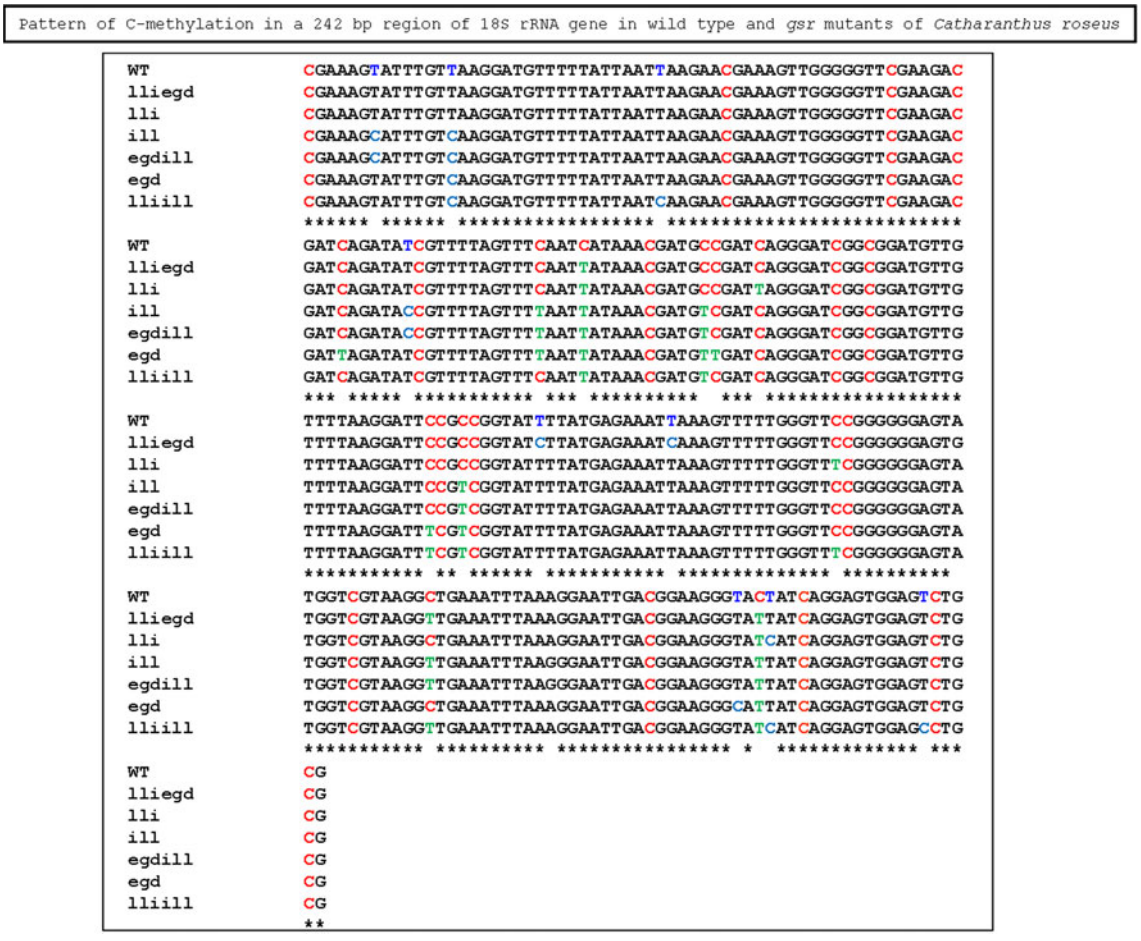

Figure 3. Clustal W alignment of bisulphite-treated DNA sequence of bases in a 242 nucleotide mRNA coding region of 18S rDNA in *C. roseus* cv Nirmal wild type and its salt tolerant-cum-morphological single mutants, *leafless inflorescence* (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*) and *lli egd*, *lli ill* and *egd ill* double mutants. Methylated cytosines are shown in red Cs, unmethylated cytosines are shown in green Ts and gained methylated cytosines are shown in blue Cs.

Table 2. List of species whose 5S rDNA sequence corresponding to 120-bp 5S rDNA of *C. roseus* cv Nirmal and its mutants was compared.

	Class	Plant name	Abbreviation	Accession no.	Gene ID	Per cent cytosine content
1	Cryptomonad	*Cryptomonas paramecium*	Cryp	NC_015331.1	10447389	26.7
2	Moss	*Brachythecium rutabulum*	BR1	FR695700.1	347626704	21.7
3		*Physcomitrella patens*	Php	NC_007945.1	3989114	26.7
4	Fern	*Equisetum hyemale*	EH	FR695694.1	347626698	28.3
5	Gymnosperm	*Cedrus deodara*	Cde	NC_014575.1	9845541	25.8
6		*Cycas taitungensis*	Cyt	NC_010303.1	5867549	27.5
7		*Pinus gerardiana*	Pg	NC_011154.4	7875156	25.8
8	Monocot	*Oryza rufipogon*	Oruf	NC_013816.1	8774356	25.0
9		*O. sativa*	OS	DQ152232.1	76468802	31.7
10		*O. sativa indica*	OSI	NC_007886.1	3950768	25.0
11		*O. sativa japonicus*	Osj	NC_011033.1	6450136	25.0
12		*Sorghum bicolor*	Sorb	NC_008360.1	4306032	25.0
13		*Spirodella polyrhiza*	SPP	NC_017840.1	12486912	25.0
14		*Triticum aestivum*	TA	NC_007579.1	3800117	25.0
15		*Zea luxurians*	Zlux	NC_008333.1	4267034	28.3
16		*Z. mays*	ZM	NC_007982.1	4055935	28.3
17		*Z. mays* subsp. *parviglumis*	ZMpar	NC_008332.1	4267078	28.3
18		*Z. perennis*	Zper	NC_008331.1	4266994	28.3
19	Eudicot	*Arabidopsis thaliana*	AT	NC_001284.2	4024964	25.8
20		*Atropa belladona*	Atb	NC_004561.1	806527	24.2
21		*Beta macrocarpa*	Bmac	NC_015994.1	11124105	25.8
22		*B. vulgaris*	Bvl	NC_015099.1	10220689	25.8
23		*Boea hygrometrica*	BH	NC_016741.1	11542637	24.2
24		*Brassica rapa subsp. campestris*	Bcamp	NC_016125.1	11272168	25.0
25		*B. napus*	Bnap	NC_008285.1	4237954	25.0
26		*B. oleracea*	Bole	NC_016118.1	11271767	25.0
27		*Carica papaya*	Cpa	NC_012116.1	7441447	25.8
28		*Citrullus lanatus*	Clan	NC_014043.1	9072749	25.8
29		*Citrus maxima*	CM	FJ356261.1	209971515	39.2
30		*Cucumis sativus*	Cucs	NC_016005.1	11123873	25.0
31		*Cucurbita pepo*	Cp	NC_014050.1	9072867	25.8
32		*Datura stramonium*	Dstr	NC_018117.1	13230378	24.2
33		*Daucus carota*	Dcar	NC_017855.1	12598520	25.8
34		*Glycine max*	Gmax	NC_007942.1	3989369	24.2
35		*Gossypium barbadense*	Gb	NC_008641.1	4575267	24.2
36		*Helianthus annuus*	Ha	NC_007977.1	4055611	24.2
37		*Hevea brasiliensis*	Hev	NC_015308.1	10352000	23.3
38		*Lotus japonicus*	LJ	NC_016743.2	11542754	24.2
39		*Medicago truncatula*	MT	NC_003119.6	5333162	35.8
40		*Mercurialis canariensis*	MC	DQ536139.1	108744202	35.8
41		*M. elliptica*	ME	DQ536135.1	108744198	35.8
42		*M. perennis*	MP	DQ536142.1	108744205	35.8
43		*M. reverchonii*	MR	DQ536137.1	108744200	35.0
44		*M. tomentosa*	MT	DQ536133.1	108744196	35.8
45		*Millettia pinnata*	Mpin	NC_016742.1	11542718	24.2
46		*Mimulus guttatus*	MG	NC_018041.1	13080227	25.0
47		*Morus indica*	Min	NC_008359.1	4290667	24.2
48		*Nelumbo lutea*	Nl	NC_015605.1	10743505	24.2
49		*Nicotiana tabacum*	Ntab	NC_006581.1	3205278	25.0
50		*Phaseolus vulgaris*	Phv	NC_009259.1	5075309	24.2
51		*Pisum sativum*	PS	NC_014057.1	9073084	25.0
52		*Ricinus communis*	Rcom	NC_015141.1	10221389	25.8
53		*Sesamum indicum*	Sin	NC_016433.2	11452489	24.2
54		*Silene latifolia*	Sil	NC_016730.1	11452489	24.2
55		*S. noctiflora*	Snoc	NC_016371.1	11353845	22.5
56		*S. vulgaris*	SVL	NC_016406.1	11447187	25.8
57		*Solanum lycopersicum*	SL	NC_007898.2	3950436	24.2
58		*Tripsacum dactyloides*	TD	NC_008362.1	4306106	28.3
59		*Vigna radiata*	VR	NC_015121.1	10215546	25.0
60		*Vitis vinifera*	VV	NC_012119.1	7498744	25.8

Table 3. List of species whose 18S rDNA sequence corresponding to 242 bp 18S rDNA of *Catharanthus roseus* cv Nirmal and its mutants was compared.

	Class	Plant name	Abbreviation	Accession no.	Gene ID	Per cent cytosine content
1	Cryptomonad	*Cryptomonas paramecium*	Crp	NC_015331.1	10447385	16.5
2	Liverwort	*Merchantia polymorpha*	Mpo1	X75521.1	547571	20.3
3		*Pleurozia purpurea*	Ple	NC_013444.1	8542323	23.6
4		*Treubia lacunosa*	Trlc	NC_016122.1	11272047	19.8
5	Hornwort	*Megaceros aenigmaticus*	Meg	NC_012651.1	7804480	22.7
6		*Phaeoceros laevis*	Phl	NC_013765.1	8746939	22.3
7	Moss	*Anomodon rugelii*	Anr	NC_016121.1	11271920	23.1
8		*Huperzia squarrosa*	Husq	NC_017755.1	12354473	23.6
9		*Physcomitrella patens*	Phy	NC_007945.1	3989112	23.1
10	Fern	*Equisetum hyemale*	EH	FR695694.1	347626698	21.1
11	Gymnosperm	*Cycas taitungensis*	Cyt	NC_010303.1	5867613	23.6
12	Monocot	*Oryza sativa japonica*	Osj	NC_011033.1	6450167	23.9
13		*O. rufipogon*	Oruf	NC_013816.1	8774318	23.7
14		*O. sativa*	OS	NC_007886.1	3950770	23.9
15		*O. sativa indica*	Osi	NC_007886.1	3950769	23.9
16		*Sorghum bicolor*	Sorb	NC_008360.1	4306030	23.9
17		*Spirodela polyrhiza*	Spirp	NC_017840.1	12486911	23.9
18		*Tripsacum dactyloides*	Trd	NC_008362.1	4306104	23.9
19		*Triticum aestivum*	TA	Z14078.1	14395	23.9
20		*Zea luxurians*	Zlux	NC_008333.1	4267032	25.6
21		*Z. mays*	ZM	NC_007982.1	4055912	26.0
22	Eudicot	*Arabidopsis thaliana*	AT	X16077.1	16506	14.5
23		*Beta macrocarpa*	Bmac	NC_015994.1	11124146	23.9
24		*B. vulgaris*	Bvl	NC_015099.1	10220733	23.9
25		*Brassica napus*	Bnap	NC_008285.1	4237952	22.7
26		*B. oleracea*	Bole	NC_016118.1	11271766	22.7
27		*B. rapa campestris*	Brcamp	NC_016125.1	11272166	22.7
28		*Carica papaya*	Carp	NC_012116.1	7441448	23.7
29		*Citrullus lanatus*	Clan	NC_014043.1	9072752	23.9
30		*Cucurbita pepo*	Cucp	NC_014050.1	9072830	23.9
31		*Daucus carota*	Dc	NC_017855.1	12598521	23.9
32		*Lotus japonicus*	LJ	NC_016743.2	11542753	23.7
33		*Millettia pinnata*	Mpin	NC_016742.1	11542719	23.9
34		*Mimulus guttatus*	Mg	NC_018041.1	13080206	23.9
35		*Nicotiana tabacum*	NT	NC_006581.1	3205239	23.9
36		*Ricinus communis*	Rcom	NC_015141.1	10221391	23.9
37		*Silene conica*	Scon	NC_016228.1	11341904	23.6
38		*S. noctiflora*	Siln	NC_016371.1	11353846	21.5
39		*S. vulgaris*	Silv	NC_016406.1	11447186	22.3
40		*Vitis vinifera*	VV	NC_012119.1	7498754	23.9

of Vecscreen (www.ncbi.nlm.nih.gov/vecscreen/vecscreen.html) of NCBI. Thus 5S rRNA and 18S rRNA sequences for the wild type and six mutants became available. The seven sequences for each kind of rDNA were submitted for multiple sequence alignment with Clustal W (www.genome.jp/tools/clustalw/) to observe the changed cytosine to thymine conversion after bisulphite treatment. To compare the 5S rDNA and 18S rDNA sequences among the seven *C. roseus* genotypes, the primer sequences were deleted visually (figures 2 and 3). For the construction of phylogenetic tree, a keyword search was performed on NCBI ref. seq. to obtain 60 sequences of 5S rRNA (table 2) and 40 sequences of 18S rRNA (table 3) which were selected for alignment along with the corresponding sequences of *C. roseus*. All the 5S rRNA and 18S rRNA FASTA sequences along with the wild type sequence of *C. roseus* were aligned using the online version of ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The alignments were used to draw phylogenetic tree using Phylip ver. 3.68 package (Felsenstein 1989). The tree was made with 1000-bootstrap value. The phylogenetic trees were visualised and the different groups were highlighted with the help of Dendroscope ver. 3.2.2 (Small *et al*. 2004; Huson and Scornavacca 2012; Satheeshkumar and Gupta 2012).

Results

As per the objectives of the study, only the bisulphite treated 5S and 18S rRNA gene sequences were studied for the presence of methylated and demethylated cytosines in the CG,

Table 4. Distribution of methylated and demethylated cytosines in 5S and 18S rDNA sequences examined in *C. roseus* cv Nirmal.

Kind of rDNA	Size of sequence observed (bp)	Total number of cytosines		Contexts of methylated cytosines©		
		Methylated	Unmethylated	CG	CHG	CHH
5S	120	7 (87.5%)	1 (12.5%)	3 (42.8%)	1 (14.3%)	3 (42.8%)
18S	242	27 (100%)	0 (0%)	15 (55.6%)	9 (33.3%)	3 (11.1%)
5S + 18S	362	34 (97.1%)	1 (2.9%)	18 (52.9%)	10 (29.4%)	6 (17.7%)

Table 5. Demethylation and remethylation of cytosines in a 120-bp 5S rDNA sequence in the salinity-tolerant-cum-morphological-cum-hypomethylation *leafless inflorescence* (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*) single mutants and *lli egd*, *lli ill* and *egd ill* double mutants in *C. roseus* cv Nirmal (wild type, WT).

Genotype	Total number of cytosine site elements	Frequency of demethylation at the various cytosine elements			Cytosine elements at which methylation gained
		CG	CHG	CHH	
WT	8	0	0	1	NA
lli	9	1	1	3	CHH
egd	8	1	0	3	NA
ill	9	0	0	3	CHH
lli egd	9	0	0	2	CHH
lli ill	8	1	1	3	NA
egd ill	8	0	0	3	NA
Total	59	3	2	18	3
Mean % frequency of demethylation and gain of methylation in cytosines		5.1	3.4	30.5	5.1
Mean % frequency of demethylated cytosines			39.0		

NA, not applicable.

Table 6. Demethylations and remethylations of cytosines in a 242-bp 18S rDNA sequence in the salinity tolerant-cum-morphological-cum-hypomethylation *leafless inflorescence* (*lli*), *evergreen dwarf* (*egd*) and *irregular leaf lamina* (*ill*) single mutants and *lli egd*, *lli ill* and *egd ill* double mutants in *C. roseus* cv Nirmal (wild type, WT).

Genotype	Total number of cytosine site elements	Frequency of demethylation at the various cytosine elements			Cytosine elements at which methylation gained
		CG	CHG	CHH	
WT	27	0	0	0	NA
lli	27	0	1	3	NA
egd	29	1	3	3	2 CHH
ill	30	0	3	3	1 CHG + 2 CHH
lli egd	29	0	1	2	2 CHH
lli ill	30	0	5	2	3 CHH
egd ill	30	0	3	3	1 CGH + 2 CHH
Total	202	1	16	16	2 CHG + 11 CHH = 13
Mean % frequency of demethylation and gain of methylation at cytosines		0.5	7.9	7.9	6.9
Mean % frequency of demethylated cytosines			16.3		

Table 7. Identity of the base in heterologous species at specific sites in 5S rDNA of *C. roseus* mutants where methylated cytosine was gained following the loss of thymine.

Position of the gained methylated cytosine in the 5S rDNA sequence studied	Number of species in which the corresponding position of heterologous 5S rDNA was occupied by			
	Cytosine (C)	Thymine (T)	Guanine (G)	Adenine (A)
17	45	8	7	–
53	19	41	–	–
93	1	40	16	3

CHG and CHH contexts, in the wild type and mutants. The salient observations are noted below. The sequences of the bisulphite-treated DNAs are given in figures 2 and 3, the corresponding sequences of nonbisulphite-treated DNAs are given in figures 2 and 3 in electronic supplementary material.

Preponderance of methylation at CG elements in rDNAs

It will be seen from table 4 that in *C. roseus*, the cytosine methylation was lower in 5S rDNA (87%) than in 18S rDNA (100%). The distribution of methylated cytosines among the three kinds of cytosine elements in 5S and 18S rDNA was respectively, CHG:CG:CHH :: 1:3:3 and CHH:CHG:CG :: 1:3:5. In 5S and 18S rDNAs, the three kinds of methylated cytosine elements fell in the following order: CG (52.9%) > CHG (29.4%) > CHH (17.7%).

Patterns of loss and gain of cytosine methylation in rDNAs

In the wild type bisulphite-treated 5S rDNA, only one out of eight cytosines was unmethylated and the context was CHH (table 5). The 5S rDNA sequences in mutants contained on average basis 8.5 cytosines. The gain of cytosines had occurred in the CHH contexts (figure 2). About 45% of the cytosines in the 5S rDNA sequences of mutants were demethylated. Of these demethylations, 77.3% had occurred in the CHH contexts. There were few instances of demethylation in CHG context in the *lli* mutant.

The mutants had on average basis, 29.1 cytosines in their 18S rDNAs as compared to 27.0 in the wild type (table 6). The gain had occurred in CHG and CHH contexts (CHG:CHH :: 1:5.5) (figure 3). About 18.9% of the 18S rRNA sequence in mutants was demethylated. These demethylations had occurred in the three cytosine elements in the proportion CG:CHG:CHH :: 1:15.8:15.8.

In the mutants, in 5S and 18S rDNAs, the proportion of cytosine demethylations was CG:CHG:CHH :: 1:4.5:8.3 and cytosine methylations gained was CG:CHG:CHH :: 0:1:7 (tables 5 and 6). The presence of *ill* mutation was causal for higher level of demethylation in the CHG context.

T → C transitions resulted in gain of cytosines in 5S and 18S rDNAs of mutants

It will be seen from figures 2 and 3 and tables 5 and 6 that in *C. roseus* mutants, cytosines had gained at three locations in 5S rDNA and at eight locations in 18S rDNA. These gains were by replacement of thymine by cytosine. In 18S rDNA, at one of the affected locations on the transition was noted in four out of six *C. roseus* mutants. At another location in 18S rDNA, two of the six *C. roseus* mutants carried the T → C transition.

Table 8. Identity of the base in heterologous species at specific sites in 18S rDNA of *C. roseus* mutants where methylated cytosine was gained following the loss of thymine.

Position of the gained methylated cytosine in the 18S rDNA sequence studied	Number of species[a] in which the corresponding position of heterologous 18S rDNA was occupied by			
	Cytosine (C)	Thymine (T)	Guanine (G)	Adenine (A)
8	37	2	–	–
15	38	–	–	–
34	2	–	34	2
71	37	1	–	–
143	12	2	1	–
155	4	34	–	–
221	3	35	1	–
224	3	–	1	–
238	39	–	–	–

[a]Species names are given in tables 1 and 2.

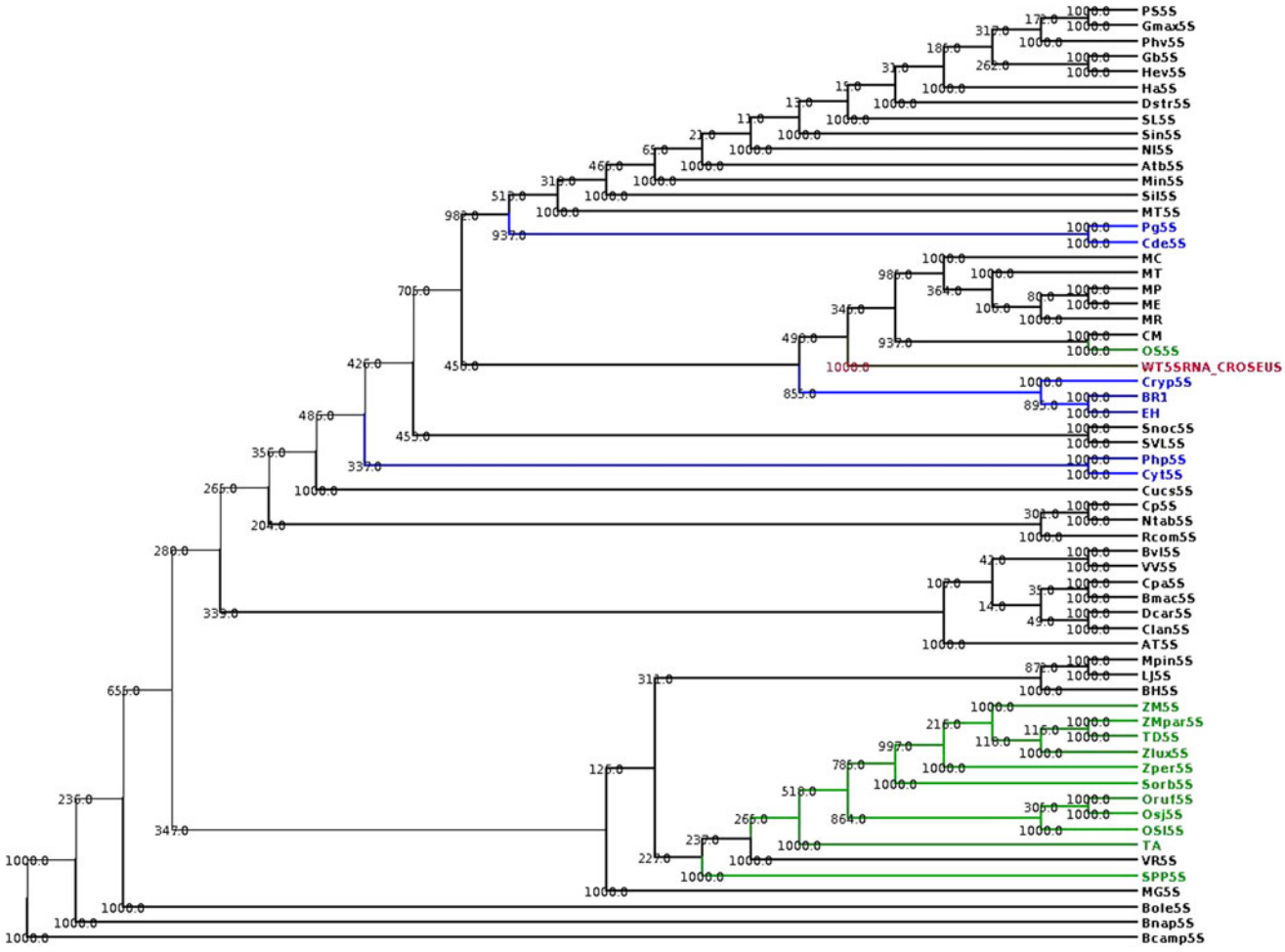

Figure 4. Phylogenetic tree prepared from nucleotide sequence of 5S rRNA gene. A total of 60 species sequences were taken from the NCBI database with 5S rDNA sequence length of 120 bp, aligned with Clustal W, the tree was made with Phylip ver. 3.68 package with 1000 bootstrap value and visualized with Dendroscope (ver. 3.2.2). The black lines represent dicotyledons, the green lines represent monocotyledons and the blue lines conifers, ferns and liverworts. The red colour is for the 5S rRNA gene sequence of *C. roseus* cultivar Nirmal (wild type). The full names of species are given in table 2.

Occurrence of cytosine in 5S and 18S rDNAs of heterologous species at the sites of cytosine gain in C. roseus mutants

Survey of 5S rDNA sequences in 60 plant species (table 2) showed that 1–45 species (table 7) possessed cytosine at the three locations of gain of cytosines in *C. roseus* mutants. Likewise a survey of 40 plant species (table 3) showed that two to 39 species carried cytosine at the eight positions in 18S rDNA where T → C transitions had occurred in *C. roseus* mutants (table 8).

Placement of C. roseus on phylogenetic trees constructed for 5S and 18S rDNAs

A phylogenetic tree was constructed using Phylip ver. 3.68 to study the relationship among *C. roseus* and other plant species on the basis of 5S (120 bp) and 18S (242 bp) ribosomal regions. In the phylogenetic tree for 5S rDNA, the *C. roseus* sequence was clustered amongst the interface of monocots and dicots, very close to those of *Oryza sativa* and *Citrus maxima* (figure 4). The position of *C. roseus* in the tree for 18S rDNA was also at the interface of monocots and dicots, very close to that of *A. thaliana* and *Oryza* spp. (figure 5).

Discussion

The results described above revealed the base compositions of 5S and 18S rDNAs of *C. roseus* cv 'Nirmal', degrees of hypomethylation and gain of methylated cytosines in the salt tolerant-cum-morphological *lld*, *egd* and *ill* mutants of *C. roseus* and the contexts of cytosine demethylations and methylated cytosine gains in mutants. These points are discussed below.

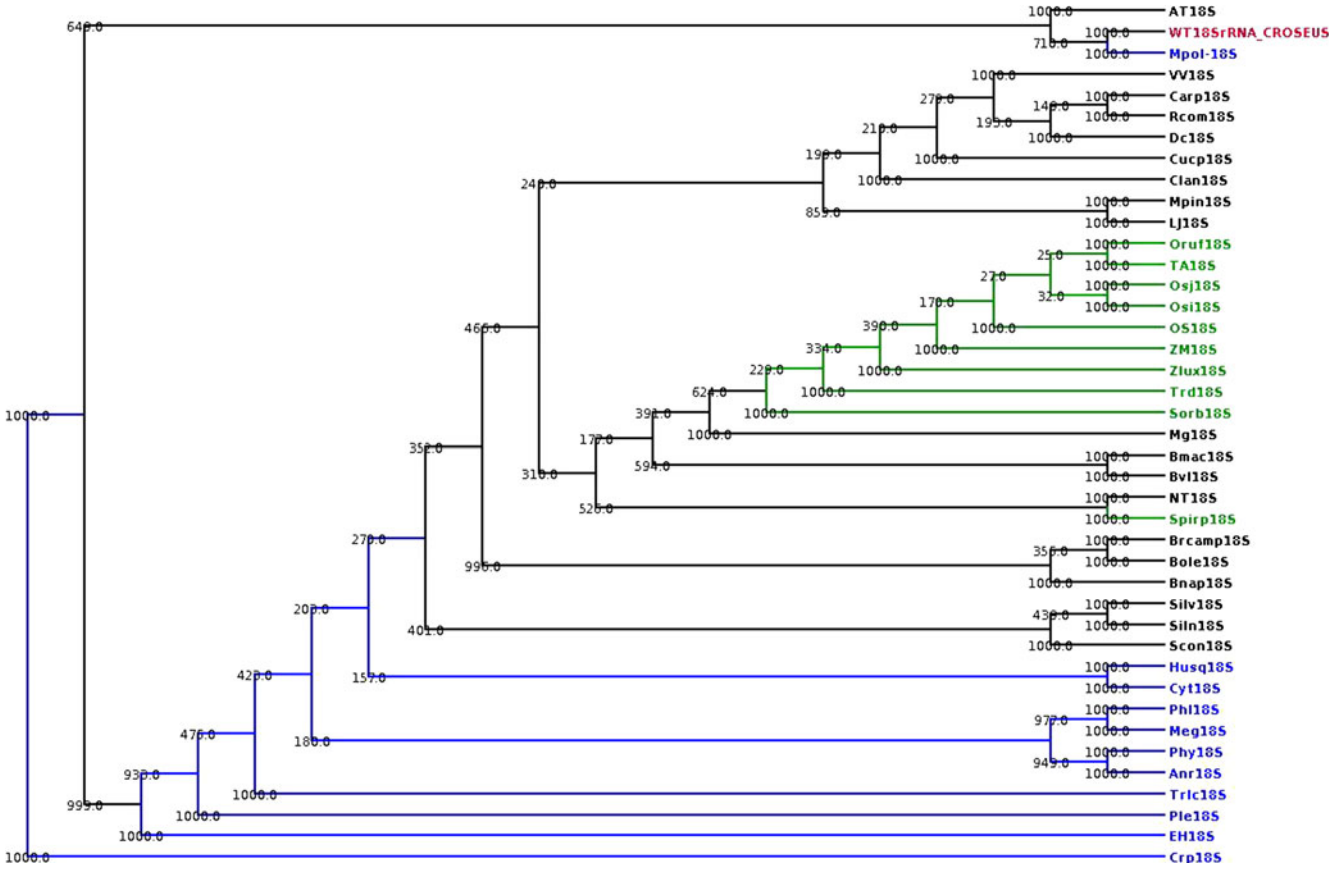

Figure 5. Phylogenetic tree prepared from nucleotide sequence of 18S rRNA gene. A total of 40 species sequences were taken from the NCBI database with 18S rDNA sequence length of 242 bp, aligned with Clustal W, the tree was made with Phylip ver. 3.68 package with 1000 bootstrap value and visualized with Dendroscope (ver. 3.2.2). The black lines represent dicotyledons, the green lines represent monocotyledons and the blue lines conifers, ferns and liverworts. The red colour is for the 18S rRNA gene sequence of *Catharanthus roseus* cultivar Nirmal (wild type). The full names of plant species are given in table 3.

Ribosomal DNAs of C. roseus have low content of cytosine

The ribosomal RNA products of 5S rDNAs and 18S rDNAs are, respectively, the components of large and small subunits of ribosomes. Their high copy number reflects their essential role in protein synthesis. The internal/coding sequences of 5S and 18S rDNAs of *C. roseus* cv Nirmal studied here proved homologous to corresponding sequences from several plant species. They demonstrated various levels of homology with a large number of dicot and monocot and lower plant species. However, both 5S and 18S rDNAs of *C. roseus* were observed to be low in their cytosine content which was only about 15.8% in both 5S and 18S rDNA, lower than many species of plants listed in the tables 2 and 3. It is known that cytosines are lost from genomes on account of spontaneous deamination (Zhang and Mathews 1994; Huen *et al*. 2008) and glycosylase-cum-lyase activities of cytosine demethylases redundantly specified by plant genomes (Zhu 2009). The deaminated 5 methyl cytosines are replaced by thymines (Pfeifer 2006; Walsh and Xu 2006). The methylated cytosines pruned from DNA by demethylases are substituted by other bases via repair of abasic sites by a variety of DNA repair mechanisms (Agius *et al*. 2006; Bhutani *et al*. 2011). The low cytosine content in rDNAs of *C. roseus* indicates role of potent cytosine methylases in the evolution of the species.

CHG and CHH cytosine elements are the preferred sites of demethylation in mutants

In the 5S and 18S rDNA sequences of *C. roseus* cv Nirmal, methylated cytosines were observed at CG, CHG and CHH elements with 53, 29 and 18% frequency, respectively. However, 60% of the cytosine demethylations in the *C. roseus* mutants were observed at CHH elements and 33% at CHG elements. These results suggested that the *lli*, *egd* and *ill* mutants were deficient in the processes that mediate maintenance and/or establishment of cytosine methylations in CHH and CHG contexts. In a part of this work being reported separately (Kumari *et al*. 2013), it was observed that the above *C. roseus* mutants were downregulated in the expression of their *RDR2* and *DRM2* (components of RdDM pathway) and *CMT3* (component of maintenance-cum-establishment pathway) genes (also see figure 1; tables 1 and 2 in electronic supplementary material). All the observations together suggest

that in the *lli*, *egd* and *ill* mutants, the lesions have altered the regulation of the DNA methylation pathways such that those involved in the methylation of cytosines in the CHG and CHH contexts are highly underexpressed. It is indicated that in *C. roseus*, there may be several novel genes, including *LLI*, *EGD* and *ILL* whose products act as activators, on the one hand, on genes such as *RDR2* and *DRM2*, which are participants of the RdDM establishment and maintenance pathway, and on the other hand, on gene such as *CMT3*, which is a participant of maintenance pathway, involved in cytosine methylation in genomic DNA.

Addition to CHG and CHH methylated sites by gain of cytosines in 5S and 18S rDNA sequences in C. roseus mutants

The 5S and 18S rDNA sequences of the *lli*, *egd* and *ill* single mutants and *lli egd*, *lli ill* and *egd ill* double mutants altogether gained 16 methylated cytosines, 14 in CHH context and two in CHG context. The cytosines were gained as T → C transitions. Such transitions can arise by the errors that occur during DNA replication followed by DNA repair. Alternatively, since 5S and 18S rDNAs possess methylated cytosines, their removal by demethylases can create opportunities for extended repair and thereby errors in repair. It is known that aberrant base pairing occurs between T and G (Mazin 1993). Repair at such sites will produce DNA molecules wherein T is replaced by C. Their selection will lead to results such as those obtained with the *C. roseus* 5S and 18S rDNA sequences in mutants. These results imply that there is a mechanism in *C. roseus* whereby loss in cytosine methylation is compensated by introduction of cytosines at new locations. The observations confirm occurrence of spontaneous mutations at high frequency in the background of extensive cytosine demethylation, as available in the *lli*, *egd*, *ill* and double mutants. A survey of 5S and 18S rDNA sequences in heterologous species revealed that Cs are present in other species at the sites of gain of Cs in mutants, indicating possibilities of selection advantage in the presence of C at the new sites.

Concluding remarks

This study characterized the 5S and 18S rDNA sequences of the wild type and salt tolerant-cum-morphological mutants of *C. roseus*. The mutants had been earlier shown to be possessing Mendelian inheritance, hypomethylated in repeat sequences and upregulated and downregulated in many genes and pleiotropically different in a variety of traits between each other and from the wild type. It was found here that *C. roseus* 5S and 18S rDNAs were low in cytosine content and cytosines were largely present in CG context. The cytosines in rDNAs of mutants were mostly demethylated in CHG and CHH contexts. The cytosines gained in the mutants were also in CHG and CHH contexts. Taking into consideration previous work on the mutants, hypomethylation was attributed to the downward expression of RdDM and CMT3 pathways for cytosine methylation establishment and maintenance. It emerged that *C. roseus* has novel genes such as *EGD*, *ILL* and *LLI* that act as positive regulators of genes that comprise the RdDM and CMT3 pathways. Loss of function mutations in *EGD*, *ILL* and *LLI* genes are viable, cytosine hypomethylated, demonstrate vastly pleiotropic phenotypes and deficiency in RdDM and CMT3 pathways of cytosine methylation establishment and maintenance.

Acknowledgements

Indian National Science Academy and Council of Scientific and Industrial Research are thanked for grant of scientistship schemes to SK, Department of Biotechnology is thanked for research grant for work on periwinkle and for postgraduate fellowship to RK, Director of NIPGR is thanked for providing research facilities and SKA Institution for Research, Education and Development is thanked for grant of a postgraduate fellowship to VS.

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Received 4 November 2012, in final revised form 13 July 2013; accepted 22 July 2013
Published on the Web: 16 December 2013