Detection and Mapping of QTLs Affecting Contents of Pharmaceutical Alkaloids in Leaf and Root of *Catharanthus roseus*

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Abstract *Catharanthus roseus* leaves and roots hyper-accumulate the terpenoid indole alkaloids, which are required for the economic production of pharmaceutical molecules vindoline (V), catharanthine (C), vinblastine (VB), vincristine (VC), ajmalicine (A) and serpentine (S). For developing marker-assisted selection (MAS) breeding in *C. roseus*, the objectives of the present study were: (a) To characterize variability and associations between six traits—V (VL), C (CL), VB + VC (VBL) contents of leaves and C (CR), A (AR) and S (SR) contents in roots, in a recombinant inbred line (RIL) population in two environments. (b) To identify and place quantitative trait loci (QTLs) associated with the traits on the map. The population of 191 RILs was found to segregate transgressively for all the six traits. The intra-organ correlations of leaf and root traits were positive and significant. The inter-organ traits were not correlated. All the traits except AR were highly heritable ($h^2 = 41–79\%$). A RIL population genetic map of 179 markers on 8 linkage groups (LGs) covering 1,786.5 cM genetic distance was constructed. The RIL-generated genetic map led to identification of 20 QTLs for 6 traits (5, 4, 1, 4, 2, and 4 QTLs, respectively for CL, VL, VBL, CR, AR, and SR) by the use of single marker regression, simple interval mapping and composite interval mapping approaches. QTLs were detected only on 4 of the 8 LGs. QTL for the same or different traits were mapped in clusters or singularly. The results suggest that a combination of MAS and phenotypic selection offer possibilities of realizing simultaneous improvement in C and V accumulation in leaves and roots of *C. roseus*.

Keywords Composite interval mapping · Linkage map · Medicinal plant · Single marker analysis · Simple interval mapping · Terpenoid indole alkaloids

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Introduction

The apocynaceous species *Catharanthus roseus* produces more than 130 terpenoid indole alkaloids (TIAs), including several useful as drugs, vinblastine (VB) and vincristine (VC) for cancer chemotherapies and ajmalicine (A) and serpentine (S) in the treatments of arterial hypertension and anxiety, respectively [25, 62, 78, 80]. Some of the TIAs have been shown to provide protection to *C. roseus* against pests [7, 47, 53, 66]. The highly valuable species specific VB and VC and their more effective synthetic analogs are semi-synthesized commercially from vindoline (V) and catharanthine (C), the natural precursors of VB and VC, purified from the leaf extracts of the cultivated *C. roseus* plants [30, 32, 65, 77]. The cost of production is high

because the V, C, VB, and VC accumulate in plant organs in very low concentrations [15, 50, 51, 58, 64, 74, 78]. The compounds V, VB, and VC have been totally synthesized but the processes are industrially inviable [31, 33, 84].

The progress in dissection of the biosynthetic pathway has shown that the process is highly complex and involves subpathways accomplished in various organelles of cells of different tissues in aerial organs and roots [20, 50, 59, 78, 79]. Central intermediate of TIAs is strictosidine [2, 18, 20, 21] formed as condensate of the indolic precursor tryptamine derived from shikimate pathway [12, 29, 68] and the terpenoid secologanin precursor derived from combination of methyl erythritol phosphate-pathway and monoterpene-secoiridoid-pathway [4, 9, 19, 25, 60]. Catenamine synthesized from strictosidine via several steps serves as the branch point for different TIA subpathways including those for V, C, and A and S [5, 22, 23, 38, 42, 46, 75]. C, A and S are synthesized in root, stem, leaves, and flowers [50, 74], while V, VB, and VC are synthesized only in leaves and flowers [74]. Many of the structural and regulatory genes, enzymes and intermediates that determine the pathway and various cell types of different organs in which specific steps of pathway are accomplished have been identified and characterized [15, 20, 50, 67]. Processes involved in the biosynthesis of V, C, VB, and VC in leaves are understood in some detail [5, 10, 22, 23, 37, 38, 42, 44, 46, 56, 57, 66, 75]. Strictosidine, is synthesized in epidermal cells, serves as a precursor for V and C synthesis in leaf [2, 18, 23, 29, 48, 49, 57, 66, 76]. C synthesized in the leaf epidermis via as yet unknown subpathway is excreted for deposition in its waxy layer [66]. Majority of the steps from catenamine to V are known [5, 42, 49, 50, 56, 57, 75, 76]. Early part of V subpathway from strictosidine is accomplished in epidermal cells [29, 57, 66] while late steps and deposition of V occurs in the inner parenchymatous cells (laticifers and idioblasts; 8). V and C translocated to a common compartment in leaf cells are dimerized for the synthesis of VB and VC [66]. Ajmalicine (A) is synthesized from catenamine via a reductive step [15, 26] and oxidation of A leads to synthesis and deposition of S in cellular vacuoles [3, 52]. The TIA biosynthetic pathway at many stages of its progression involves translocation steps whereby intermediates are shuttled between cell types and intra-cellular compartments [4, 22, 50, 57, 66, 67, 76].

Until recently, a large body of work on *C. roseus* has remained focused on metabolic engineering to develop resources for V and C that may be more economic than their conventional resource, the roots and/or leaves produced on field grown *C. roseus* plants [78, 85, 87]. Metabolic engineering efforts to develop seeds, shoot, hairy root, cell suspension cultures, and heterologous organisms competent to hyper-synthesize V, C, and VB have been largely unsuccessful [17, 28, 52, 55, 63, 69, 82, 83].

Economic harvest of TIA high value products by metabolic engineering awaits understanding of TIA pathway in much greater details [50, 63, 75]. Emphasis is required on the application of genetics-based breeding procedures for improving the V, C, VB, and VC contents and yield of the organs that accumulate them in *C. roseus*.

Application of the conventional breeding procedures requires selection of segregants or recombinants in populations derived from the designed hybrids, in which the desired alkaloid(s) are accumulated in abundance. The breeding work has been apparently constrained by the expensive and time consuming nature of the organ-wise alkaloid profiling procedures [27, 74]. In these circumstances, QTL identification and mapping is a powerful genetic approach to tag favorable genes/alleles of traits of interest with DNA markers [45]. Subsequently, it is possible to introgress desirable alleles associated with QTLs into medicinal germplasm by marker-assisted selection (MAS) breeding [45]. Availability of germplasm lines of *C. roseus* with high content of C, V, VB, S, and/or A will lower the cost of production of TIA drugs. Accumulation of an alkaloid in *C. roseus* organ may consist of three broad components: synthesis [15], deposition [41], and loss by catabolism [14]. Each of these is expected to be under polygenetic control and therefore alkaloid concentration in an organ of *C. roseus* is expected to be amenable for QTL analysis. Markers linked to QTLs can be scored at early stage of plant growth [74] and breeding lines containing low levels of alkaloids can be eliminated early in breeding if MAS is used. Besides, MAS would allow simultaneous selection of breeding lines for several traits [11, 45].

The objective of present study was to develop a segregating population of large size, to use it on the one hand to develop a frame work genetic map of DNA markers and on the other hand to characterize the variation in the mapping population by phenotypic analysis of the traits of interest, identify QTLs contributing to the variation in the traits and map the QTLs with reference to DNA markers. For the first time, 20 QTLs have been identified for 3 leaf traits in *C. roseus*—percent contents of C, V, and VB in leaves and 3 root traits—percent contents of C, A, and S in roots. Nine of the 10 QTLs identified for the contents of V, C, and VB in leaves and 4 out of 4 QTLs for C content in roots proved effective in the isolation a genotype from the mapping population leaves and roots of which were rich in the valuable alkaloids.

Materials and Methods

Mapping Population

The mapping population of 191 recombinant inbred lines (RILs) derived by single seed descent from a pair cross

between the line called ‘*lli*’ [36] and the floricultural cultivar ‘Delhi Pink’ [54] was used in the present study. The ‘*lli*’ line is the renamed *gsr-8* ethylmethanesulfonate-induced mutant in the *glycophytic salinity response-8* (*GSR-8*) gene in the background of white flower bearing and *Pythium aphinadermatum* tolerant medicinal cultivar ‘WH40’ or ‘Nirmal’ [1, 35, 61]. The cv ‘Delhi Pink’ bears pink color flower petals. In its compound, racemose inflorescence flowers are borne in pairs subtended by one of the two leaves per node. Moreover, the altered inflorescence of *lli* plants has axes that are much branched with leaves missing from the flowering nodes. Thus, *LLI* and *lli* inflorescence phenotypes serve as morphological marker. The parental lines have been shown to be genetically distant by morphological, biochemical, and molecular analysis [54, 70, 74]. The genomic DNA of ‘*lli*’ is hypomethylated as compared to that of ‘Delhi Pink’ (unpublished observation). Both the parents had been shown to synthesize and accumulate pharmaceutically important TIAs, ‘Delhi Pink’ plants at higher level than the ‘*lli*’ plants. An F_1 product of mating was self pollinated to produce 212 F_2 progeny. Few RILs that produced sick or poorly fertile plants or continued to segregate (for *LLI* and *lli* habit or flower color) were rejected. The $F_{2:6}$ and $F_{2:7}$ RIL populations were phenotyped in the year 2007 and 2008, respectively. Leaf samples for genotyping were taken from $F_{2:7}$ RILs grown separately.

Field Trials

Parents and all of 191 RILs were phenotyped for six traits; namely, percent vindoline concentration in leaves (VL), percent catharanthine concentration in leaves (CL) and roots (CR), percent vinblastine and vincristine concentrations in leaves (VBL), percent serpentine (SR) and ajmalicine (AR) in roots. Field trials for this purpose were conducted at the institute’s (NIPGR) farm at New Delhi (latitude 28°36 N; longitude 77°12 E; altitude 216 m). The fields used in 2007 and 2008 trials were different and had been vacated by garden pea and wheat crops, respectively. Each trial was laid in randomized block design, replicated two times. There were 5 plants/genotype in a 1.5 m row and the distance between rows was 0.5 m (~66 thousand plants ha^{-1}), in each replication. Each year seeds were sown in nursery in the middle of February in clay pots and transplanted in the field in mid-April. Three plants were harvested per genotype per replication in the middle of September. The period from seed sowing to harvest spanned spring, summer and monsoon seasons. The crop growth period in the year 2008 was relatively warmer and wetter. There were 220.6 thermal degree days (TDD) in 2008 as compared to 210.7 in 2007 during 15 April–15 September. The total rainfall from April to September in

the years 2007 and 2008 was 49.7 and 84.9 cm, respectively. The N, P, K, and S fertilizers were applied to the fields at the rate of 80, 40, 40, and 10 kg ha^{-1} , respectively. The cultivation conditions standardized earlier were used [34, 54]. The plants identified for harvesting were scored for *lli/LLI* and petal color features, dug out, their roots washed free of adhering soil, placed in paper bags and kept for drying in a shed which was circulated with stream of air at room temperature. Leaves and roots from the three dry plants of a replication of a genotype were pooled and weighed. The replication-wise leaf and root materials of RILs, and parents were sampled for alkaloid extraction and quantification.

Quantification of Alkaloids

The alkaloids were extracted from leaf and root samples at room temperature and analyzed by RP-HPLC [73], with some modifications. Each sample was extracted and analyzed twice. For extraction of alkaloid content, finely powdered 1 g each of leaves and roots were left overnight in methanol (3×30 mL), filtered, and concentrated under vacuum to dryness. The extract, defatted with hexane (3×10 mL), was acidified by addition of 3 % HCl (15 mL). The acidified extract, made alkaline (pH 8–9) by dropwise addition of liquor ammonia (about 25 % NH_3), was further extracted with chloroform (3×30 mL), washed with water, then dried over anhydrous sodium sulfate and finally concentrated in vacuo to give the alkaloid extract. The extracts were stored at 4 °C till further investigation. For LC analysis, each extract was redissolved in 1 and 5 mL HPLC grade methanol.

Chromatographic separation of the alkaloids was performed through a Shimadzu (Kyoto, Japan) LC-10ATvp gradient HPLC equipped with two LC—10ATvp pumps, controlled by a SCL-10A interface module, and SIL-10ADvp autoinjector. For identification of peak(s) corresponding to the alkaloid(s) under investigation and checking its peak purity, a SPD—M 10Avp PDA detector (Shimadzu, Japan) was used. A Phenomenex (CA, USA) Luna C18 (2) (250×4.6 mm; 5 μm) reversed-phase column was used for all the LC analysis. The mobile phase consisting of acetonitrile and 0.1 M phosphate buffer (pH 4.9) was deployed as: linear gradient from 20:80 to 65:35 (v/v) in 0–30 min and 65:35 (v/v) to 20:80 (v/v) in 30–35 min (column rinsing); 35–40 min, isocratic elution with 20:80 (v/v) (column equilibration). A constant flow rate of 1.2 mL/min was maintained during analysis and all injections were of 10 μL . The detection of peaks was carried out at a wavelength of 220 nm. Identification and quantification of the alkaloids were achieved by comparison of their absorbance with that of the external standards. Standard stock solutions containing 1 mg/mL of VC, VB,

A (Sigma Aldrich, USA), C (Alexis Corporation, Switzerland), V, and S (gifted by R. Verpoorte) were prepared in methanol and stored at -20°C . These stock solutions were suitably diluted to prepare calibration plots for estimation of the percent content of the individual alkaloids in leaf and root extract.

Statistical Analysis

The software SPSS16.0 (SPSS, Chicago, USA) was used for the analysis of variance (ANOVA), application of t test and estimation of correlation coefficients. Because the phenotypic trait values for genotypes for 2007 and 2008 were significantly correlated and trait-wise mean values of RILs for 2007 and 2008 did not differ significantly, the trait-wise ANOVA was performed on the combination of 2007 and 2008 data to estimate variation due to genotypes, year (season) and genotype $\times$ season interaction. The broad-sense heritability (h^2) estimates were also derived from the combined data of 2007 and 2008 seasons.

Framework Genetic Linkage Map Construction

A framework genetic linkage map of the mapping population with 172 molecular markers and the *lli/LLI* morphological marker was already available [6]. The map of 8 linkage groups is based exclusively on the DNA markers that segregant-wise repeatedly gave the same result and segregated in Mendelian fashion among the 191 RILs of the mapping population. The map has markers elicited by 84 randomly amplified polymorphic DNA (RAPD) primers (called OP, followed by a capital letter identifying the primer set markers), 11 inter simple sequence repeat (ISSR) primers (called U markers), 39 simple sequence repeat (SSR) primer pairs (called CrSSR markers), 30 established sequence tag (EST)-SSR primer pairs (called CrES markers) and 8 microRNA primers (called CrMR markers). In addition, 37 CrES primers were used to detect polymorphism using the PCR and electrophoresis conditions similar to those described by Chaudhary et al. [6]. Previously mapped markers together with the new markers were used to perform the linkage analysis in the mapping population using MAPMAKER 3.0 [40, 43]. Marker order and distances between markers were calculated using the Kosambi mapping function.

QTL Analysis

A framework map with an average marker density of 10.0 cM was used for QTL dissection of quantitative traits. QTL analyses were implemented in QTL Cartographer Version 2.5 (<http://statgen.ncsu.edu/qtlcart/WQTLCart.htm>) [81]. First, data were analyzed to identify markers associated with variation for each trait by use of single marker

analysis (SMA) using all loci at a statistical threshold of $P < 0.01$. Second, trait data were analyzed with simple interval mapping (SIM) [24, 39] and composite interval mapping (CIM) [86] to identify and confirm the presence of QTLs. The maximum LOD score of association between the genotype and trait data were calculated for SIM and CIM, and QTL predictions were accepted for SIM for values greater than a threshold value of 2.5. The CIM analysis was run using Model 6 with forward and backward stepwise regression, a window size of 10 cM, and 2 cM walking speed along chromosomes. The genome-wide LOD score thresholds ($P < 0.05$) for QTL detection were determined with 1,000 permutations [8, 13]. The location of a QTL, LOD score, and percent phenotypic variance (R^2) were estimated by CIM for each QTL. The QTL nomenclature was the following: q the abbreviation for quantitative trait, followed by abbreviation for trait name and by the number of QTL for that trait. For example, the QTL *qVLI* means QTL number 1 for the trait percent vindoline content in leaves and the QTL *qCR2* means QTL number 2 for percent catharanthine content in roots.

Results

Variation for Alkaloid Concentrations in the RIL Population

The mean values and ranges of the six alkaloid traits for the two parental lines and 191 $F_{2:6}$ and $F_{2:7}$ RILs based on the field trials conducted in 2007 and 2008 are shown in Table 1. The table also presents the means and ranges separately for the RILs bearing *lli* and *LLI* phenotypes. The results of analysis of variance on the RILs for the six traits are shown in Table 2. The frequency distributions of all the traits showed continuous variation (Fig. 1). Transgressive segregation was observed in both the directions. The analysis of variance indicated existence of highly significant variation among RILs for all the traits. These features of RILs showed that multiple genes were involved in the determination of the traits and the population of RILs was suitable for QTL mapping of the traits under examination.

The C, V, and VB concentrations in leaves and S and C concentrations in roots were similar in 2007 and 2008. The A content in roots differed between 2007 and 2008. The coefficients of correlation between RIL-wise observations recorded in 2007 and 2008 for all the alkaloid traits were positive and significant indicating general concord between the corresponding year-wise data. Between the parents VL, VBL, and CR were at higher levels in “Delhi Pink” than “*lli*”, and AR is somewhat higher in “*lli*” than in “Delhi Pink.” However, these differences were not statistically significant. Mean values of RIL population were intermediate of parental values for VL,

Table 1 Percent content of pharmaceutically important terpenoid indole alkaloids in leaves and roots of 'lil' and 'Delhi Pink' parents and a recombinant inbred line (RIL) mapping population derived from their cross, evaluated in field trials conducted during the years 2007 and 2008

Trait (organ-wise percent alkaloid concentration) ($\times 10^{-2}$)	Year	Parental line	Variation in RIL population				t_{186} for the difference between <i>LLJ</i> and <i>lil</i>		
			Entire population			Sub-population of <i>lil</i> phenotype			
			' <i>lil</i> '	'Delhi Pink'	Mean (range)			t_{380} for the difference between the values of 2 years	Correlation coefficient between the values of 2 years
Catharanthine in leaves (CL)	2007	0.30 $\pm$ 0.02	0.29 $\pm$ 0.03	0.34 $\pm$ 0.01	0.55	0.781*** (0.0001)	0.31 $\pm$ 0.02 (0.05–1.01)	0.38 $\pm$ 0.02 (0.08–1.42)	2.25* (0.03)
	2008	0.26 $\pm$ 0.10	0.24 $\pm$ 0.04	0.33 $\pm$ 0.01 (0.05–1.17)			0.31 $\pm$ 0.02 (0.05–1.14)	0.36 $\pm$ 0.02 (0.09–1.17)	1.98* (0.049)
Vindoline in leaves (VL)	2007	1.76 $\pm$ 0.09	3.43 $\pm$ 0.43	2.20 $\pm$ 0.06	0.30	0.862*** (0.0001)	1.96 $\pm$ 0.06 (0.46–3.94)	2.41 $\pm$ 0.09 (0.46–5.74)	4.04*** (0.0001)
	2008	1.81 $\pm$ 0.23	2.12 $\pm$ 0.20	2.17 $\pm$ 0.06 (0.68–6.46)	(0.77)		1.92 $\pm$ 0.05 (0.76–3.22)	2.42 $\pm$ 0.08 (0.68–6.46)	4.65*** (0.0001)
Vinblastine + vincristine in leaves (VBL)	2007	0.49 $\pm$ 0.01	0.67 $\pm$ 0.04	0.66 $\pm$ 0.02	0.14	0.785*** (0.0001)	0.60 $\pm$ 0.02 (0.18–1.45)	0.72 $\pm$ 0.03 (0.16–1.52)	3.37*** (0.001)
	2008	0.54 $\pm$ 0.01	0.78 $\pm$ 0.03	0.65 $\pm$ 0.02 (0.13–1.55)	(0.89)		0.59 $\pm$ 0.02 (0.22–1.35)	0.72 $\pm$ 0.03 (0.13–1.55)	3.51** (0.001)
Serpentine in roots (SR)	2007	33.8 $\pm$ 3.80	33.4 $\pm$ 4.8	35.10 $\pm$ 0.53	0.30	0.316*** (0.0001)	34.4 $\pm$ 0.70 (18.50–55.65)	35.67 $\pm$ 0.80 (11.60–59.85)	1.14 (0.26)
	2008	36.5 $\pm$ 2.40	34.5 $\pm$ 3.10	34.92 $\pm$ 0.44 (19.50–52.05)	(0.77)		34.2 $\pm$ 0.54 (19.50–48.40)	35.57 $\pm$ 0.69 (20.55–52.05)	1.56 (0.12)
Ajmalicine in roots (AR)	2007	3.1 $\pm$ 0.50	2.75 $\pm$ 0.45	3.83 $\pm$ 0.09	1.95*	0.381*** (0.0001)	3.46 $\pm$ 0.10 (1.50–7.80)	4.18 $\pm$ 0.15 (0.55–8.60)	4.01*** (0.0001)
	2008	3.35 $\pm$ 0.05	3.25 $\pm$ 0.25	3.61 $\pm$ 0.07 (1.25–8.80)	(0.05)		3.38 $\pm$ 0.08 (1.25–7.05)	3.82 $\pm$ 0.12 (1.65–8.80)	2.97** (0.003)
Catharanthine in roots (CR)	2007	1.45 $\pm$ 0.15	1.85 $\pm$ 0.75	2.19 $\pm$ 0.08	0.37	0.499*** (0.0001)	2.08 $\pm$ 0.10 (0.20–4.25)	2.31 $\pm$ 0.12 (0.15–8.15)	1.40 (0.16)
	2008	2.25 $\pm$ 0.35	2.55 $\pm$ 0.25	2.24 $\pm$ 0.07 (0.20–8.90)	(0.71)		2.15 $\pm$ 0.08 (0.20–4.05)	2.32 $\pm$ 0.11 (0.55–8.90)	1.22 (0.23)

*, **, and *** = significant at 5, 0.1 and 0.01 % levels, respectively; *P* values are given in parenthesis

Table 2 Significance of variation due to genotypes, seasons and seasons $\times$ genotypes and estimates of broad-sense heritability for the alkaloid traits in the trial of 191 F_{2:6} and F_{2:7} recombinant inbred linesdeveloped from the cross ‘Ili’ $\times$ ‘Delhi Pink’ in *Catharanthus roseus* (f and h^2 values are provided)

Trait ^a	Analysis of variance components in RILs			Heritability h^2 (%)
	Genotype $f_{190:381}$	Season $f_{1:381}$	Genotype $\times$ season $f_{190:381}$	
CL	9.55*	1.63	1.21 ^{NS}	64.6
VL	9.92*	0.47	0.74 ^{NS}	79.2
VBL	9.90*	0.11	1.19 ^{NS}	41.3
SR	5.95*	0.40	3.13*	50.1
AR	7.12*	19.80*	3.26*	15.4
CR	11.5*	1.05	3.87*	61.0

NS not significant ($P > 0.05$)* Significant at $P < 0.0001$ probability level^a The description of the six traits is given in the Table 1

VBL, and CR and higher than parental values for CL and AR. Among the traits studied, VL was the most highly expressed (from 0.0046 to 0.0646 %). Leaf traits could be arranged in the following order in terms of their level of expression: VL > VBL > CL. Among the root traits, SR expression was the highest (from 0.1161 to 0.5985 %). The root traits fell in the following order in terms of their relative expression: SR > CR > AR. On average basis the RILs of LLI phenotype had significantly higher contents of leaf and root alkaloids, except for S in roots; both Ili and LLI RILs had similar SR expression. The RILs of LLI and Ili phenotype separately and together demonstrated enormous variation in both the directions of mean values for all the six alkaloid traits studied.

Heritability of the Alkaloid Traits

The broad-sense estimates of the heritability of the six alkaloid traits are presented in Table 2. The heritability (h^2) values indicate the proportion of genetic variation in the expression of the total phenotypic variation in RILs for individual traits. The heritability ranged from 15.4 % for AR to 79.2 % for VL. The traits could be arranged in the following order in terms of their heritabilities: VL (79.2 %) > CL (64.6 %) > CR (61.0 %) > SR (50.1 %) > VBL (40.3 %) > AR (15.4 %). As is evident from the above data the heritability of AR was significantly lower than all the other studied alkaloids.

Correlations of the Alkaloid Concentrations in RIL Population

Correlation estimates are presented in the Table 3. Phenotypic correlation analysis revealed that correlations were positive and significant between the VL, CL, and VB (+VC) leaf traits in all pair-wise combinations. The pair-

wise correlations between SR, AR, and CR were also positive and significant. However, the correlations of the leaf traits VL and VBL with root traits (SR, AR, and CR) were weakly positive but insignificant. Contrastingly, the correlations of CL with root alkaloid traits were negative, but feeble and insignificant. The correlation analysis showed that accumulation of the studied alkaloids in leaves was independent of that in roots and vice versa.

Advancement in the Framework Genetic Linkage Map

The genetic linkage framework map of *C. roseus* constructed earlier by us had 172 DNA markers and 1 morphological marker [6]. By following the procedures briefly described here and in detail previously [6], 6 CrES markers were added into the map of 8 linkage groups (LGs): CrES361, CrES392 and CrES401 into LG1, CrES364 into LG2, CrES377 into LG5 and CrES390 into LG8. Table S1 gives a list of all the CrES primer pairs that were tested and sequences of those which led to addition of markers on the map. Figure 2 shows the advanced version of the framework genetic linkage map (with QTLs placed on it). As a result of the advancement, the map has 84 RAPD, 11 ISSR, 39 SSR (anonymous) markers, 8 microRNA, 36 EST-SSR (coding region) markers and 1 morphological marker. The total map length now measures 1,786.5 cM, with an average distance of 10.0 cM between markers. The longest linkage group has 45 markers covering a genetic distance of 320.1 cM. The shortest linkage group of 89.9 cM genetic distance has 12 markers. The different types of markers on LGs are in proportion of the genetic distance between their markers. Three markers on LG3 have genetic distance >25 cM (26.2–28.1 cM). None of the markers are ≥ 30 cM apart. All of 179 markers are uniquely located on the map.

Fig. 1 Frequency distributions of the terpenoid indole alkaloid content traits measured during 2007 and 2008 in the ‘Ili’ × ‘Delhi Pink’ $F_{2.6}$ and $F_{2.7}$ population(s). Arrows indicate the alkaloid levels

in the parental lines. Dashed and solid arrows indicate the ‘Ili’ parent and ‘Delhi Pink’ parent, respectively

Table 3 Coefficients of correlation between the percent contents of vindoline (VL), catharanthine (CL), and vinblastine + vincristine (VBL) in leaves and catharanthine (CR), ajmalicine (AR) and serpentine (SR) in roots, among the recombinant inbred lines of the cross ‘Ili’ × ‘Delhi Pink’ in *Catharanthus roseus*

Trait	VL	CL	VBL	SR	AR
CL	0.487**				
VBL	0.387**	0.417**			
SR	0.090	−0.025	0.034		
AR	0.071	−0.021	0.058	0.337**	
CR	0.005	−0.059	0.052	0.214*	0.569**

* and ** significant at $P < 0.01$ and $P < 0.001$ probability level, respectively

Detection of Leaf and Root Alkaloid QTLs

Quantitative trait loci analysis was performed on all the six alkaloid traits in the RIL mapping population using the 179 markers distributed over 8 linkage groups (LGs) on the map. Application of all the three analytical methods—SMA, SIM and CIM—identified a total of 20 QTLs across the traits, 10 QTLs each for the leaf—and root—organ related alkaloid traits. Significant associations were detected between the six traits and markers located at various positions on the LGs 1, 3, 4, and 6 while markers on LGs 2, 5, 7, and 8 of the map were not found associated with any of the six traits (Table 4; Fig. 2). Of the total QTLs, 11

Fig. 2 Framework genetic linkage map of *C. roseus* showing 20 quantitative trait loci (QTLs) identified for the terpenoid indole alkaloid content traits using single marker analysis (SMA), simple interval mapping (SIM) and composite interval mapping (CIM) in the leaves and roots of 191 recombinant inbred lines of the cross “*lil*” × “*Delhi Pink*”. The QTLs shown in map are for the traits: catharanthine (CL), vindoline (VL), vinblastine + vincristine (VBL) content in leaves and serpentine (SR), ajmalicine (AR), catharanthine (CR) content in roots. QTLs were designated using *q* as abbreviation for QTL, followed by an abbreviation of the trait name in upper case

and a number identifying the QTL number for the trait. For each QTL, its name and the peak region on the concerned LG has been differentially indicated by a color: green for CL, violet for VL, blue for VBL, brown for SR, pink for AR and orange for CR. Graphical representations from QTL Cartographer using SMA (as red peak), SIM (as green peak) and CIM (as blue peak) for QTLs depicted by at least two methods are shown near the closest linked marker. The QTLs identified by either SMA or SIM only has been denoted by a asterisk and bullet, respectively. (Color figure online)

QTLs were found located on LG1, 5 QTLs on LG3 and 2 QTLs each on LG4 and LG6.

Leaf Alkaloid Traits

CL

A total of 5 QTLs were identified for CL. Of these, only two, *qCL1* on LG4 and *qCL2* on LG6, demonstrated significance with all the three analytical methods. The *qCL3* and *qCL4* detected on LG1 and LG3, respectively, were not significantly detected by CIM method. The *qCL5* was not

detected to be significant by SMA and CIM methods. Individually, the 5 QTLs accounted for the phenotypic variance in the range of 11.9–18.9 %.

VL

In all 4 QTLs were identified for VL. Among them *qVL1* and *qVL2* were identified by all of SMA, SIM, and CIM methods. The *qVL3* was identified by SMA and SIM methods while *qVL4* was identified only by SMA method. The VL QTLs were responsible for up to only 12.6 % phenotypic variance. All the four VL QTLs were located on LG1.

Table 4 Map locations and summary of the quantitative trait loci (QTLs) identified for six traits by applying single marker analysis (SMA), simple interval mapping (SIM) and composite interval mapping (CIM) analyses using QTL Cartographer software in a RIL mapping population of the cross “*lli*” × “Delhi Pink” in *Catharanthus roseus*. QTLs identified as significant are given in bold

Trait	QTL	Linkage group	QTL Cartographer		CIM		Marker(s) in interval ^c (donor parent) ^d				
			SMA (<i>P</i> < 0.0001)	SIM	CIM		LOD	LOD peak position (cM)	LOD	LOD peak position (cM)	<i>R</i> ² (%) ^a
					<i>R</i> ² (%) ^a	LR threshold ^b					
CL	<i>qCL1</i>	4	75.1–107.3	14.10	95.3	29.95	13.66	8.58	93.3	12.1	CrES52 (<i>lli</i>), CrES62 (DP), CATR28 (DP)
	<i>qCL2</i>	6	126.8–149.2	7.98	138.6	17.41	13.66	10.24	138.6	14.8	OPD20 (<i>lli</i>), U842 (DP), OPM14 (DP)
	<i>qCL3</i>	1	11.8–19.7	8.03	14.1	17.58	13.66	0.10	15.1	0.1	CrSSR65 (DP), OPC12 (<i>lli</i>), OPT18 (<i>lli</i>)
	<i>qCL4</i>	3	139.2–150.1	3.36	146.3	11.84	13.66	0.10	149.4	0.1	CrSSR98 (DP), CrES267 (DP)
	<i>qCL5</i>	3	222.9–254.7 ^e	4.31	254.7	18.88	13.66	nd	nd	nd	OPO3 (DP), OPD20 (<i>lli</i>)
VL	<i>qVL1</i>	1	55.8–76.9	3.93	63	9.47	13.59	6.25	65	12.6	CrMR165a (DP), CrSSR56 (DP), CrES361 (DP), U835 (<i>lli</i>), OPX3 (DP), U825 (<i>lli</i>)
	<i>qVL2</i>	1	135.7–165.8	6.28	143.1	20.49	13.59	3.58	148.1	7.6	OPA18A20 (<i>lli</i>), OPU13 (<i>lli</i>), CrSSR199 (DP), OPA3 (DP)
VBL	<i>qVL3</i>	1	35.0–43.2	4.95	40.2	11.37	13.59	1.80	40.2	2.89	OPU13 (<i>lli</i>), OPY1 (DP)
	<i>qVL4</i>	1	232.3–309.7	2.52	264.6	5.92	13.59	1.00	264.6	1.54	CrSSR67 (DP), OPR12 (DP), CrSSR183 (DP), OPG13 (<i>lli</i>)
	<i>qVBL1</i>	1	138.2–166.1	7.77	153.9	22.3	13.03	11.59	153.9	30.9	CrSSR199 (DP), OPA3 (DP)
	<i>qSR1</i>	3	196.4–240.7	16.91	222.4	35.86	13.33	16.87	222.4	31.9	OPO3 (DP)
SR	<i>qSR2</i>	1	11.5–20.0	4.37	13.8	12.19	13.33	0.12	14.1	0.2	CrSSR65 (DP), OPC12 (<i>lli</i>), OPT18 (<i>lli</i>)
	<i>qSR3</i>	3	252.1–289.6 ^e	3.17	276.7	14.46	13.33	0.24	276.7	0.6	U812 (DP)
	<i>qSR4</i>	4	108.2–114.7 ^e	3.81	110.1	15.03	13.33	0.48	110.1	0.1	OPC11 (<i>lli</i>)
	<i>qAR1</i>	3	26.4–51.4	7.69	35.3	17.39	13.02	6.13	35.3	9.9	CrSSR272 (DP), CrES27 (<i>lli</i>)
AR	<i>qAR2</i>	6	35.2–61.5	8.22	44.5	18.07	13.02	10.43	43.1	23.8	CrES231 (<i>lli</i>), OPE15 (DP)
	<i>qCR1</i>	1	185.2–206.0	10.76	200	24.34	12.12	12.78	200	27.3	CrMR390a (DP), CrSSR108 (DP), CrES248 (<i>lli</i>)
	<i>qCR2</i>	1	206.6–218.9	9.84	208.7	25.29	12.12	11.92	208.7	28.3	OPO6 (DP), OPX4 (DP)
	<i>qCR3</i>	1	151.1–169.9	9.05	165.5	19.55	12.12	0.18	165.5	0.2	CrSSR199 (DP), OPA3 (DP)
CR	<i>qCR4</i>	1	172.9–185.8	9.79	175.9	20.91	12.12	0.61	175.9	0.4	CrES36 (DP), OPT1 (DP), CrSSR207 (DP), CrSSR154 (DP)

nd not detected

^a The amount of phenotypic variation explained by each identified QTL^b Threshold determined by 1,000 permutations^c Among the markers in the interval, the underlined marker is closer to the QTL position^d Donor parent in parenthesis, *lli* or DP (Delhi Pink)^e Significant at $P < 0.01$ probability level

VBL

Only one QTL *qVBL1* located on LG1, was detected for the VBL trait. This *qVBL1* must be a major QTL since it explained 40 % of the phenotypic variance detected in the expression of VBL.

Root Alkaloid Traits

CR

A total of 4 QTLs were detected, *qCR1* and *qCR2* by the SMA, SIM, and CIM methods; and *qCR3* and *qCR4* by SMA and SIM methods only. All the qCRs were mapped on LG1. They individually explained 19.6–28.3 % of the observed phenotypic variance in this trait.

AR

Two qARs, one each on LG3 and LG6 were detected by all the three analytical methods. The *qAR1* and *qAR2* were respectively responsible for 9.9 and 23.8 % of the phenotypic variance in the trait.

SR

Four QTLs were identified for this trait, *qSR1* on LG3 by all the three detection methods, *qSR2* on LG1 by SMA and SIM methods and *qSR3* on LG3 and *qSR4* on LG4 by the SIM method. These 4 QTLs explained 12.2–31.9 % of the observed phenotypic variance for SR.

Co-Localization of QTLs on Linkage Groups

The QTLs for the same and/or different traits were observed to be co-located on the linkage groups (Fig. 2). On LG1, of the 11 QTLs, 10 fell into three regions. All the QTLs related to CR fell in a 65.9 cM region on LG1 from 152.1 to 218.9 cM. An interval of 30.4 cM from 135.7 to 166.1 cM included two QTLs, *qVL2* and *qVBL1*, both for leaf alkaloids. There was an overlap of 27.6 cM between the *qVL2* (135.7–165.8 cM) and *qVBL1* (138.2–166.1 cM). Another region of 65.4 cM spanning 11.5–76.9 cM distance on the upper part of LG1 contained four QTLs, namely *qSR2* (peaking at 14.1 cM location), *qCL3* (15.1 cM), *qVL3* (40.2 cM) and *qVL1* (65 cM), three QTLs for leaf traits and a QTL for root trait. On LG3, a 93.2 cM region from 196.4 to 289.6 cM, included *qSR1* (peak at 222.4 cM), *qCL5* (254.7 cM) and *qSR3* (276.7 cM), QTLs for leaf and root traits. Another coincidental location of leaf and root QTLs was *qCL1* (93.3 cM) and *qSR4*

(110.1 cM) in a 39.6 cM region from 75.1 to 114.7 cM on LG4.

Relative Contribution of Favorable QTL Alleles for Leaf and Root Traits by Parents

Table 4 lists the markers that fall in the interval that defines the location of each of the 20 QTLs. The donor of each marker is also specified. It is seen that of the 45 markers that are listed against the 20 QTLs, 30 were from the Delhi Pink parent and 15 from the *Ili* parent. Delhi Pink contributed more favorable alleles as compared to the *Ili*. If the marker closest to the QTL was treated as index of donor, it appeared that *Ili* and Delhi Pink, respectively, contributed 6 and 14 favorable QTL alleles.

Discussion

Medicinal cultivars rich in the pharmaceutically important terpenoid indole alkaloids (TIAs) are required in *C. roseus*. To enable the marker-assisted improvement of TIAs in *C. roseus*, 20 QTLs that affect the content of 5 important TIAs in roots and/or leaves have been identified. This is perhaps the first study of identification, mapping and validation of QTLs using combination of SMR, SIM and CIM analytical procedures in *C. roseus*. Some aspects of the analyses are discussed below.

Comparative Properties of the Alkaloid Traits in the Mapping Population

The contents of a specific TIA in different organs of *C. roseus* represent the organ-wise net accumulation of the TIA, outcome of the amount synthesized and saved from possible catabolism. The progress in the molecular genetic dissection of the TIA pathway in *C. roseus* has shown that the processes involved are under multigenic control [15, 20, 79]. The content of a TIA in an organ is therefore expected to be high in genotypes in which combination of alleles of the concerned genes favor increased synthesis and storage of the alkaloid in the organ. Genotypes harboring combinations of favorable alleles for improved expression of a trait (such as the content of TIA in an organ) are expected to occur in segregating populations from crosses between genetically variant germplasm. The mapping population of RILs characterized in this study, developed from the cross between “*Ili*” (an inflorescence mutant of the medicinal cultivar “Nirmal”) and “Delhi Pink” (a floricultural cultivar), indeed represents such a population. The RIL population demonstrated considerable variation in all the traits studied: VL, CL, VBL, CR, SR, and AR. Transgressive segregation was observed in the RIL population for all the traits. There were

RILs in which the SR, VL, VBL, AR, CR, and CL traits were expressed at 1.6, 2.2, 2.4, 2.8, 4.0, and 4.6-fold higher level, respectively, as compared to the corresponding mean value of the parents. For each trait, a large component of the observed variation was of genetic nature, even though the parents of the mapping population did not differ significantly in the expression of all the six traits. The heritability estimates for all the traits except AR, were moderate to high in range. Interestingly, the ranges of variation were wide, and heritabilities for CL, CR, and VL traits were higher than for other traits. This result may be of much economic importance. VB and other more effective anticancer molecules are commercially semi synthesized from V and C which are also the natural precursors of VB [15, 58, 64]. The commercial processes uses V and C extracted from the leaves of the cultivated plants of *C. roseus* medicinal varieties. The detection of highly heritable genetic variation in the CL and VL traits in the RIL population indicates that the segregating populations from the cross “*lli*” × “*Delhi Pink*” offer possibilities for selection of genotypes bearing V and C rich leaves. The properties of CR trait in the RIL population also suggest possibilities of selection of genotypes bearing roots highly rich in C. The *C. roseus* leaves contain several times (8×) more V than C. The commercial extracts of leaves therefore have relatively more V than C, although semi-synthesis of VBL requires V and C in equimolar concentrations. The imbalance between the quantities of C and V from leaves may be narrowed/overcome by extraction of C and V from whole plants of genotypes that bear hyper-C-rich roots, such as those available among the “*lli*” × “*Delhi Pink*” segregants. The studied RIL population indeed harbors individual RILs in which VL, CL, VBL, and CR are simultaneously very high (D54, VL 3.14, CL 1.32, VBL 0.95 and CR 5.10×10^{-2} %).

In the RIL mapping population, the leaf traits CL, VL, and VBL were highly correlated with each other. Like wise the root traits were pair-wise correlated. Interestingly, the leaf traits did not correlate with the root traits. These results confirm similar conclusions arrived in an earlier study [74]. All these results have important implications. The absence of correlations between leaf and root traits means that the synthesis and storage of the C, V, and VB TIAs in leaf is autonomous of the corresponding processes for C, A, and S in roots. This inference is in conformity with the previous study of the expression of specific genes and/or enzymes involved in the synthesis of V in various tissues of leaves and roots borne on *C. roseus* plants and in in vitro cultures of hairy roots of *C. roseus* [15, 16, 20, 23, 50, 60, 78]. There is evidence that 16-methoxytabersonine, a precursor of vindoline synthesized in leaf epidermal cells is secreted into the cells of inner mesophyll tissue where V is synthesized and stored [20, 22, 23, 66]. V is not synthesized in normal roots and hairy roots [20, 63, 74]. C in leaves is

synthesized in epidermal cells and accumulates in the wax associated with the surface of epidermis [66]. The assembly of VB from the C and V components probably occurs in the leaf epidermis [10, 66]. The mechanism that shuttles V and C back into epidermal cells to enable VBL synthesis is presently obscure. VB is not synthesized in roots as V is not available there [78]. There is no evidence of any connection/transport of TIAs between root and leaf. The contents of V and C in leaves are expected to be correlated with each other because they are synthesized from a common precursor, the strictosidine [20–22, 66, 79]. The CL and VL are correlated with VBL because VBL is assembled from dimerization of V and C [15, 66]. Since the correlations between V and C, V and VB and C and VB are high, it may be possible to produce genotypes, leaves of which are rich in all the three pharmaceutical TIAs.

C, A, and S are known to be synthesized in root tips [15, 20, 50, 63, 72, 74, 78]. A and S share a subpathway such that S is the product of A [15]. The common precursor for C and A is cathenamine [16, 20, 21, 50, 66, 79]. A is derived from cathenamine in one step [20] while C is synthesized from cathenamine perhaps in multiple steps which remain to be understood. The AR and SR are expected to be correlated since they comprise a subpathway of TIA synthesis [15]. The CR and AR/SR are correlated because their precursor is common [15, 20, 79]. Among C, S, and A, the C and S are end products and A is the intermediate for S. This may be the reason for high heritabilities for CR and SR as compared to AR. It is possible to visualize that selection for CR or SR may result in co-selection for CR, AR and SR traits.

The existence of strong correlations among leaf traits on the one hand and root traits on the other hand raises the possibility that screening for a selected one or two traits might result in improvement of the organ-wise correlated traits. The autonomy in the expression of TIA pathway for C in roots and leaves is confirmed. Possibility of selection of genotypes simultaneously improved in VL, CL and CR is indicated.

Suitability of Framework Genetic Linkage Map for QTL Analysis

This study has used a 179 point genetic linkage map consisting of dominant and co-dominant markers. The map is based exclusively on the markers that segregated repeatedly according to Mendelian expectations among 191 F_{2:7} RILs. The markers were placed on 8 LGs based on stringent mapping conditions. The total map length is 1,786.5 cM. The map distances on individual LGs range from 89.9 to 320.1 cM. The average inter-marker distance is 10 cM. The map has suitable number of evenly distributed markers on each LG. Although relatively unsaturated,

the framework map is quite robust and thus suitable for QTL analysis [11]. It has 17 of the 92 markers mapped on a framework map using a different mapping population [71].

Detection and Validation of QTLs for Alkaloid Accumulation Traits

Detection of QTLs affecting the percent contents of pharmaceutically important TIAs in leaves and roots in this study has augmented the already available information on the genetic architecture of TIA accumulation in *C. roseus* organs. A total of 20 QTLs were identified. Considering the complexity of the processes involved in TIA accumulation (TIA synthesis, deposition and transformation/consumption by catabolism) the number of QTLs identified appears to be small. It is possible that QTLs affecting the most decisive steps of TIA accumulation for which the parents of RILs varied genetically got identified. Among the 20 QTLs identified, 5 were for CL, 4 each for VL, CR and SR, 1 for VBL and 2 for AR. The detection of QTLs may have been in proportion of the total QTLs affecting each of the trait. This idea is corroborated by existing information that many more steps are involved in the synthesis of V from the TIA central intermediate strictosidine and its deposition in leaf idioblast and laticifers (several genes for synthesis of tabersonine from strictosidine, six genes from tabersonine to V, several genes for inter- and intra-cellular transport of intermediates and possibly a few genes each for stable deposition and regulation of the processes) [4, 5, 20, 22, 57, 60, 66] than those involved for synthesis of VB from V and C and its accumulation (several steps including the dimerization reaction leading to α -3',4'-anhydrovinblastine, a few genes for the transport of C and V to the site of VB synthesis and possibly a few genes for the stable deposition of VB and regulation of the processes) [10, 16, 22, 23, 60, 66]. Since A is an intermediate for S, detection of 4 QTLs for SR and 2 QTLs for AR is also in agreement with the suggestion that the number of QTLs detected for a trait indicates the degree of complexity in its genetic determination. Presently, there is little information about the genetic architecture of subpathway that is responsible for synthesis of C from strictosidine, the intermediate shared with S subpathway in roots and V pathway in leaves. The detection of 5 QTLs for CL and 4 QTLs for CR traits indicates existence of multiple steps in the determination of CL and CR, perhaps of the same magnitude as for the VL trait. To reveal new and larger arrays of QTLs for the presently studied traits, segregating populations from crosses between parents having more divergent expression of traits, and construction of more detailed genetic linkage map by integration of additional DNA markers, especially SSR and SNP markers, may be examined in future studies.

Linkage relationships of QTLs with each other and with co-located markers are multifariously informative. The QTLs for all the six traits studied here were mapped on 4 out of a total of 8 LGs in the genome of *C. roseus* ($2n = 2x = 16$). QTLs for 5 of the 6 traits, except for AR were mapped on LG1. All the QTLs for three traits VBL, VL, and CR were mapped on LG1. For the CL, AR, and SR, the QTLs were mapped on two or more of LGs. Co-localization of two QTLs of different traits may be indicative of involvement of common function in the determination of the traits. In the present study, there are two such examples. The QTLs *qCL3* and *qSR2* were found linked to the same marker OPC12 on LG1. This indicates that a gene located at the site of OPC12 perhaps determines both CL and SR traits. It could be any of the genes among those that determine TIA synthesis steps up to cathenamine, a post-strictosidine intermediate both for C in leaves and S in roots of *C. roseus*. Another set of two QTLs for distinct traits, namely *qVBL1* and *qVL2* were found linked to the same marker CrSSR199 in an interval of 30.4 cM on LG1. Among all the QTLs, *qVBL1* accounted for the highest (>30 %) phenotypic variability demonstrated in its trait. The *qVBL1* and *qVL2* perhaps represent gene(s) concerned with the transport of V from its site of accumulation in leaf laticifers and idioblasts to the site of VB synthesis, most likely leaf epidermis. Interestingly, all the 4 QTLs for CR trait formed a cluster in a region of 66.8 cM distance on LG1. This might suggest that the genes located at the sites of CR QTLs share regulatory mechanism(s). Contrastingly, the 5 QTLs for the CL traits were observed to be located on 4 different LGs. The *qCL3* was located far removed from the QTL cluster of CR trait on LG1. These observations imply that although CL and CR traits are concerned with accumulation of C, albeit in different organs, their QTLs defined here apparently do not concern common gene functions. This finding supports the conclusion that C synthesis-cum-accumulation pathway involves many steps. Another interesting observation was that even though all of the QTLs for VL and CR were located on LG1, yet the VL and CR traits were not correlated. Since recombination frequency/chromosome/generation is usually low, an implication of co-occurrence of QTLs for different alkaloid accumulation traits on same LG is high level of co-inheritance of the QTLs/concerned traits. Location of QTLs for all the traits on 4 LGs of 1,074.4 cM genetic distance, with the total exclusion of 4 LGs of 712 cM genetic distance, indicates that perhaps *C. roseus* acquired TIA biosynthetic ability as a consequence of inter-species hybridization followed by selection of a TIA^+ ($n = 8$) diploid fertile derivative.

The mapping population of RILs demonstrated transgressive segregation for all the studied traits, even though the parents did not differ much in the phenotypic

expression of the traits. Therefore, both the parents were expected to contribute favorable alleles for the expression of different traits. The observations confirmed that both the parents contributed favorable QTLs. However, it was found that “Delhi Pink” contributed more favorable alleles of QTLs than “*Ili*.” All the favorable QTLs for the CR trait and the only one for VBL were contributed by Delhi Pink. The floricultural cultivar Delhi Pink was identified as resource of alleles for increased accumulation of pharmaceutically important alkaloids in *C. roseus*.

Feasibility of Marker-Assisted Breeding for Improvement of Alkaloid Contents

The characteristics of variation for 3 leaf and 3 roots TIA content traits in the mapping population indicate that the segregating populations of the “*Ili*” × “Delhi Pink” cross may prove suitable for selecting superior genotypes for all the 6 traits. The TIAs V, C, VB, A, and S are materials for the production of drugs for three different kinds of clinical conditions. Unlike A and S, for which there are alternate resources, *C. roseus* roots and/or leaves are the only resource for C, V and VB. Phenotyping of the 191 RILs had revealed a line designated D54 which had V, VB, and C in high concentrations in leaves and C in high concentrations in roots. Genotyping showed that D54 was positive for 13 out of 14 QTL alleles detected as favorable for CL (4/5), VL (4/4), VBL (1/1) and CR (4/4) traits. D54 possessed 9 out of 14 DNA markers identified closest to *qCL2-qCL4*, *qVL2-qVL4*, *qVBL1*, *qCR2*, and *qCR4* and 4 markers identified close to *qVL1*, *qCR1*, and *qCR3*; it did not possess the markers identified close to *qCL1*. D54 exemplified the possibilities of exercising marker-assisted selection (MAS) for genotypes having improved VL, CL, and CR traits. A strategy combining MAS and phenotypic selection seems most appropriate at the current level of QTL analysis and relatively low density of markers on the genetic linkage map underlying the QTL analysis. It is suggested that the segregating populations of “*Ili*” × “Delhi Pink” cross at young stages of growth in early generation(s) may be first subjected to genotyping in respect of markers close to the VL, CL, and C QTLs. The smaller populations enriched via selection for favorable QTLs based on markers may then be phenotyped for the VL, CL, and CR traits to finally select genotypes simultaneously improved for the VL, CL, and CR traits.

Summarized conclusion

In *C. roseus*, a total of 20 QTLs for 3 leaf and 3 root TIA traits related to the economics of the production of important TIA drugs were identified. The QTLs were detected by combined use of the SMA, SIM, and CIM analytical procedures on a transgressively segregating

population that also served as the genetic linkage mapping population for placement of 179 markers on the 8 LGs of the *C. roseus* genome. The QTLs were mapped only on 4 of the 8 LGs, 2 each on two LGs and 5 and 11 on the other two LGs. Clusters of QTLs affecting the same or different traits were also detected. A two-step selection scheme combining the use of markers linked to QTL followed by phenotypic selection has been proposed for isolation of genotypes simultaneously improved for VL, CL, VBL, and CR traits from the segregating population(s) of the cross that gave the mapping population.

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