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RESEARCH ARTICLE

Construction of genetic linkage map of the medicinal and ornamental plant *Catharanthus roseus*

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Abstract

An integrated genetic linkage map of the medicinal and ornamental plant *Catharanthus roseus*, based on different types of molecular and morphological markers was constructed, using a F_2 population of 144 plants. The map defines 14 linkage groups (LGs) and consists of 131 marker loci, including 125 molecular DNA markers (76 RAPD, 3 RAPD combinations; 7 ISSR; 2 EST-SSR from *Medicago truncatula* and 37 other PCR based DNA markers), selected from a total of 472 primers or primer pairs, and six morphological markers (stem pigmentation, leaf lamina pigmentation and shape, leaf petiole and pod size, and petal colour). The total map length is 1131.9 cM (centiMorgans), giving an average map length and distance between two markers equal to 80.9 cM and 8.6 cM, respectively. The morphological markers/genes were found linked with nearest molecular or morphological markers at distances varying from 0.7 to 11.4 cM. Linkage was observed between the morphological markers concerned with lamina shape and petiole size of leaf on LG1 and leaf, stem and petiole pigmentation and pod size on LG8. This is the first genetic linkage map of *C. roseus*.

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Introduction

Catharanthus roseus is simultaneously an ornamental and a medicinal plant species. It belongs to the family Apocynaceae, consisting of 411 genera and 4650 species, many of them are of ornamental and medicinal values (Simpson 2006). The genus *Catharanthus* includes seven species besides *C. roseus*, namely *C. coriaceus, C. lanceus, C. longifolius, C. ovalis, C. pusillus, C. scitulus* and *C. trichophyllus*, of which *C. pusillus* is endemic to India whereas others are endemic to Madagascar. The species *C. roseus* and *C. trichophyllus* are crosshybridizable. Several scores of ornamental cultivars of *C. roseus*, bred for differing shoot habit, time of blooming, petal coloration and suitability for cultivation in homes and gardens are in vogue worldwide (Snoeijer 2001; van der Heijden *et al*. 2004). An important breeding objective for the genetic improvement of ornamental cultivars is the incorporation of resistance towards the fungal pathogens *Pythium aphanidermatum* and *Phytopthera nicotinae* to which most of the ornamental cultivars are susceptible. *C. roseus* has potent secondary metabolism responsible for monoterpenoid glucosides and other terpenoid compounds, steroids, phenolics, flavanoids, anthocyanins and 130 terpenoid indole alkaloids (TIAs) (Facchini 2001; van der Heijden *et al*. 2004; Pandey-Rai *et al*. 2006). The widely used anticancer TIA drugs vinblastine and vincristine (Leveque *et al*. 1996) are semisythesized from their natural precursors vindoline and catharanthine that are obtained from *C. roseus* root and shoot organs. Ajmalicine, a cardiactonic TIA drug is also extracted from *C. roseus* roots (Leveque *et al*. 1996). The high cost of production of pharmaceutically important TIAs is related to their low level of accumulation in *C. roseus* organs. Thus, to increase the concentrations of TIAs in plant organs is another important objective of genetic work on *C. roseus*.

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C. roseus is one of the highly investigated plant species, with genetic, proteomic, metabolomic and biotechnological studies in progress (Kulkarni *et al*. 1999; van der Heijden *et al*. 2004; Jacobs *et al*. 2005; Memelink 2005; Ledue *et al*. 2006; Rischer *et al*. 2006). Some of the features of *C. roseus* that make it a suitable genetic system are the biannual, seed cyclable, herbaceous perennial habit, diploidy ($2n = 16$; 1500 Mbp = 12 × *Arabidopsis thaliana* genome) and amenability to controlled pollination and micropropagation (Mishra and Kumar 2000; van der Fits *et al*. 2000; Debnata *et al*. 2006). Many of the genes involved in TIA biosynthesic pathway of *C. roseus* have been cloned and sequenced for the analysis of their expression in various plant organs (Geerlings *et al*. 2001; van der Fits and Memelink 2001; Facchini and St-Pierre 2005; Mahroug *et al*. 2006). A large number of proteins/enzymes involved in the primary metabolism of *C. roseus* have been shown to be highly homologous to corresponding ones already characterised and sequenced in one or more other plant species (Jacobs *et al*. 2005). Several mutant loci affecting the development of organs have been reported in *C. roseus* (Pandey-Rai and Kumar 2000, 2001; Pandey-Rai *et al*. 2003). A genetic linkage map of *C. roseus* constructed with the use of molecular and morphological markers is required to eventually localise the loci concerned with TIA yield, disease resistance and ornamental features on the map for their possible assistance in the breeding programmes.

A variety of DNA markers, including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), simple sequence repeats (SSR), amplified fragment length polymorphism (AFLP) and their variants have proved useful for studying segregation in mapping populations and help in genetic linkage determination for map construction (Lörz and Wenzel 2005). F_2, double haploid (DH) and recombinant inbred lines (RILs) derived from crosses between genetically divergent parents have been successfully used as mapping populations in a large number of food and industrial plants (Lörz and Wenzel 2005; Meksem and Kahl 2005).

In *C. roseus*, a number of microsatellite markers have recently been developed and deployed for the study of intraspecific and interspecific as well as intrageneric and intergeneric genetic polymorphism (Shokeen *et al*. 2005, 2007). In the present work we studied the segregation of RAPD, ISSR, EST-SSR and sequence-specific markers in the F_2 progeny plants of a cross between two morphologically differentiable and genetically distant lines to construct a framework genetic map of *C. roseus*.

Materials and methods

Plant materials and DNA extraction

We used 144 F_2 plants of *C. roseus*, developed by crossing the accession 'Pink Delhi' (pink coloured flower petals and stem, dark green leaf lamina bearing elliptic apex and borne on small petiole, long pods, tall habit, less salt and drought sensitivity, and high in alkaloid yield) and accession *gsr8* (white flower petals, yellow green stem and leaf lamina bearing oblong apex and borne on large petiole, small pods, semidwarf habit, more salt and drought tolerance, low in alkaloid yield) as mapping population. Genomic DNA was extracted from leaf material of field grown plants using a CTAB method (Saghai-Maroof *et al*. 1984). The DNA was RNAase treated and subsequently quantified on agarose gel by comparison with standard lambda DNA marker (Amersham Biosciences, USA).

RAPD and ISSR marker analysis

A total of 260 decamer RAPD primer (sets A to M, Operon Technologies Inc., Alameda, CA, USA) and 42 ISSR primers (UBC Primer Set No. 9, Vancouver, BC, Canada) were screened on the two parents ('Pink Delhi' and *gsr8*) to determine their potential of clear polymorphisms and reproducibility. The selected primers were examined on 144 F_2 plants. The PCR reactions were carried out in a 25 μl volume containing 1 U of *Taq* polymerase (Invitrogen Corporation, USA), 50 ng of genomic DNA, 0.80 μM of RAPD and ISSR primers, 0.1 mM of each dNTPs, 2.5 μl of 10× PCR reaction buffer (500 mM KCl, 200 mM Tris-HCl (pH 8.4)), 1% Triton × (for ISSR) and 3 mM $MgCl_2$. DNA amplifications were carried out in a iCycler thermal controller (Bio-Rad). The following two steps were used for RAPD: 1 cycle consisting of 60 s at 94°C, 30 s at 36°C and 60 s at 72°C followed by 45 cycles of 5 s at 94°C, 15 s at 36°C and 60 s at 72°C, and a final cycle of 7 min at 72°C. The ISSR amplifications were carried out with a preliminary cycle of 2 min at 94°C, followed by 35 cycles of 20 s at 94°C, 50 s at 50°C, and 90 s at 72°C, and a final cycle of 7 min at 72°C. The amplification products were resolved on 1.2% (for RAPD) and 1.5% (for ISSR) agarose gels in Tris-borate EDTA buffer (45 mM Tris-borate and 1 mM EDTA) and stained with ethidium bromide.

EST-SSR marker analysis

We used 42 barrel medic (*Medicago truncatula*) EST-derived SSR primers to screen for polymorphism between the two parents ('Pink Delhi' and *gsr8*). Eight of the EST-SSR primers that were detected for polymorphism between the two parents were used to assess the mapping population. The reactions were carried out in a 20 μl volume containing 1 U of *Taq* polymerase (Invitrogen Corporation, USA), 50 ng of genomic DNA, 0.80 μM of each primer, 0.2 mM of each dNTPs, 2.0 μl of 10× PCR reaction buffer (500 mM KCl, 200 mM Tris-HCl (pH 8.4)), and 3 mM $MgCl_2$. DNA amplifications were carried out in a MyCycler thermal controller (Bio-Rad). EST-SSR amplifications were performed using a preliminary step of 3 min at 94°C, followed by 45 cycles of 60 s at 94°C, 60 s at 50°C and 120 s at 72°C, and a final

step of 10 min at 72°C. The amplification products were resolved on 1.5% agarose gels in Tris-borate EDTA buffer (45 mM Tris-borate and 1 mM EDTA) and stained with ethidium bromide.

Other PCR-based sequence-specific markers

We developed 49 primers named as 'designed' and 55 primers named as 'designed forward/reverse + RAPD' for screening the genetic resources of *C. roseus*, and these primer sets were also used as DNA markers in the present study. The nomenclature for these primers is given in the section called 'marker nomenclature'. Primer sequences, their resources and product sizes for the above mentioned primer sets are presented in table 1. The reactions were carried out in 25 μl volume containing 1 U of *Taq* polymerase (Invitrogen Corporation, USA), 50 ng of genomic DNA, 0.80 μM of designed or designed forward/reverse + RAPD primers, 0.1 mM of each dNTPs, 2.5 μl of 10× PCR reaction buffer (500 mM KCl, 200 mM Tris-HCl (pH 8.4)), and 3 mM $MgCl_2$. DNA amplifications were carried out in a iCycler thermal controller (Bio-Rad). Designed primer based amplifications were carried out in three steps: first step consisted of a cycle of 30 s at 94°C second step consisted of a cycle of 30 s at 94°C, 60 s at 45°C and 60 s at 72°C for 10 cycles followed by 35 cycles of 30 s at 94°C, 60 s at 52°C and 60 s at 72°C and a final step of a cycle of 10 min at 72°C. The designed + RAPD primer based amplifications were also carried out in 3 steps: first step consisted of a cycle of 60 s at 94°C, 30 s at 50°C, 60 s at 72°C; second step consisting of a cycle of 60 s at 94°C, 30 s at 36°C, 60 s at 72°C followed by 45 cycles of 5 s at 94°C, 15 s at 36°C and 60 s at 72°C and a final step of a cycle of 7 min at 72°C. The amplification products were resolved on 1.5% agarose gels in Tris-borate EDTA buffer (45 mM Tris-borate and 1 mM EDTA) and stained with ethidium bromide. The parents were screened for the presence/absence of bands and polymorphic, repeatable and clear markers were used on the whole of F_2 population.

Table 1. Designed, designed forward/reverse + RAPD and EST-SSR primer sequences investigated for use in genetic map construction in *Catharanthus roseus.*

Locus	Primer sequences (5′ to 3′)[a]	Gene product for which primer designed and used, and identity of the RAPD primer used[f]	Size(s) (bp) of the DNA marker band(s) realized[g]	Linkage group (LG) number on which mapped
SGA_07	TCCGGTTCCCCACCACN[b]AAY[c]CAR[d]AC GGGACCGGTAGAACTTGGCRAAYTTNTC	PUTATIVE CYTOCHROME-C OXIDASE (COX)	250	LG3
SGD_21	CCATCCGGTTCCTGGCNATH[e]GAYGC GGCCTCCTTGGAGATGCCYTCCATYTG	TRANSKETOLASE 1 (TKL 1)	250	LG2
SGD_28	GCCCACCATCGTCACCAAYGCNGARGG CCGGAGGTGGACAGCACYTCRAANAC	HEAT SHOCK PROTEIN 70 (HSP70)	750	LG2
SGD_32	GGACGTGCAGCGGATCATHAAYGARCC CTCGGACACGACCTCCTGNACYTTNGG	HEAT SHOCK PROTEIN 68 (HSP68)	800	LG2
SGD_36	GCAACGCCCTGATGCARGAYCC AGGGTCACGGCGGCNCCRTTYTC	ANKYRIN-REPEAT PROTEIN HBP1 (ANK HBP1)	500 900	LG2
SGD_42	ACCGTGAACGAGTGGGGNTGGTG GCAGCTCCAGGCCCTCYTTRTTYTC	ENOLASE (ENO)	1200	LG2
SGD_43	CCGTGGTGCTGGCCAARGTNGAYGC CCAGGGTGTGGCCGAANTCRTARTC	DISULPHIDE ISOMERASE (DI)	1100	LG2
SGD_44	GGGCGGCAACTGGAARTGYAAYGG CAGGGCGTAGGCGGCYTTYTTNCC	PUTATIVE TRIOSEPHOSPHATE ISOMERASE (TRI)	800	LG2
SGD_48	CACCCCCTACTGCATCATGTTYGGNCC GGGCTTCCATGGGCCYTTRTAYTC	CALRETICULIN (CRT)	1800	LG2
SSG_01	GGAATTCCTAGCTTGTGTGGCAAGA GTGTGCCCCA	ANTHRANILATE SYNTHASE (ANS); OPD8	1000	LG1
SSG_02	CAAAGTTTCCACACAGCACA GGCTGCAGAA	TRYPTOPHAN DECARBOXYLASEF (TDC); OPF13	1200, 900	LG1

SSG_03	GTGAAAGAATTACCAGCTCA TGCTGCAGGT	GERANYL GERANYL PYROPHOSPHATE SYNTHASE (GGP); OPF14	2200	LG1
SSG_04	GGAATTCCGTGTATGCATGATTCTGG TGCTGCAGGT	EMBRYONIC FLOWER-2 (EMF2); OPF14	1200	LG1
SSG_05	AGTTTACAAAGGAGAAGGCA GGAAGCTTGG	REVOLUTA (REV); OPF10	1500	LG1
SSG_06	AGTAGTGAAGAAGAAGTTGCACA ACCCGGTCAC	FIMBRIATA (FIM); OPD20	1800	LG1
SSG_07	CGGGATCCCGTTGTTTTGTTTGGTTCT3 GGAAGCTTGG	APETALA-1 (APA1); OPF10		LG1
SSG_08	GGAATTCCTGTATGTGGCAAACTGT GGAAGCTTGG	APETALA-1 (APA1); OPF10	2000, 1200, 700	LG1
SSG_09	GCAAAAGTGAGTGAAGATG	PHOSPHOENOLPYRUVATE (PEP)	1300	LG1, LG7
SSG_11	GTACATTCCATCTGCCATGA CACCAGGTGA	KNOX (KNX); OPE10	1000	LG1
SSG_12	CCCACTTCAGGGGCTTCACNACYTCCC GGAAGCTTGG	ALDEHYDE DEHYDROGENASE1 (ALDH1); OPF10	400	LG1
SSG_13	ATAAAGCTACAGGTGATGCC GGAAGCTTGG	ISOPENTENYL PYROPHOSPHATE ISOMERASE (IPPI); OPF10	1800	LG1
SSG_14	GGTGCAGTGCAACATGGGNGCNAA GGAAGCTTGG	S-ADENOSYL-L-METHIONINE SYNTHETASE(SAMS); OPF10	500 1000	LG1
SSG_15	GGAATTCCAGAGAGAAGAATATGGCG GTGCCTAACC	APETALA 3 (APA3); OPG6	700	LG1
SSG_16	GTATTTTCGGTCAAACCACA CTTCACCCGA	UNIFOLIATA (UNI); OPE9	800	LG1
SSG_17	GGAATTCCTAAGAACCAACCAACAC GGCACTGAGG	CYTOCHROME P450 REDUCTASE (CPR); OPG2	1500 1800	LG1
SSG_18	GGAATTCCAAGTAGCCGACAAGTCA GGAAGCTTGG	FLOWERING LOCUS C (FLC); OPF10	400 1200	LG1
SSG_19	GGAATTCCAGACTTGTGTCAGGTGA GAATCGGCCA	SUPPRESSOR OF CLR (SLR); OPH18	500	LG2
SSG_20	CTCTCTATCTCTCTCTCTCTCA CTTCACCCGA	EARLY FLOWERING-4 (ELF4); OPE9	1200	LG3
SSG_21	AAGAAGCTGAGAATCACTGA TGCCCGTCGT	EARLY FLOWERING-3 (ELF3); OPG11	1200	LG2
Mt_NC - PGR_02	GCATGCATTTTGTTGACCAC GCCACCCAATAATCCAATGT	*Medicago turncatula* EST_SSR	400	LG2
Mt_NC - PGR_34	ATCGAATCCCACCATTACCA ATCTCAATCAAAGGCATGGG	*Medicago turncatula* EST_SSR	300	LG2

a, Annealing temperatures: for SGD primer 45°C (10 cycles) followed by 52°C (35 cycles); for SSG primer 50°C (1 cycle), 36°C (1 cycle) followed by 36°C (35 cycles); b, N = A/T/G/C; c, Y = C/T; d, R = A/G; e, H = A/C/T; f, These designations are the same as given by the supplier, Operon, Technologies Inc., Alameda, CA, USA; g, The parental origin of each band is shown in the figure 2 by the letter P for Pink Delhi and letter G for *gsr8*, placed next to the values of band size in brackets.

DNA marker nomenclature

Each RAPD markers were designated using the primer with which the polymorphism was observed (capital letter followed by a two digit number which corresponds to an Operon Technologies primer). RAPD primers, which are monomorphic, were also used in pairs for detection of polymorphism. Paired RAPD primers were designated using a plus sign differentiating individual RAPD primers. The markers

namely designed, designed + RAPD and ISSR were named as 'SGD', 'SSG' and 'U' followed by two or three digit numbers, respectively. The two mapped EST derived microsatellite markers (from *M. truncatula*) were named as 'Mt_ESSR' followed by a two digit number. The subscript of each DNA marker gives the size of the marker in terms of base-pair length.

Morphological markers

The six morphological markers for which the F2 mapping population was scored are shown in figure 1 and table 2. The morphological observations were recorded on twenty week old plants. Three letter designations assigned to the different characters (table 2) were placed on the genetic map.

Inheritance and linkage analysis

The markers were analysed by a chi-square test for goodness-of-fit to the expected Mendelian segregation ratio (3:1) of a dominant locus in an F_2 population ($P < 0.01$). Linkage analysis was performed using the software MAPMANAGER QTX v0.30 (Manly *et al*. 2001). Linkage groups were established at a lod score of 3.0 by using the command "Make Linkage Groups". The order of the loci within the linkage group was refined by the "ripple" command. The Kosambi mapping function (Kosambi 1944) was used to convert the recombination fractions into additive genetic distances in cM. Final linkage maps were drawn with the help of graphical package MapChart (Voorrips 2002).

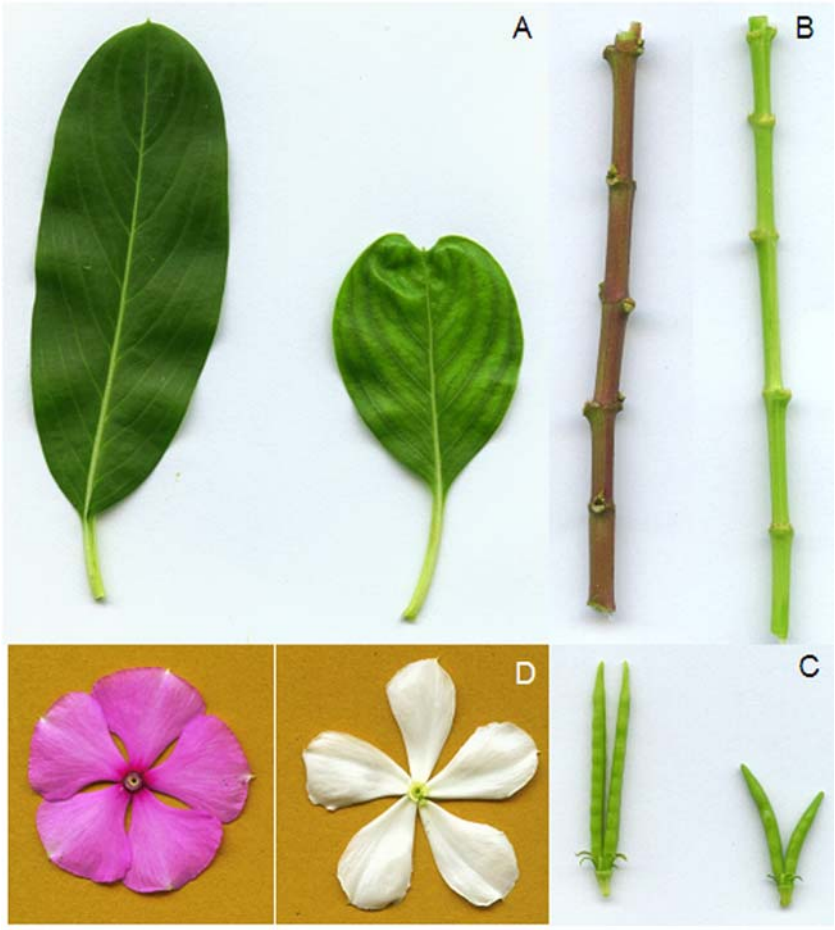

Figure 1. Morphological features of leaf, stem, flower and fruit in the 'Pink Delhi' (left) and *gsr8* (right) accessions of *Catharanthus roseus* which were used as the parents for deriving the mapping population of F2 progeny plants. (A) Leaf, dark green lamina, elliptic apex and short petiole in 'Pink Delhi', and yellow green lamina, oblong apex and long petiole in *gsr8*, (B) Stem, pigmented in 'Pink Delhi' and light green in *gsr8*, (C) Fruit, long pods in 'Pink Delhi' and short pods in *gsr8* and (D) Flower, pink coloured flower petals in 'Pink Delhi' and white petals in *gsr8*.

Table 2. Patterns of inheritance and linkage relationships of morphological markers in *Catharanthus roseus*.

Morphological character	Marker designation	Morphologically distinguishable classes	Observed distribution	χ^2 for Mendelian segregation	Linkage groupe (LG) on which located	Nearest molecular marker			
						Upword[a]		Downward[b]	
						Designation	Distance (cM)	Designation	Distance (cM)
Stem pigmentation	STP	Pigmented pink	104	0.6	LG8	LLP	9.1	POL	7.1
		Nonpigmented	40						
Leaf lamina pigmentation	LLP	Dark green	103	0.9	LG8	$C12_{800}$	1.4	STP	9.1
		Yellowish green	41						
Leaf lamina shape	LLS	Elliptic apex	101	0.2	LG1	$A13_{2000}$	3.9	LPS	3.1
		Oblong apex	37						
Leaf petiole size	LPS	Short	101	0.2	LG1	LLS	3.1	$B18_{300}$	3.8
		Long	37						
Corolla petal colour	CPC	Rosy pink	97	4.5	LG8	$E2_{1000}$	0.7	$E2_{800}$	8.0
		White	47						
Pod length	POL	Long	99	2.5	LG8	STP	7.1	$E2_{1000}$	11.4
		Short	44						

[a] Nearest marker above the concerned morphological marker, as in figure 1;
[b] Nearest marker below the concerned morphological marker, as in figure 2.

Results

Mapping of molecular markers

All 472 DNA primers were screened for finding out if they generated polymorphisms between 'Pink Delhi' and *gsr8*, the parents of the *C. roseus* mapping population used (table 3). Out of 260 RAPD primers, 69 revealed polymorphism between the parents and generated 110 markers (approximately 1.5 markers per primer). Eleven of the 24 RAPD combination primer pairs also demonstrated polymorphisms between the parents and gave 18 additional RAPD markers. A set of 20 markers became available from 14 ISSR primers which distinguished the parents. Although, 42 EST-SSR primers were screened against parents only eight EST-SSR primer pairs gave as maximum markers. Twenty one and 57 markers became available from among the 49 designed and 55 designed+RAPD primer pairs, respectively. Thus altogether 234 DNA markers became available for screening against the F_2 mapping population. The sizes of these markers varied from 100 ($D13_{100}$) to 3500 ($D5_{3500}$) bp. However, only 125 (53.4%) of these markers could actually be placed on the linkage groups of *C. roseus*. The success in placement of different kinds of polymorphic DNA primers/markers on the map varied. They could be arranged in the following order in terms of their observed mapping potential: RAPD (69.1%) > designed (47.6%) > designed forward/reverse + RAPD (47.4%) > ISSR (35%) > EST-SSR (25%) > RAPD combination (16.6%). The segregation patterns of 76 of the 125 mapped markers deviated significantly from the expected 3:1 Mendalian ratio ($P < 0.01$). The markers showing distorted segregation pattern were not more specific to either of the parents. Among the 125 DNA markers that could be mapped following was the break up of the different kinds of markers: RAPD (60.8%); RAPD combination (2.4%); ISSR (5.6%); EST-SSR (1.6%); designed (8.0%); and designed+RAPD (21.6%). A noteworthy feature of the mapped ISSR markers was the presence of (GA) or (AC) repeat motif in them.

Mapping of morphological markers

The F_2 mapping population was also scored for a set of six morphological markers for which the parental lines were distinguishable (figure 1; table 2). Six characters that were studied—corolla petal colour (CPC), leaf lamina pigmentation (LLP), leaf lamina shape (LLS), leaf petiole size (LPS), pod length (POL), and stem pigmentation (STP)—demonstrated Mendalian (3:1) segregation pattern in the F_2 mapping population. The observations allowed placement of all the six morphological markers on the linkage groups of *C. roseus* (table 3).

Linkage map

Of the 240 DNA and morphological markers screened in F_2 population, 131 (54.6%) were mapped and 109 remained

Table 3. Molecular and morphological markers used for the construction of genetic linkage map of *catharanthus roseus.*

Marker type	Primers/primer pairs screened	Number of polymorphic primers/ primer pairs	Polymorphic markers	Mapped markers
RAPD	260	69	110	76
RAPD combination	24	11	18	3
ISSR	42	14	20	7
EST-SSR	42	8	8	2
Designed	49	19	21	10
Designed + RAPD	55	38	57	27
Morphological	6	-	6	6
Total	478	159	240	131

unlinked. Fourteen linkage groups were formed as opposed to the expected eight on the basis of haploid chromosome number of the species (figure 2). The linkage groups varied widely in length, from 4.2 to 387.8 cM (table 4). Two linkage groups (LG1 and LG2), were longer than 300 cM, whereas 9 linkage groups (LG3 to LG11), were less than 55 cM but longer than 20 cM. Only three linkage groups (LG 12 to LG14), were less than 20 cM in length. The map covered a total length of 1131.9 cM with an average marker density of 8.6 cM between two adjacent markers. A total of 45 gaps (a gap is equal to the distance between two adjacent markers) were found, of which 31 and 14 are of the sizes 10 to 20 cM and 20 to 30 cM, respectively (table 4). The maximum number of markers and gaps were found in the linkage groups LG1 and LG2, respectively. Among the 131 markers placed on the genetic linkage map of *C. roseus* (figure 2), 49 are distributed on LG1, 27 on LG2, 8 on LG7, 7 on LG6 and LG8, 5 on LG9, 4 on LG5, LG10, LG11 and LG13, 3 on LG3, LG4, LG12 and LG14. The six morphological markers got placed on 2 linkage groups, LLS and LPS on LG1 and LLP, STP, POL and CPC on LG8.

Discussion

Detailed genetic linkage maps of crop plant, on which molecular DNA markers are tightly placed and in which loci for morphological features, quantitative traits and specific genes have been located with reference to molecular markers are very useful, especially for cloning of desired chromosome segment/gene(s) and early selection of desirable recombinants in cross breeding programmes. Such highresolution map of a species evolves by progressive integration of maps developed by the study of several to many mapping populations having origin in different intraspecies and interspecies crosses, to maximise the coverage of genetic polymorphisms prevalent in the species. Induced mutations are also used to enrich the genetic variability of crop species where the genetic resources demonstrate narrow spectrum of genetic polymorphism. Additionally, all possible kinds of DNA markers are deployed to densely cover both euchromatin and heterochromatin rich genome regions. A detailed genetic linkage map is required for efficient implementation of breeding programmes aimed at incorporation of disease resistance and high alkaloid yield related traits in *C. roseus*. The present work achieved its goal to develop a primary map of *C. roseus*, which may serve as the backbone for future work. The genetic linkage map of *C. roseus* presented here was constructed using an intraspecies F_2 population. The map consists of 125 molecular and six morphological markers and covers 1132 cM of the genome in two large (LG1 and LG2) and 12 small linkage groups (LG3 to LG14). Thus 14 numbers of linkage groups exceed the haploid number of chromosomes ($n = 8$) of the species. The initial maps

Table 4. Summary of genetic linkage map of *Catharanthus roseus.*

Linkage group	Length (cM)	Number of markers	Number of gaps[a] of different lengths (cM) 10–20	20–30
LG1	387.8	49	14	0
LG2	372.3	27	11	77
LG3	54.0	3	0	2
LG4	47.4	3	0	2
LG5	45.0	4	1	1
LG6	43.5	7	0	1
LG7	41.5	8	0	1
LG8	37.7	7	1	0
LG9	28.9	5	2	0
LG10	25.8	4	1	0
LG11	20.1	4	1	0
LG12	12.0	3	0	0
LG13	11.7	4	0	0
LG14	4.2	3	0	0
Total	1131.9	131	31	14

[a] Gap is the distance between two adjacent markers.

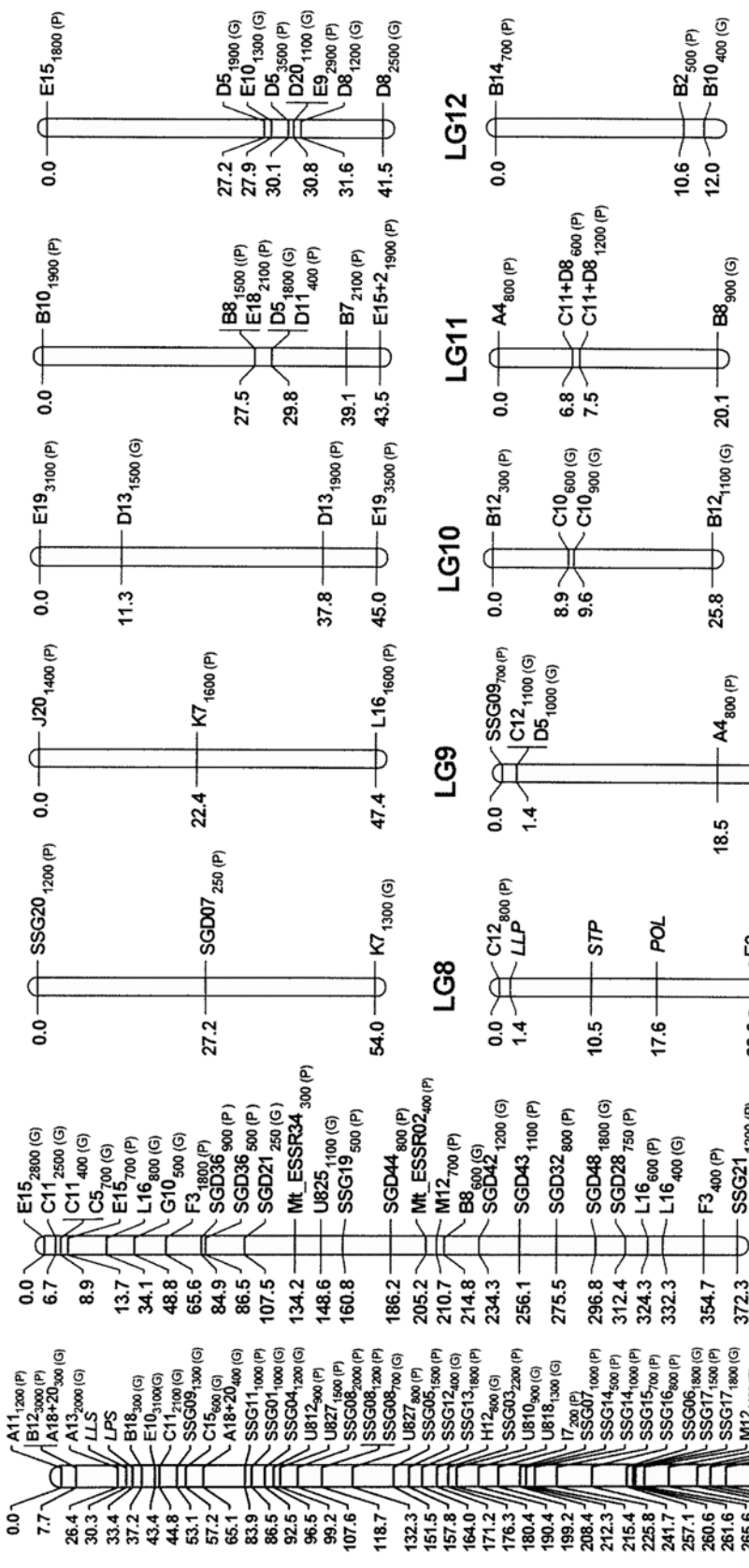

Figure 2. Linkage map of *Catharanthus roseus* in the F_2 population of the cross 'Pink Delhi' × *gsr8*. Loci names and cumulative genetic distances, in cM are indicated on the right and left side of the vertical bars, respectively. RAPD and ISSR markers, are represented as the primer name and number is followed by band size in subscript. Other PCR based markers (designed and designed forward/reverse + RAPD) are also represented by the primer name (SGD and SSG, respectively) and number followed by band size in subscript. The parental origin of each band is shown in the figure by the letter P for 'Pink Delhi' and letter G for *gsr8* placed next to the values of band size in brackets. The acronyms of morphological markers have been used as shown in table 2.

of several plant species, including pea (*Pisum sativum*), oat (*Avena sativa*) and Bengal gram (*Cicer arietinum*), also exhibits this feature (Weeden *et al*. 1996; Laucou *et al*. 1998; Loridon *et al*. 2005; Weising *et al*. 2005; Becher 2007). For example, the high density integrated map of *Cicer arietinum* ($n = 8$) based on RAPD, ISSR, AFLP, SSR and sequenced specific markers defined 13 linkage groups (Weising *et al*. 2005). This effect arises on account of large gaps between markers. It is expected that some of the linkage groups will coalesce when maps constructed by use of several other mapping populations, and with wider range and larger number of DNA markers, are integrated to develop a more detailed genetic linkage map. *C. roseus* is diploid with $2n = 16$ and genome size approximately 12 times bigger than that of *A. thaliana*, which is also diploid but has $2n = 10$. It is expected that the present form of *C. roseus* genome (chromosome complement) must be an outcome of duplications, deletions and a variety of rearrangements in the course of its evolution. The observed preponderance of mapped markers on LG1 and LG2 may be a consequence of the past events related to the evolution of the structures of individual chromosomes of *C. roseus* chromosomal complement. Further work, specially phylogenomic studies in *C. roseus* and the other model systems, will be helpful in revealing the bases of differential distribution of euchromatin and heterochromatin among sister chromosomes of a complement.

The molecular markers mapped in this study were of the RAPD, ISSR, EST-SSR and sequence specific types. The fingerprinting RAPD and ISSR markers are known to be useful in increasing the map density in both euchromatic and heterochromatic regions. Indeed, 86 (68.8%) of the 125 DNA markers placed on the *C. roseus* map are of RAPD and ISSR kinds. The sequence specific and EST-SSR markers are known to cover the euchromatic regions of genome/map. A total of 12 such markers were placed on the map. Thirty markers have their origin in RAPD combinations (3) and RAPD + sequence specific combinations (27). The combinations apparently gave markers by promoting new amplifications in regions not covered by a single RAPD and sequence specific primers. The sequence specific + RAPD markers are also expected to have covered the euchromatic regions of *C. roseus* map. This study showed that combining of RAPD with other kinds of primers can be a reliable means to generate new markers.

Interestingly, none of the ISSR primers possessing $(AT)_8$ or $(TA)_8$ repeat sequence produced amplification products in *C. roseus*. A similar result was also reported in lentil *Lens culinaris* (Rubeena *et al*. 2003). The result was surprising since (AT) repeats are most abundant repeat type in plant genomes (Wang *et al*. 1994; Gupta and Varshney 2000). In the present study, six out of eight mapped ISSR markers were obtained, three each from (GA) and (AC) repeat motifs. The (GA) and (AC) repeat motifs were also found to be abundant and useful for mapping in Bengal gram, lentil, wheat (*Triticum aestivum*) and other plant species (Ratnaparkhe *et al*. 1998; Kojima *et al*. 1998; Gupta and Varshney 2000; Ammiraju *et al*. 2001).

Morphological features of the kind included in this study are expected to be a result of coordinated expression of multigenes. However, the genetic backgrounds of the parents may be polymorphic for only one to several genes determining the traits. Segregation patterns of the six characters studied fitted the Mendelian 3:1 ratio. In this study the inheritance pattern of the corolla petal colour, known to be multigenic in other studies (Kulkarni *et al*. 2005), was seen to be monogenic. The distances between morphological markers that colocalized on LG1 and LG8 was large (from 3.1 to 9.1 cM), such that their coordinated regulation based on linkage is not indicated.

The linkage map constructed from the cross between 'Pink Delhi' × *gsr8* using PCR-based markers is the first to be reported for *C. roseus*. It is hoped that the genetic map will prove useful in locating and manipulation of genes of interest and in selection of TIA yield determining traits found linked with molecular markers in segregating populations.

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