RESEARCH ARTICLE

Characterization of variation and quantitative trait loci related to terpenoid indole alkaloid yield in a recombinant inbred line mapping population of *Catharanthus roseus*

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Abstract

Improved *Catharanthus roseus* cultivars are required for high yields of vinblastine, vindoline and catharanthine and/or serpentine and ajmalicine, the pharmaceutical terpenoid indole alkaloids. An approach to derive them is to map QTL for terpenoid indole alkaloids yields, identify DNA markers tightly linked to the QTL and apply marker assisted selection. Towards the end, 197 recombinant inbred lines from a cross were grown over two seasons to characterize variability for seven biomass and 23 terpenoid indole alkaloids content-traits and yield-traits. The recombinant inbred lines were genotyped for 178 DNA markers which formed a framework genetic map of eight linkage groups (LG), spanning 1786.5 cM, with 10.0 cM average intermarker distance. Estimates of correlations between traits allowed selection of seven relatively more important traits for terpenoid indole alkaloids yields. QTL analysis was performed on them using single marker (regression) analysis, simple interval mapping and composite interval mapping procedures. A total of 20 QTL were detected on five of eight LG, 10 for five traits on LG1, five for four traits on LG2, three for one trait on LG3 and one each for different traits on LG three and four. QTL for the same or different traits were found clustered on three LG. Co-location of two QTL for biomass traits was in accord of correlation between them. The QTL were validated for use in marker assisted selection by the recombinant inbred line which transgressively expressed 16 traits contributory to the yield vinblastine, vindoline and catharanthine from leaves and roots that possessed favourable alleles of 13 relevant QTL.

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Introduction

Plant species of different families are known to synthesize more than 2000 terpenoid indole alkaloids (TIA) (Gerasimenko *et al.* 2002; Barleben *et al.* 2007; Ziegler and Facchini 2008; Guirimand *et al.* 2010a,b). The natural synthesis of TIA has been recognized as a part of plant's defence mechanism against pests and diseases (Meisner *et al.* 1981; Chockalingam *et al.* 1989; Luijendijk *et al.* 1996; Roepke *et al.* 2010). More than 130 TIA are produced in the apocynaceous species, *Catharanthus roseus* (van der Heijden *et al.* 2004; Wang *et al.* 2011a). Among the TIA of *C. roseus*, several are valuable pharmaceuticals. Vinblastine (VB) and vincristine (VC) are extensively used in anticancer chemotherapeutics (Tellingen *et al.* 1993; Leveque *et al.* 1996; Schmeller and Wink 1998; Gidding *et al.* 1999; Pasquier and Kavallaris 2008), ajmalicine (A) is used as antihypertensive and serpentine (S) is used in the treatment of anxiety (Hedhili *et al.* 2007). Among these, VB and VC are species specific, no other plant species is known to synthesize VB and VC (van der Heijden *et al.* 2004). *C. roseus* leaves and flowers synthesize and accumulate VB and VC in very low concentrations (Singh *et al.* 2008). Total synthesis of VB and VC is known at the laboratory scale (Yokoshima *et al.* 2002; Kuboyama *et al.* 2004). VB and VC are commercially produced semisynthetically by dimerization of their natural precursors vindoline (V) and catharanthine (C) (Langlois *et al.* 1976; Potier 1980; Ishikawa *et al.* 2008; Tam *et al.* 2010). The TIA V and C are also species specific (van der Heijden *et al.* 2004). These are extracted from dried leaves harvested from *C. roseus* plants (Potier 1980; Kruczynsky and Hill 2001; Ishikawa *et al.* 2008; Tam *et al.* 2010). The ultimate aim of genetical research on *C. roseus* is to develop a biological resource which produces valuable TIA in high yields.

A priori, two approaches are available for reducing the cost of production of precious TIA: (i) breeding of improved

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cultivars of *C. roseus*; and (ii) metabolic engineering of *C. roseus* and construction of TIA^+ heterologous organisms for increasing the yield of desired TIA. Metabolic engineering of the TIA biosynthetic pathway by way of overexpression of specific genes, treatment with hormone(s) and elicitors and precursor feeding has been pursued in cultured cells, tissues and organs and in whole plants of *C. roseus* (Moreno *et al.* 1995; Canel *et al.* 1998; Sevon and Oksman-Caldentey 2002; Whitmer *et al.* 2002, 2003; Peebles *et al.* 2006). Heterologous transgenic cells/organs overexpressing critical steps of TIA pathway have been constructed (McKnight *et al.* 1991; Geerlings *et al.* 1999; Hong *et al.* 2006). However, despite much emphasis on the application of metabolic and genetic engineering for increasing the yield of species-specific TIA of *C. roseus*, potential of these technologies remains to be realized. As yet no biological material which outyields leaves of the existing genotypes of *C. roseus* has been reported (Pasquali *et al.* 2006; Facchini and De Luca 2008; Guirimand *et al.* 2010b). Although considerable progress has been made, yet the constraint has been poor understanding of the genetic control of TIA pathway. The current level of genetic dissection of TIA pathway has indeed shown that it is highly complex. A very large number of genes are involved and many remain to be defined (van der Heijden *et al.* 2004; Rischer *et al.* 2006; Shukla *et al.* 2006; El-Sayed and Verpoorte 2007; Mahroug *et al.* 2007; Murata *et al.* 2008; Ziegler and Facchini 2008; Guirimand *et al.* 2010b; Verma *et al.* 2011). Various known genes of the pathway express in a tissue specific manner and their products function in specific organelles (De Luca and St-Pierre 2000; Irmler *et al.* 2000; Burlat *et al.* 2004; Kutchan 2005; Murata and De Luca 2005; Oudin *et al.* 2007; Mahroug *et al.* 2007; Levac *et al.* 2008; Murata *et al.* 2008; Guirimand *et al.* 2009). The intermediates of the pathway shuttle intracellularly between organelles and intercellularly between tissues (Facchini and De Luca 2008). The cellular sites of synthesis of intermediates are often different from those for synthesis and deposition of the products of pharmaceutical importance (Bird and Facchini 2001; Bock *et al.* 2002; Alcantara *et al.* 2005; Murata and De Luca 2005; Mahroug *et al.* 2006; Samanani *et al.* 2006; Roytrakul and Verpoorte 2007; Hedhili *et al.* 2007; Oudin *et al.* 2007; Ziegler and Facchini 2008; Costa *et al.* 2008; Guirimand *et al.* 2009, 2011a,b). Many steps of TIA pathway are differentially regulated (Ouwerkerk and Memelink 1999; Van der Fits and Memelink 2001; Papon *et al.* 2005; Mahroug *et al.* 2007; Hedhili *et al.* 2007; Campos-Tamayo *et al.* 2008). Until the biotechnological engineering can be accomplished, the use of plant breeding procedures to improve the yield of TIA from *C. roseus* plant organs needs emphasis.

Among the various plant breeding procedures available, the selection breeding involves isolation of individual plants bearing superior phenotype from the segregating population (Lorz and Wenzel 2005). Hybrids between parents possessing desirable features are used to generate segregating populations. The inbred progenies of the plants isolated in early segregating generations are used to obtain pure breeding lines for the improved phenotype(s). The selection breeding brings together genes/alleles favourable for the improved expression of the traits of interest, from diverse parents, into the selected genotype(s). In *C. roseus*, to isolate genotypes abundant in desired TIA, the members of the segregating populations must be organwise extracted for alkaloids and extracts analysed for the quantities of desired TIA. The breeding of medicinal cultivars in *C. roseus* was deterred by the expensive and time consuming nature of TIA extraction and analytical procedures (Facchini and De Luca 2008; Singh *et al.* 2008; Guirimand *et al.* 2011a,b). Since it is known that biosynthesis and accumulation of TIA occurs by multigenic processes (El-Sayed and Verpoorte 2007), the TIA amounts in *C. roseus* plant organs can be treated as quantitative traits (QT). The QT are amenable to marker assisted selection (MAS) (Dekkers and Hospital 2002; Lorz and Wenzel 2005). In MAS, first the DNA markers linked to QT are identified to define QT loci (QTL)/genes/alleles favourable for the expression of trait in desired direction. This is achieved by segregational analysis of DNA markers in a relatively small segregating population which is also phenotyped for trait(s) of interest. Second, for the exercise of actual selection in large segregating population(s), the population is genotyped only with DNA markers already found linked to QT of interest or QTL. Application of this technology in *C. roseus* will allow tracking of QTL that control the TIA yield determining traits in large segregating populations without having actually quantitate them analytically for TIA. The present work is perhaps the first study in the direction of definition and validation of the use of QTL in selection breeding in *C. roseus*.

Here, we have used a population of recombinant inbred lines (RILs) originating from the cross '*lli*' (a medicinal-cum-floricultural line) × 'Delhi Pink' (a floricultural cultivar) which demonstrated segregation for a variety of traits. A part of this population (191 RIL) had been used by us to place 178 DNA markers and one morphological marker on eight linkage groups (LG) of *C. roseus* ($2n = 2x = 16$) (Chaudhary *et al.* 2011). In this work, 197 RIL were evaluated for 30 QT related to the biomass of leaves, stems, roots and whole plant, harvest indices of organs and contents and yields of total and specific TIA from plant organs. The population was genotyped for all the 178 mapped DNA markers. QTL analysis of seven QT (selected on the basis of inter-trait correlation analysis) allowed identification of 20 QTL together with positions of DNA markers linked to them on the genetic map. The efficacy of QTL in the identification of superior genotype(s) was validated. Our work is perhaps the first study of QTL in *C. roseus*.

Materials and methods

Germplasm and population development

The mapping population of RIL was developed from the germplasm '*lli*' and 'Delhi Pink', which had been shown

to be genetically distant by morphological, biochemical and molecular analyses (Mishra *et al.* 2001; Shokeen *et al.* 2007; Singh *et al.* 2008). RIL was developed by single seed descent starting from F_2 generation of the cross *lli* × Delhi Pink. The '*lli*' plants are abiotic stress-tolerant and have altered inflorescence due to a mutation in the *GLYCOPHYTIC SALINITY RESPONSE-8* (*GSR-8*) gene (Pandey-Rai *et al.* 2003; Kumar *et al.* 2007). Unlike LLI (Delhi Pink) plants which bear flowers in pairs in the axil of one of the two leaves per node, the *lli* (leaf-less inflorescence) plants do not possess leaves on the flowering nodes (Kumar *et al.* 2007; Chaudhary *et al.* 2011). The flowers of 'Delhi Pink' have pink coloured petals while '*lli*' flowers bear white petals. A total of 212 RIL were developed. Of these 197 RIL were used in the present study and 191 RIL had been used in the construction of genetic linkage map (Chaudhary *et al.* 2011). A small number of RIL were omitted from consideration for sundry reasons, including poor growth, susceptibility to infection by phytoplasma and segregation for flower colour and *lli*/*LLI* traits.

Experimental design and plant growth measurements

Parents and 197 RIL were evaluated organwise for biomass and TIA from 2007 and 2008. In both years, the field trials were laid in different fields, at the institute's experimental farm in New Delhi (latitude 28°36N; longitude 77°12E; and elevation 216 m). Trials from 2007 to 2008 were performed in fields vacated by garden pea and wheat, respectively. The crop season (summer – monsoon – autumn) in 2008 was relatively warmer and received more monsoon rains than in 2007. Each trial was in randomized block design replicated twice. Each genotype was planted in one row of 1.5 m with five plants per replication (∼66 thousand plants ha^{-1}). The plants were raised according to the already standardized conditions (Kulkarni *et al.* 1999; Mishra *et al.* 2001). Genotypes were sampled replicationwise when their plants had attained an age of about 28 weeks (22 weeks after transplantation of nursery-raised seedlings in field). Three central plants per row had been tagged and scored for flower colour and inflorescence features before they were dug out along with root system. After the washing of roots to remove adhering soil, individual whole plants were placed in paper bags for drying. The plants were dried under circulated air at room temperature in shade and weighed. The leaves (+flowers and fruits), stems and roots separated from the individual dried plants of a genotype from a replication were pooled and weighed to obtain average single plant dry weights of organs. The leaf, stem and root materials were extracted and profiled for estimating the contents of total TIA and specific TIA.

Quantification of TIA

The method described by Singh *et al.* (2004, 2008) with some modifications was used for the extraction of alkaloids from the dry samples of leaves, stems and roots and analysis of extracts by HPLC. Each sample of plant material was extracted and analysed twice. The extraction procedure used gave extracts that were principally TIA. The procedure consisted of suspending 1 g finely powdered material in methanol (3 × 30 mL) overnight followed by concentration of filtrate to dryness under vacuum. To obtain the alkaloid extract, crude extract was sequentially treated as follows: It was defatted with hexane (3 × 10 mL), acidified (15 mL, 3% HCl), made alkaline (pH 8–9) by dropwise addition of liquor ammonia (about 25% NH_3), extracted with chlorophorm (3 × 30 mL), washed with water, dried over sodium sulphate (anhydrous) and concentrated under vacuum. The alkaloid extracts so obtained were stored at 4°C. Extracts redissolved in HPLC grade methanol were used in liquid chromatographic (LC) analysis.

Shimadzu (Kyoto, Japan) LC-10A Top gradient HPLC, equipped with two LC-10ATvp pumps and each of SCL-10A interface module and SIL-10A Dvp autoinjector, was used for the chromatographic separation of alkaloids. A SPD-M10 Avp PDA detector (Shimadzu, Kyoto, Japan) was used to identify peak(s) relating to specific TIA of interest. LC-analysis was performed using a Phenomenex (Torrance, California, USA) Luna C18 (2) (250 × 4.6 mm; 5 μm) reversed-phase column. Acetonitrile and 0.1 M phosphate buffer (pH 4.9) comprised the mobile phase applied as: linear gradient from 20 : 80 (v/v) 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). All injections were of 10 μL and during analysis flow rate of 1.2 mL/min was maintained. Peaks were detected at 220 nm wavelength. External standards guided the identification and quantification of alkaloids. Calibration plots of individual alkaloids were used to estimate per cent contents of TIA of interest in the experimental extracts. The caliberation plots were prepared by diluting the stocks of V, C, VB, VC, A and S maintained in methanol at −20°C. VC, VB and A, and C were purchased from Sigma (USA) and Alexis Corporation (Switzerland), respectively; V and S were gifted by R. Verpoorte of the Institute of Biology, Leiden University, The Netherlands.

Description, nomenclature and abbreviations of phenotypic traits

The single plant dry weights (g) of the organs obtained as described above were called root dry weight (RDW), stem dry weight (SDW) and leaf dry weight (LDW). The whole plant dry weight was called as total dry weight (TDW). The harvest indices (%) of the plant organs calculated as dry weight of organ (roots, stems or leaves) divided by the total dry weight × 100 were called harvest index root (HIR), harvest index stem (HIS) and harvest index leaf (HIL). Total contents of alkaloids present in organs (expressed as % of dry weight of organ) were called as total alkaloid content in leaves (TL), total alkaloid content in stems (TS) and total alkaloid content in roots (TR). The organwise total alkaloid yield (mg) was calculated as dry weight of organ ×

total alkaloid content divided by 100; the values were called total alkaloid yield from leaves (TAYL), total alkaloid yield from roots (TAYR) and total alkaloid yield from stems (TAYS). The whole plant total alkaloid yield was calculated as TAYL+TAYR+TAYS and abbreviated as TAYW. The organwise content (%) of a TIA was estimated as weight of alkaloid extracted from organ divided by dry weight of organ × 100; the values were called per cent serpentine in leaves (SL), per cent serpentine in roots (SR), per cent ajmalicine in roots (AR), per cent vindoline in leaves (VL), per cent vinblastine (+ vincristine) in leaves (VBL), per cent catharanthine in leaves (CL) and per cent catharanthine in roots (CR). The yield of a TIA from an organ (mg) was calculated using the formula: per cent content of alkaloid in organ × dry weight of organ divided by 100. The organwise alkaloid yields were called yield of serpentine from leaves (SLY), yield of serpentine from roots (SRY), yield of serpentine from leaves and roots (SLY+SRY=SLRY), yield of ajmalicine from roots (ARY), yield of vindoline from leaves (VLY), yield of vinblastine (+vincristine) from leaves (VBLY), yield of catharanthine from leaves (CLY), yield of catharanthine from roots (CRY) and yield of catharanthine from leaves and roots (CLY+CRY=CKRY).

Statistical analysis

Statistical analyses were performed by using various modules of the software SPSS 16.0 (SPSS, Chicago, USA). Histograms and ANOVA were used to describe the variation in phenotypic traits, and latter in terms of genotypic, seasonal and season × genotype interaction components. Pearson's phenotypic correlations (r) were estimated to examine associations between traits. The broad sense heritability (H^2) was estimated as % proportion of genotypic variation divided by phenotypic variation. The r and H^2 were estimated from the combined data of seasons 2007 and 2008.

Genetic linkage map and QTL analysis

Construction of a genetic linkage map of *C. roseus* ($2n = 2x = 16$) has been described previously (Chaudhary *et al.* 2011). The map originally contained 172 DNA markers and one morphological marker. To this six DNA markers have since been added in the course of the present work. The available framework map is based on segregational analysis of a very large number of markers in 191 of the 197 RIL used for the phenotypic analysis in the present study. The mapped DNA markers include 134 anonymous markers (84 RAPD, 11 ISSR and 39 SSR) and 43 coding region markers (eight microRNAs and 36 EST-SSR). With 10 cM as the average distance between adjacent markers, the map of eight LG measures 1786.5 cM. The genetic distance of the eight LG varies from 89.9 cM (12 markers) to 320.1 cM (45 markers). All marker loci are uniquely located on the map. Although as yet far from saturated, the available map is robust since it is exclusively based on markers that demonstrated Mendelian segregation in the mapping population of 191 RIL. All the markers placed on the framework map were used for QTL analysis (figure 1). The Window QTL Cartographer v2.5 (http://statgen.ncsu.edu/qtlcart/WQTL Cart.htm) (Wang *et al.* 2011b) was used to identify and locate QTL linked to molecular markers. First, single marker regression analysis (SMA) was deployed to identify the significant variation associated with the DNA markers. Second, the QTL present were identified and confirmed by simple interval mapping and composite interval mapping (CIM). The CIM Model 6 was run for each trait yearwise and on the combined data of 2007 and 2008 by performing forward and backward stepwise regression, with window size of 10 cM and 2 cM walking speed along the LG. The genomewide traitwise LOD score thresholds for QTL detection were determined by performing a 1000 permutations test at significance level of $P \leq 0.05$ (Churchill and Doerge 1994; Deorge and Churchill 1996). For each QTL, its location, LOD score, and per cent phenotypic variation explained (R^2) were given by the software of CIM model. The QTL were named as follows: q the abbreviation for QTL locus, abbreviated QT name and then a number indicating the serial order of QTL for the specific QT. For example qTL1 means QTL number 1 for the trait total alkaloid content in leaves (%) and qTL2 means QTL number 2 for the QT TL.

Results

Variation for TIA yield related traits in RIL population

Tables 1 and 2 present the results of field trials conducted in 2007 and 2008 seasons, in terms of phenotypic expression of 30 alkaloid-yield-related traits in parents and RIL (table 1) and analysis of variance and heritability estimates for all the traits (table 2). Table 1 gives traitwise (i) mean values of parents and significance of difference between them; (ii) means and ranges for all the 197 RIL and separately for the RIL bearing lli and LLI phenotypes; (iii) correlation coefficient and significance of difference between mean values of 197 RIL for 2007 and 2008; and (iv) significance of difference between mean values of *lli* and *LLI* RIL. Frequency distributions of RIL population for the various traits are shown in figure 2. The RIL mean values for seasons 2007 and 2008 were significantly different for only nine of the 30 traits (SDW, TDW, HIR, HIS, HIL, TL, TAYL, CR and CRY), and coefficients of correlation between expression values in 2007 and 2008 seasons were positive and highly significant for all the 30 traits (table 1). These observations indicated broad agreement between the expressions of traits in the two seasons. The mean values of 197 member RIL population were intermediate of the parental values for 25 of the 30 traits, higher than parental values for four traits (HIR, HIL, VBL and CL) and lower than parental values for only one trait (TR) (table 1). RIL population demonstrated continuous variation and transgressive segregation in both the directions for all the traits (figure 2; table 1). The ANOVA

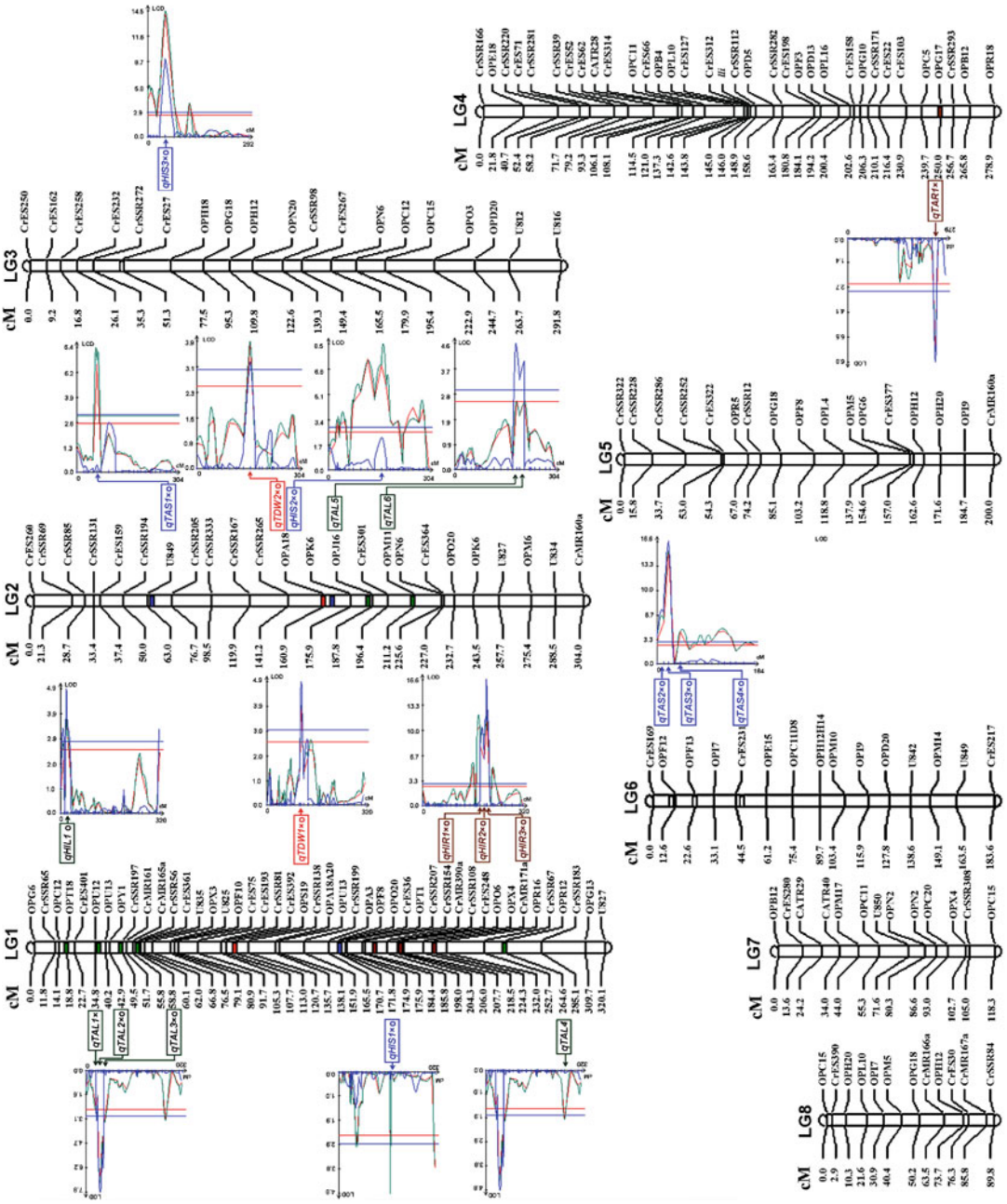

Figure 1. The *lli* × Delhi Pink RIL population linkage map of *Catharanthus roseus* of 178 DNA markers and one morphological marker placed on eight linkage groups showing the locations of 20 QTL for the seven traits analysed during 2007 and 2008. All QTL shown were detected by single marker (regression) analysis (SMA), simple interval mapping (SIM) and/or composite interval mapping (CIM) using data for 2007 and 2008 trials. QTL Cartographer plots using SMA as red peak, SIM as green peak and CIM as blue peak for QTL identified are shown near the closest linked marker. The name and the peak region for each QTL on the respective LG has been differentially indicated by a colour: QTL for leaf traits by green colour, stem traits by blue colour, root traits by brown and red colour for total dry matter trait, respectively. QTL was designated using q for QTL, followed by an abbreviation of the trait name in upper case and a number identifying QTL number for the trait. The QTL name is followed by x and/or o which indicate(s) the significance in 2007 and 2008 trials, respectively.

Table 1. Expression levels of traits related to the yield of terpenoid indole alkaloids in individual organs and whole plants of *lli* and Delhi Pink, the parents, and a recombinant inbred line mapping population derived from their cross, quantitatively evaluated in annual field trials over two years (seasons) in *Catharanthus roseus*.

					Variation in RIL population					
		Parental line			Entire population			Subpopulation of		
Trait	Year	*lli*	Delhi Pink	t_2 For the difference between the mean of two years of the parents	Mean (range)	t_{392} For the difference between the values of 2 years	Correlation coefficient between the values of 2 years	*lli* phenotype	*LLI* phenotype	t_{195} For the difference between *LLI* and *lli*
Root dry weight (RDW) (g)	2007	5.00 ± 0	7.75 ± 0.25	10.00** (0.01)	6.87 ± 0.23 (1.00–19.00)	1.02 (0.31)	0.89 (0.000)	4.95 ± 0.22 (1.50–10.50)	8.55 ± 0.31 (1.00–19.00)	9.22**** (0.0001)
	2008	5.00 ± 0	7.25 ± 0.25		6.54 ± 0.21 (1.00–18.00)			4.72 ± 0.20 (1.00–10.00)	8.14 ± 0.27 (1.00–18.00)	9.86**** (0.0001)
Stem dry weight (SDW) (g)	2007	51.50 ± 3.00	99.00 ± 9.50	3.00 (0.10)	58.62 ± 1.74 (10.50–116.50)	2.89** (0.0041)	0.81 (0.000)	45.71 ± 2.20 (10.50–109.00)	69.94 ± 2.08 (17.00–116.50)	7.99**** (0.0001)
	2008	41.25 ± 1.25	75.00 ± 18.00		51.72 ± 1.64 (4.50–116.00)			38.09 ± 1.73 (10.50–93.00)	63.66 ± 2.08 (4.50–116.00)	9.31**** (0.0001)
Leaf dry weight (LDW) (g)	2007	30.25 ± 0.25	56.00 ± 4.50	3.04 (0.09)	38.20 ± 1.27 (8.00–95.00)	0.80 (0.43)	0.81 (0.000)	28.21 ± 1.39 (8.00–69.00)	46.95 ± 1.62 (10.50–95.00)	8.66**** (0.0001)
	2008	26.50 ± 1.00	43.50 ± 9.50		36.74 ± 1.31 (2.50–93.50)			24.61 ± 1.15 (8.00–71.50)	47.37 ± 1.65 (2.50–93.50)	11.0**** (0.0001)
Total dry weight (TDW) (g)	2007	86.75 ± 3.25	163.25 ± 14.25	3.08 (0.09)	103.69 ± 3.13 (20.00–224.00)	1.98* (0.05)	0.83 (0.000)	78.86 ± 3.68 (20.00–181.50)	125.44 ± 3.81 (28.50–224.00)	8.73**** (0.0001)
	2008	72.75 ± 0.25	125.75 ± 27.75		95.00 ± 3.07 (8.00–215.50)			67.41 ± 2.95 (19.50–169.00)	119.18 ± 3.82 (8.00–215.50)	10.51**** (0.0001)
Harvest index root (HIR) (%)	2007	5.78 ± 0.22	4.79 ± 0.29	1.38 (0.30)	6.80 ± 0.15 (2.34–15.82)	2.01* (0.04)	0.54 (0.000)	6.71 ± 0.23 (2.70–15.82)	6.87 ± 0.18 (2.34–13.27)	0.57 (0.57)
	2008	7.01 ± 0.03	6.02 ± 1.13		7.23 ± 0.15 (2.38–15.83)			7.44 ± 0.25 (2.38–15.38)	7.04 ± 0.17 (2.38–13.62)	1.44 (0.15)
Harvest index stem (HIS) (%)	2007	59.38 ± 1.22	61.01 ± 0.41	2.65 (0.12)	56.64 ± 0.31 (47.05–71.43)	4.34**** (0.0001)	0.55 (0.000)	35.78 ± 0.41 (47.05–68.59)	55.95 ± 0.38 (47.12–71.43)	2.36* (0.02)
	2008	56.57 ± 1.53	59.41 ± 1.24		54.69 ± 0.32 (44.02–69.27)			56.03 ± 0.48 (45.55–67.15)	53.51 ± 0.40 (44.02–69.27)	4.05**** (0.0001)
Harvest index leaf (HIL) (%)	2007	34.84 ± 1.00	34.20 ± 0.13	4.09* (0.05)	36.57 ± 0.28 (24.15–46.77)	3.57*** (0.0004)	0.45 (0.000)	57.42 ± 0.05 (24.86–44.58)	37.17 ± 0.39 (24.15–46.77)	2.31* (0.02)
	2008	36.42 ± 1.56	34.58 ± 0.11		38.08 ± 0.31 (21.89–48.33)			36.53 ± 0.44 (25.50–45.05)	39.44 ± 0.41 (21.89–48.33)	4.87**** (0.0001)
Total alkaloid content in leaves (TL) (%)	2007	1.35 ± 0.06	1.49 ± 0.19	0.48 (0.68)	1.47 ± 0.02 (0.92–2.12)	4.38**** (0.0001)	0.71 (0.000)	1.42 ± 0.02 (1.09–2.00)	1.51 ± 0.03 (0.92–2.12)	2.68** (0.01)
	2008	1.26 ± 0.03	1.35 ± 0.29		1.36 ± 0.02 (0.65–2.11)			1.31 ± 0.02 (0.78–1.74)	1.41 ± 0.03 (0.65–2.11)	2.77** (0.01)
Total alkaloid content in roots (TR) (%)	2007	4.02 ± 0	4.22 ± 0.36	4.28* (0.05)	3.29 ± 0.07 (1.18–5.58)	2.59 (0.01)	0.31 (0.000)	3.42 ± 0.09 (1.28–5.19)	3.18 ± 0.10 (1.18–5.58)	1.75 (0.08)
	2008	4.02 ± 0	4.54 ± 0.52		3.51 ± 0.05 (1.64–5.50)			3.64 ± 0.06 (2.10–5.45)	3.39 ± 0.07 (1.64–5.50)	2.65** (0.01)

Table 1 (*contd*).

Trait	Year	Parental line			Variation in RIL population					
					Entire population			Subpopulation of		
		lli	Delhi Pink	t_2 For the difference between the mean of two years of the parents	Mean (range)	t_{392} For the difference between the values of 2 years	Correlation coefficient between the values of 2 years	*lli* phenotype	*LLI* phenotype	t_{195} For the difference between *LLI* and *lli*
Total alkaloid content in stems (TS) (%)	2007	1.73 ± 0	1.29 ± 0.02	6.57* (0.02)	1.51 ± 0.03 (0.62–3.11)	1.48 (0.14)	0.79 (0.000)	1.50 ± 0.04 (0.74–2.64)	1.52 ± 0.04 (0.62–3.11)	0.39 (0.69)
	2008	1.68 ± 0.01	1.29 ± 0.11		1.59 ± 0.04 (0.56–4.32)			1.58 ± 0.06 (0.56–4.32)	1.59 ± 0.06 (0.65–4.14)	0.06 (096)
Total alkaloid yield from leaves (TAYL)	2007	4.04 ± 0.11	8.44 ± 1.73	1.69 (0.23)	5.67 ± 0.20 (1.07–15.60)	2.13* (0.04)	0.81 (0.000)	4.05 ± 0.22 (1.07–10.17)	7.09 ± 0.26 (1.15–15.60)	8.68**** (0.0001)
(× 10^2 mg)	2008	3.33 ± 0.03	6.08 ± 2.50		5.06 ± 0.20 (0.38–14.37)			3.21 ± 0.15 (0.96–8.58)	6.67 ± 0.27 (0.38–14.37)	10.71**** (0.0001)
Total alkaloid yield from roots (TAYR)	2007	2.01 ± 0	3.27 ± 0.38	20.82** (0.002)	2.23 ± 0.09 (0.24–7.56)	0.36 (0.72)	0.73 (0.000)	1.67 ± 0.09 (0.29–4.72)	2.71 ± 0.13 (0.24–7.56)	6.30**** (0.0001)
(× 10^2 mg)	2008	2.01 ± 0	3.28 ± 0.26		2.27 ± 078 (0.30–6.27)			1.72 ± 0.08 (0.35–3.82)	2.75 ± 0.11 (1.30–6.27)	7.40**** (0.0001)
Total alkaloid yield from stems (TAYS)	2007	8.91 ± 0.55	12.90 ± 1.00	2.60 (0.12)	8.96 ± 0.33 (1.27–24.77)	1.24 (0.22)	0.81 (0.000)	7.00 ± 0.43 (1.27–21.07)	10.67 ± 0.44 (1.43–24.77)	5.92**** (0.0001)
(× 10^2 mg)	2008	6.85 ± 0.18	9.46 ± 1.50		8.33 ± 0.38 (1.05–36.23)			6.13 ± 0.40 (1.05–21.31)	10.26 ± 0.55 (1.22–36.23)	5.92**** (0.0001)
Total alkaloid yield from whole plant	2007	14.97 ± 0.44	24.61 ± 3.12	2.37 (0.14)	16.85 ± 0.56 (3.47–39.49)	1.48 (0.14)	0.82 (0.000)	12.73 ± 0.66 (3.50–32.70)	20.47 ± 0.71 (3.47–39.49)	7.92**** (0.0001)
(TAYW) (× 10^2 mg)	2008	12.20 ± 0.16	18.82 ± 3.74		15.65 ± 0.59 (2.10–50.28)			11.06 ± 0.58 (2.66–28.72)	19.68 ± 0.80 (2.10–50.28)	8.51**** (0.0001)
% Serpentine in leaves (SL)	2007	2.04 ± 0.35	0.74 ± 0.04	1.95 (0.19)	1.46 ± 0.05 (0.17–3.70)	0.86 (0.39)	0.81 (0.000)	1.55 ± 0.07 (0.40–3.32)	1.39 ± 0.07 (0.17–3.70)	1.56 (0.12)
(× 10^{-2})	2008	2.12 ± 0.90	0.95 ± 0.28		1.40 ± 0.05 (0.14–3.15)			1.50 ± 0.06 (0.44–3.03)	1.31 ± 0.06 (0.14–3.15)	2.09* (0.04)
% Serpentine in roots (SR)	2007	36.40 ± 2.60	35.83 ± 0.02	3.34 (0.08)	35.10 ± 0.51 (11.60–59.85)	0.46 (0.64)	0.31 (0.000)	34.47 ± 0.68 (11.60–59.85)	35.64 ± 0.76 (11.60–59.85)	1.34 (0.26)
(× 10^{-2})	2008	34.50 ± 1.98	32.53 ± 1.98		34.78 ± 0.43 (19.50–52.05)			34.20 ± 0.52 (19.50–48.40)	35.29 ± 0.67 (20.55–52.05)	1.25 (0.20)
Yield of serpentine from leaves	2007	6.04 ± 0.85	4.11 ± 0.21	0.77 (0.52)	5.53 ± 0.27 (0.60–22.77)	1.27 (0.20)	0.77 (0.000)	4.36 ± 0.29 (0.66–16.01)	6.55 ± 0.42 (0.60–22.77)	4.18**** (0.0001)
(SLY) (mg)	2008	5.44 ± 02.08	4.46 ± 2.17		5.05 ± 0.25 (0.44–22.70)			3.80 ± 0.28 (0.70–17.49)	6.15 ± 0.37 (0.44–22.70)	4.90**** (0.0001)
Yield of serpentine from roots	2007	18.20 ± 1.30	27.65 ± 0.93	11.14** (0.008)	24.25 ± 0.91 (1.16–67.04)	0.99 (0.33)	0.82 (0.000)	16.96 ± 0.81 (5.65–41.45)	30.63 ± 1.27 (1.16–67.04)	8.80**** (0.0001)
(SRY) (mg)	2008	17.26 ± 0.99	23.71 ± 2.32		23.02 ± 0.85 (2.28–72.49)			16.15 ± 0.71 (3.66–35.70)	29.04 ± 1.18 (2.28–72.49)	9.04**** (0.0001)

Table 1 (*contd*).

Trait	Year	Parental line			Variation in RIL population					
					Entire population			Subpopulation of		
		lli	Delhi Pink	t_2 For the difference between the mean of two years of the parents	Mean (range)	t_{392} For the difference between the values of 2 years	Correlation coefficient between the values of 2 years	*lli* phenotype	*LLI* phenotype	t_{195} For the difference between *LLI* and *lli*
SLY+SRY (=SLRY) (mg)	2007	24.24 ± 0.45	31.76 ± 0.72	2.83 (0.11)	29.77 ± 1.05 (2.03–30.60)	1.18 (0.24)	0.85 (0.000)	21.31 ± 0.96 (7.02–47.79)	37.18 ± 1.43 (2.03–30.60)	8.96**** (0.0001)
	2008	22.70 ± 3.06	28.17 ± 4.49		28.07 ± 1.00 (3.07–84.59)			19.94 ± 0.86 (4.36–42.80)	35.19 ± 1.39 (3.07–84.59)	9.05**** (0.0001)
% Ajmalicine in roots (AR) ($\times 10^{-2}$)	2007	1.58 ± 0.13	2.25 ± 0.40	4.92* (0.04)	2.19–0.08 (0.15–8.15)	0.29 (0.77)	0.49 (0.000)	2.07 ± 0.10 (0.20–4.25)	2.30 ± 0.12 (0.15–8.15)	1.49 (0.14)
	2008	2.18 ± 0.08	2.05 ± 0.50		2.22 ± 0.07 (0.20–8.90)			2.15 ± 0.08 (0.20–4.05)	2.29 ± 0.11 (0.55–8.90)	1.00 (0.30)
Yield of ajmalicine from roots (ARY) (mg)	2007	0.79 ± 0.06	1.72 ± 0.24	7.90* (0.02)	1.52 ± 0.08 (0.03–8.15)	0.12 (0.90)	0.70 (0.000)	1.02 ± 0.07 (0.05–3.90)	1.96 ± 0.12 (0.03–8.15)	6.46**** (0.0001)
	2008	1.09 ± 0.04	1.49 ± 0.41		1.51 ± 0.08 (0.03–8.90)			1.05 ± 0.06 (0.03–3.18)	1.91 ± 0.13 (0.13–8.90)	5.80**** (0.0001)
% Vindoline in leaves (VL) ($\times 10^{-2}$)	2007	1.58 ± 0.19	2.61 ± 0.83	0.15 (2.32)	2.19 ± 0.06 (0.46–5.74)	0.36 (0.72)	0.85 (0.000)	1.96 ± 0.06 (0.46–3.92)	2.40 ± 0.09 (0.46–5.74)	3.98**** (0.0001)
	2008	1.64 ± 0.17	1.98 ± 0.36		2.16 ± 0.05 (0.68–6.46)			1.90 ± 0.05 (0.76–3.22)	2.39 ± 0.09 (0.68–6.46)	4.75**** (0.0001)
Yield of vindoline from leaves (VLY) (mg)	2007	4.75 ± 0.51	15.00 ± 5.86	2.31 (0.15)	8.67 ± 0.41 (0.63–35.62)	0.61 (0.54)	0.78 (0.000)	5.69 ± 0.37 (0.63–20.86)	11.28 ± 0.59 (1.76–35.62)	7.80**** (0.0001)
	2008	4.29 ± 0.22	8.07 ± 0.17		8.30 ± 0.44 (0.63–43.40)			4.62 ± 0.24 (1.20–12.22)	11.53 ± 0.63 (0.63–43.40)	9.5**** (0.0001)
% Vinblastine + vincristine in leaves (VB+VC=VBL) ($\times 10^{-2}$)	2007	0.44 ± 0.05	0.56 ± 0.14	0.71 (0.55)	0.66 ± 0.02 (0.16–1.52)	0.06 (0.954)	0.78 (0.000)	0.60 ± 0.02 (0.18–1.45)	0.71 ± 0.03 (0.16–1.52)	3.08*** (0.003)
	2008	0.45 ± 0.09	0.59 ± 0.19		0.66 ± 0.02 (0.13–1.55)			0.59 ± 0.02 (0.22–1.35)	0.71 ± 0.03 (0.13–1.55)	3.34*** (0.001)
Yield of VB+VC (VBLY) (mg)	2007	1.32 ± 0.15	3.19 ± 1.02	1.40 (0.30)	2.56 ± 0.12 (0.27–11.19)	0.39 (0.69)	0.827 (0.000)	1.69 ± 0.10 (0.27–4.83)	3.32 ± 0.19 (0.67–11.19)	7.33**** (0.0001)
	2008	1.18 ± 0.20	2.71 ± 1.38		2.49 ± 0.14 (0.21–10.63)			1.45 ± 0.09 (0.32–5.58)	3.39 ± 0.20 (0.21–10.63)	8.27**** (0.0001)
% Catharanthine in leaves (CL) ($\times 10^{-2}$)	2007	0.29 ± 0.01	0.28 ± 0.01	0.93 (0.45)	0.34 ± 0.01 (0.05–1.42)	0.54 (0.59)	0.78 (0.000)	0.31 ± 0.02 (0.05–1.01)	0.37 ± 0.02 (0.08–1.42)	2.16* (0.03)
	2008	0.27 ± 0.01	0.27 ± 0.03		0.33 ± 0.01 (0.05–1.17)			0.31 ± 0.02 (0.05–1.14)	0.35 ± 0.02 (0.09–1.17)	1.91 (0.06)
% Catharanthine in roots (CR) ($\times 10^{-2}$)	2007	3.18 ± 0.18	3.85 ± 1.10	0.75 (0.53)	3.83 ± 0.09 (0.55–8.60)	2.11* (0.04)	0.37 (0.000)	3.44 ± 0.10 (1.50–7.80)	4.17 ± 0.14 (0.55–8.60)	4.18**** (0.0001)
	2008	3.08 ± 0.28	3.15 ± 0.10		3.59 ± 0.07 (1.25–8.80)			3.37 ± 0.08 (1.25–7.05)	3.78 ± 0.11 (1.65–8.80)	2.86** (0.005)

Table 1 (*contd*).

Trait	Year	Parental line			Variation in RIL population					
					Entire population			Subpopulation of		
		lli	Delhi Pink	t_2 For the difference between the mean of two years of the parents	Mean (range)	t_{392} For the difference between the values of 2 years	Correlation coefficient between the values of 2 years	*lli* phenotype	*LLI* phenotype	t_{195} For the difference between *LLI* and *lli*
Yield of catharanthine from leaves (CLY) (mg)	2007	0.88 ± 0.01	1.56 ± 0.17	3.61 (0.07)	1.34 ± 0.07 (0.12–5.46)	0.85 (0.39)	0.75 (0.000)	0.88 ± 0.06 (0.12–3.30)	1.74 ± 0.11 (0.19–5.46)	6.72**** (0.0001)
	2008	0.70 ± 0.10	1.13 ± 0.12		1.25 ± 0.07 (0.09–6.20)			0.76 ± 0.05 (0.09–3.08)	1.69 ± 0.10 (0.09–6.20)	7.91**** (0.0001)
Yield of catharanthine from roots (CRY) (mg)	2007	1.59 ± 0.09	2.93 ± 0.73	3.66 (0.07)	2.77 ± 0.13 (0.06–10.57)	1.95* (0.05)	0.75 (0.000)	1.72 ± 0.09 (0.41–4.61)	3.69 ± 0.20 (0.06–10.57)	8.63**** (0.0001)
	2008	1.54 ± 0.14	2.29 ± 0.16		2.43 ± 0.11 (0.26–10.38)			1.60 ± 0.08 (0.34–4.24)	3.16 ± 0.17 (0.26–10.38)	8.03**** (0.0001)
CLY+CRY (=CLRY) (mg)	2007	2.46 ± 0.08	4.49 ± 0.56	11.47** (0.008)	4.11 ± 0.17 (0.24–12.14)	1.83 (0.07)	0.78 (0.000)	2.60 ± 0.13 (0.80–5.72)	5.43 ± 0.23 (0.24–12.14)	10.32**** (0.0001)
	2008	2.24 ± 0.04	3.42 ± 0.28		3.68 ± 0.16 (0.41–12.81)			2.35 ± 0.11 (0.82–5.66)	4.85 ± 0.22 (0.41–12.81)	9.74**** (0.0001)

*, **, ***, **** Significant at 5%, 1%, 0.1% and 0.01% levels, respectively; *P* values are given in parenthesis.

Table 2. Significance of variance (mean square) due to genotypes, seasons (years) and seasons × genotypes (f) and broad sense heritability (h^2) estimates for the traits related to yield of terpenoid indole alkaloids from organs and whole plant in the trial of 197 recombinant inbred line developed from the cross *lli* × Delhi Pink in *Catharanthus roseus*.

	Analysis of variance components in RIL (n = 197)			
	Genotype	Season	Genotype × season	Heritability
Trait	$f_{196:393}$	$f_{1:393}$	$f_{196:393}$	h^2 (%)
RDW	52.04***	28.93***	3.13***	56.97
SDW	36.52***	168.23***	3.86***	17.07
LDW	36.93***	12.99***	3.80***	67.48
TDW	43.63***	93.40***	3.95*	30.30
HIR	9.54***	27.14***	2.79***	23.29
HIS	6.74***	82.09***	1.99***	7.06
HIL	5.61***	47.89***	1.90***	9.21
TL	5.69***	63.73***	0.97	5.35
TR	8.99***	46.71***	4.96***	12.99
TS	6.51***	8.11***	0.93	36.43
TAYL	23.31***	59.12***	2.45***	23.60
TAYR	23.87***	1.74	3.92***	52.91
TAYS	11.74***	9.96***	1.26	37.93
TAYW	20.80***	24.99***	2.12***	40.68
SL	8.08***	3.34	0.90	35.82
SR	5.63***	0.93	3.01***	48.70
SLY	11.22***	10.30***	1.47***	33.44
SRY	25.18***	13.45***	2.51***	50.94
SLRY	26.49***	19.80***	2.17***	52.11
AR	11.08***	0.62	3.80***	62.75
ARY	20.69***	0.17	3.66***	64.14
VL	9.89***	0.68	0.80	78.69
VLY	22.99***	4.62*	2.06***	75.07
VBL	10.03***	0.02	1.22*	39.36
VBLY	17.65***	1.50	1.71***	56.80
CL	9.54***	1.57	1.20	64.78
CR	6.93***	22.59***	3.22***	13.94
CLY	10.14***	4.15*	1.45**	60.31
CRY	23.06***	52.54***	3.54***	22.18
CLRY	22.62***	42.74***	2.80***	26.74

***, **, * Significant at $P < 0.0001$, $P < 0.001$ and $P < 0.05$ probability levels, respectively.
The description of traits is given in table 1.

analysis showed that the variation due to genotypes was highly significant for all the traits (table 2). These characteristics of variation in RIL showed that all the traits were polygenetically determined and that the population was indeed suitable for the purposes of QTL mapping.

The expression levels were higher in *LLI* RIL than in *lli* RIL for 24 of the 30 traits and equal among *LLI* and *lli* RIL for three traits (HIR, TS and SR), irrespective of seasons. However, seasonal differences were noted to have differentially affected expression levels in *LLI* and *lli* RIL for three traits (HIL, TR and SL).

Among the parents of RIL population, 'Delhi Pink' expression levels were higher than those of '*lli*' for a large majority of traits (23/30). The expression levels of the parents were nearly equal for three traits (HIL, SR and CL). Four traits were expressed at higher level in '*lli*' plants than in 'Delhi Pink' plants (HIR, TS, SL and SLY). However, the differences in expression between 'Delhi Pink' and '*lli*' were significant for only eight traits (RDW, TR, TS, TAYR, SRY, AR, ARY and CLRY).

Biomass accumulation in LLI and lli plants

Twenty-eight weeks old plants of parental and RIL genotypes accumulated biomass in the range of 8 to 224 g. On an average basis the *lli* plants accumulated about 1.74 fold more biomass than *LLI* plants (76.4 g versus 133.2 g). The major organs of the entire population accumulated biomass in the proportion root: leaf: stem :: 1 : 5.9 : 9.4; the corresponding proportions in the *lli* and *LLI* genotypes were 1.0 : 5.6 : 9.0 and 1.0 : 6.1 : 9.7, respectively. Between the *LLI* and *lli* genotypes, *LLI* plants accumulated 1.61, 1.77 and 1.73 times more biomass than *lli* plants in their root, leaf and stem organs, respectively. Despite the inflorescence being leafless in *lli*, they accumulated biomass in their leaves in approximately the same proportion as in *LLI* plants.

Heritability of the traits

There were wide differences in the heritability of traits. The heritability ranged from 5.4% for TL to 78.7% for VL. The traits could be arranged in the following groups in terms of their heritability: ≤25% (SDW, HIR, HIS, HIL, TL, TR, TAYL, CR and CRY), 26–50% (TDW, TS, TAYS, TAYW, SL, SR, SLY, VBL and CLRY), and >50% (RDW, LDW, TAYR, SRY, SLRY, AR, ARY, VL, VLY, VBLY, CL and CLY).

Organwise distribution and yield of alkaloids

Among the organs, roots were richest in the content of all alkaloids (total TIA) and yield of TIA was highest from stems. Considering the entire experimental population(s), the organs accumulated TIA (% total TIA content) in the proportion TL : TR : TS :: 1.00 : 2.57 : 1.14; and the organwise TIA yields were in the proportion TAYL : TAYR : TAYS :: 1.00 : 0.44 : 1.64. The average alkaloid yield from a plant was 1.653 g. The per cent content of pharmaceutically important TIA in roots (S, A and C) and leaves (S, C, V and VBL) was 0.406 and 0.044, respectively, nine fold more in roots than in leaves. It was 0.402 and 0.408 in the roots of *lli* and *LLI* plants, respectively, and 0.044 and 0.042 in the leaves of *lli* and *LLI* plants, respectively. In the roots the proportion AR : SR : CR was 1.00 : 1.67 : 16.23 and in the leaves the proportion CL : VBL : VL : SL was 1.00 : 2.03 : 6.61 : 4.64. Per cent contentwise, C in roots (CR) was about 11.6 fold more than in leaves (CL). Likewise, S content of roots (SR) was 24.2 fold more than that in leaves (SL). In roots, the content of S (SR) was 16.2 fold more than that of A (AR). The proportion of the % content of V, C and VBL in leaves was VL : CL : VBL :: 1.00 : 0.15 : 0.30. The yields of S, C, VBL, V and A from leaves and roots per plant were respectively

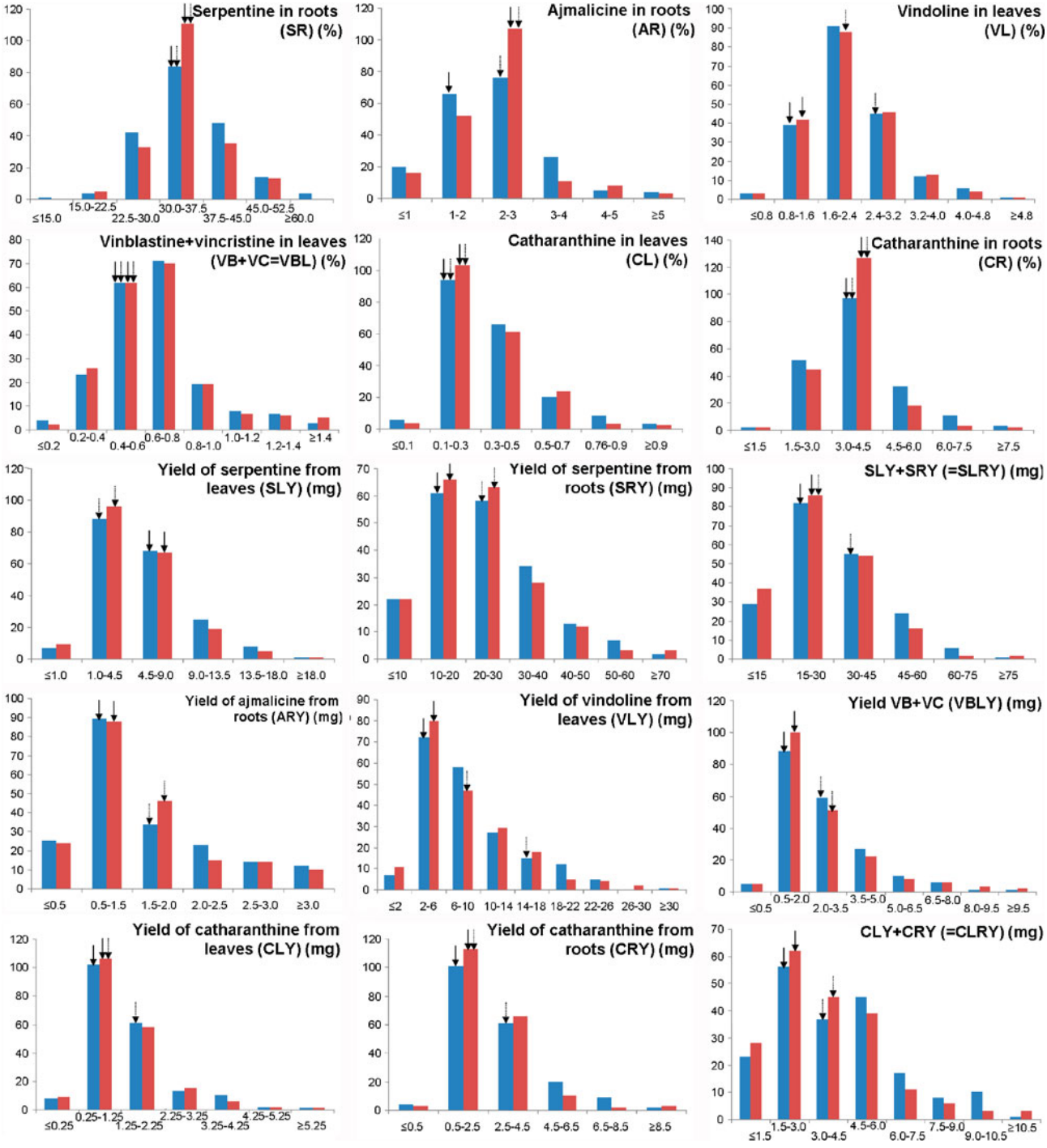

Figure 2 continues in next page.

25.5 (SLRY), 3.7 (CLRY), 2.4 (VBLY), 2.1 (VLY) and 1.6 (ARY) mg. The *LLI* plants gave higher yields of all the above pharmaceutical alkaloids as compared to *lli* plants.

Correlations between traits in parents and RIL population

The relationships between traits were tested pairwise by estimation of Pearson's coefficients of correlation. The *r* values for associations between different pairs of traits are presented in the tables 3–7. It will be seen from table 3 that correlations between the whole plant biomass (dry weight; TDW) and biomass (dry weight) of the component organs, leaves (LDW), stems (SDW) and roots (RDW) were all positive and significant. The correlation coefficients were positive and significant between harvest index of leaf (HIL) and LDW and harvest index of root (HIR) and RDW. The harvest indices for the organs (HIL, HIS and HIR) were

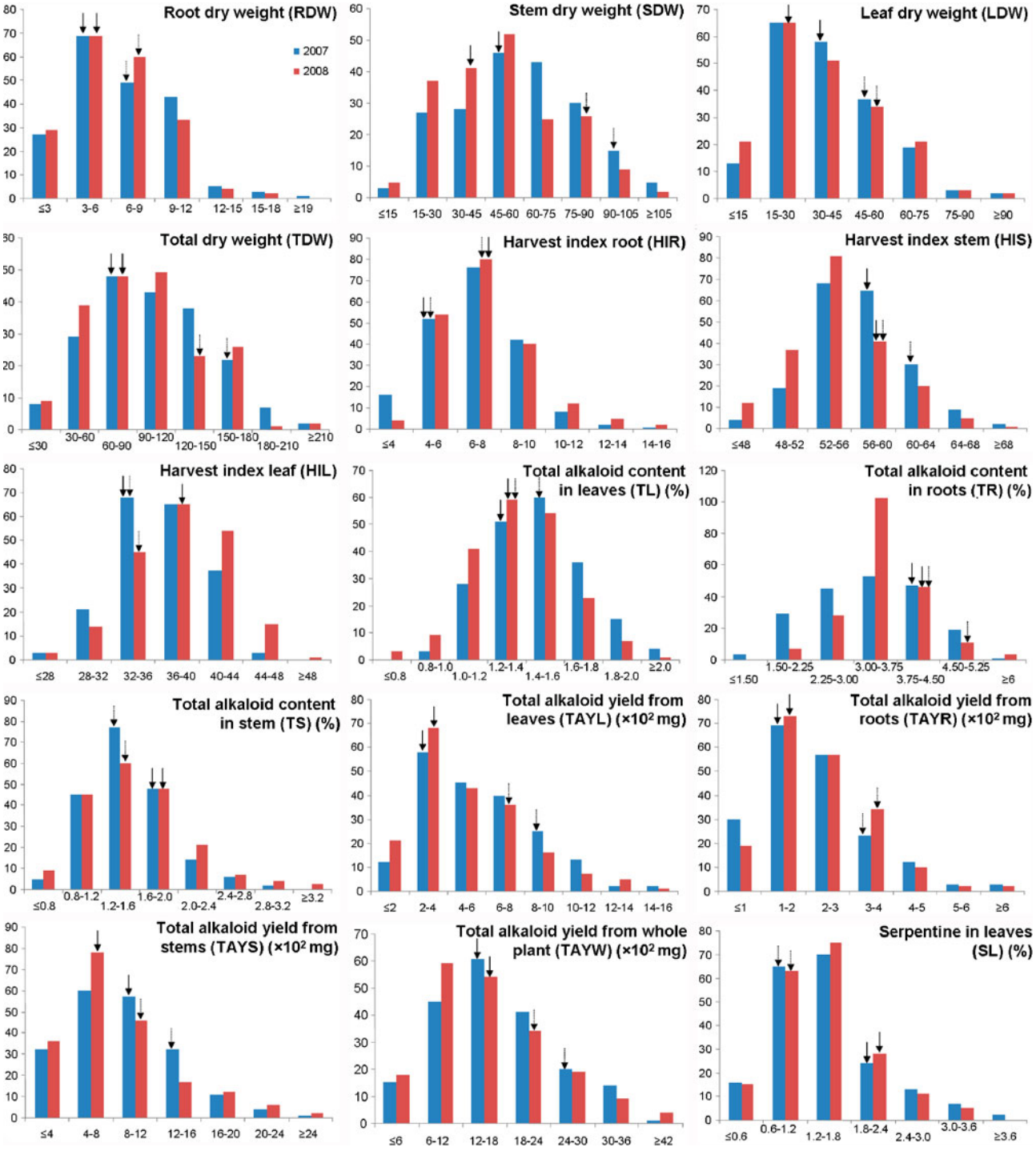

Figure 2. Frequency distributions of 30 terpenoid indole alkaloid (TIA) yield-related traits, measured during 2007 and 2008 in field trials of 197 recombinant inbred lines (RIL) derived from the cross *lli* × Delhi Pink. The blue and red bars relate to the data of 2007 and 2008 seasons respectively. The solid and dashed arrows indicate the positions of *lli* and Delhi Pink parents, respectively.

negatively correlated with each other; correlations between HIS and HIL and HIS and HIR were highly significant. HIR was negatively correlated with the traits TDW, SDW and LDW.

The correlation coefficients for relationships between per cent total alkaloids (TIA) in leaves (TL), stems (TS) and roots (TR) and total yield TIA from leaves (TAYL), stems (TAYS) and roots (TAYR) are given in table 4, while the relationships between TL and TS and TL and TR were insignificant, that between TS and TR was negative and significant. TL, TS and TR were highly correlated, respectively with TAYL, TAYS and TAYR. Both TAYL and TAYS were negatively correlated with TR. TAYL, TAYS and TAYR were highly correlated with each other.

Table 3. Coefficients of correlation between dry weights of leaf, stem and root organs, whole plant dry weight and harvest indices for the three organs estimated for the *lli* × Delhi Pink derived recombinant inbred line population evaluated for the traits over two annual field trials, in *Catharanthus roseus*.

Trait	SDW	LDW	RDW	HIS	HIL	HIR
TDW	0.97*** (0.000)	0.98*** (0.000)	0.85*** (0.000)	−0.12 (0.625)	0.14 (0.056)	−0.20* (0.005)
SDW		0.93*** (0.000)	0.81*** (0.000)	0.03 (0.65)	−0.09 (0.188)	−0.26*** (0.000)
LDW			0.82*** (0.000)	−0.29*** (0.000)	0.42*** (0.000)	−0.22** (0.002)
RDW				−0.28*** (0.000)	0.15* (0.03)	0.29*** (0.000)
HIS					−0.89*** (0.000)	−0.38*** (0.000)
HIL						−0.09 (0.222)

*, ** and ***, significant at $P < 0.05$, $P < 0.01$ and $P < 0.0001$ probability level, respectively (P values are given in parenthesis). The description of traits is given in table 1.

Table 5 presents correlations between leaf and root contents of pharmaceutical alkaloids and organwise per cent total contents and yields of all alkaloids (TIA). It will be seen that per cent contents of leaf alkaloids (SL, CL, VL and VBL) are all interrelated (r values +ve and highly significant). Likewise per cent contents of root alkaloids (SR, CR and AR) were correlated with each other highly and positively. The contents of leaf alkaloids (SL, CL, VL and VBL) were not correlated with contents of root alkaloids (SR, CR and AR). Except for SL, the VL, CL and VBL were correlated with TL and TAYL. CR was also correlated with TAYL. VL, VBL, SR, AR and CR were all correlated with TAYR.

The associations between organwise contents and yields of pharmaceutical TIA are shown in the table 6. The yields of individual TIA whether from leaf or root are all positively and highly correlated. Leaf contents of V (VL) and VB (VBL) were correlated with yields of individual TIA from leaves and roots (SLY, CLY, VLY, VBLY, SRY, ARY, CRY, SLRY and CLRY). However, CL was correlated only with SLY, CLY, VLY and VBLY and SL with only SLY and CLY. Root contents of SR, AR and CR were correlated with SRY, ARY, CRY, SLRY and CLRY. CR was also correlated with SLY, VLY and VBLY and AR with SLY.

Table 7 provides the coefficients of correlations between yields of individual alkaloids from leaves and roots (SLY, CLY, VLY, VBLY, SRY, ARY, CRY, SLRY and CLRY), per cent contents of all alkaloids from leaves (TS) and roots (TR) and yields of all alkaloids from leaves (TAYL) and roots (TAYR). It will be seen that TAYL and TAYR are strongly and positively correlated with each of the individual alkaloid yield traits. The relationship of each of leaf alkaloid traits (SLY, CLY, VLY and VBLY) with TL was positive and significant. The relationship between SLY, CLY, VLY, VBLY and TR was negative and significant, except for that between VLY and TR although negative was not significant. The yield traits SRY, ARY, CRY, SLRY and CLRY were not significantly correlated with TR.

Identification and mapping of QTL for selected traits

A set of seven TIA yield related quantitative traits were selected for the detection and mapping of loci (QTL) involved in their determination. Three of the selected traits concerned total contents (%) of alkaloids (TIA) in leaves (TL), stems (TS) and roots (TR). The rationale for selecting these traits was that TL, TS and TR were strongly correlated respectively with the alkaloid yield traits TAYL, TAYS and TAYR. TL was strongly correlated with the contents (VL, CL and VBL) and yields (VLY, CLY and VBLY) of the pharmaceutically important TIA. Another set of the selected traits comprised of HIL, HIS and HIR, respectively, related to the proportion of biomass (dry weight) due to leaves, stems and roots in the whole plant biomass (TDW). TDW, the sum of dry weights of individual organs leaves (LDW), stems (SDW) and roots (RDW), was the seventh trait. The selected traits were apparently related to all the other traits directly or indirectly.

All markers that had been placed on the genetic linkage map were used in the QTL analysis of the seven traits. The application of SMA, SIM and CIM procedures on two year field trial date identified a total of 20 QTL, 11 concerning alkaloid contents in plant organs: leaves (*qTAL*), stems (*qTAS*) and roots (*qTAR*), seven for harvest index traits (*qHIL*, *qHIS* and *qHIR*) and two for the whole plant biomass trait (*qTDW*) (table 8). The identified QTL were found associated with only five of the eight LG (table 8; figure 1). Of the 20 QTL, 10 were found located on the LG1, five on LG2, three on LG6 and one each on LG3 and LG4; none of the QTL were mapped for LG5, LG7 and LG8.

Table 4. Coefficients of correlation between the per cent contents of total alkaloids (mainly terpenoid indole alkaloids) in leaves, stems and roots, and yield of total alkaloids from these individual organs and whole plant in *Catharanthus roseus*.

Trait[a]	TS	TR	TAYL	TAYS	TAYR	TAYW
TL	0.07 (0.338)	−0.004 (0.961)	0.45*** (0.000)	0.11 (0.126)	0.14 (0.058)	0.25*** (0.001)
TS		−0.17* (0.015)	0.13 (0.075)	0.65*** (0.000)	0.09 (0.202)	0.46*** (0.000)
TR			−0.19** (0.007)	−0.19** (0.007)	0.31*** (0.000)	−0.14* (0.05)
TAYL				0.70*** (0.000)	0.64*** (0.000)	0.88*** (0.000)
TAYS					0.56*** (0.000)	0.95*** (0.000)
TAYR						0.72*** (0.000)

*, **, *** Significant at $P < 0.05$, $P < 0.01$ and $P < 0.0001$ probability level, respectively (P values are given in parenthesis). The description of traits is given in table 1.

Table 5. Coefficients of correlation between the per cent contents of specific and total terpenoid indole alkaloids in leaves and roots and yield of total TIA from the organs in *Catharanthus roseus*.

Trait[a]	CL	VL	VBL	SR	AR	CR	TL	TR	TAYL	TAYR
SL	0.28***	0.03**	0.26***	0.10	0.13	0.07	0.13	−0.01	−0.02	−0.07
	(0.000)	(0.649)	(0.000)	(0.171)	(0.078)	(0.315)	(0.062)	(0.936)	(0.837)	(0.302)
CL		0.49***	0.42***	−0.02	−0.05	−0.02	0.35***	−0.08	0.20**	0.03
		(0.000)	(0.000)	(0.766)	(0.454)	(0.824)	(0.000)	(0.270)	(0.006)	(0.049)
VL			0.40***	0.09	0.02	0.08	0.52***	0.03	0.42***	0.28***
			(0.000)	(0.202)	(0.821)	(0.267)	(0.000)	(0.676)	(0.000)	(0.000)
VBL				0.04	0.06	0.06	0.45***	−0.07	0.33***	0.16*
				(0.602)	(0.388)	(0.375)	(0.000)	(0.344)	(0.000)	(0.027)
SR					0.22**	0.34*	−0.002	0.10	0.06	0.16*
					(0.002)	(0.000)	(0.977)	(0.185)	(0.444)	(0.028)
AR						0.57***	0.021	0.12	0.08	0.20**
						(0.000)	(0.771)	(0.102)	(0.292)	(0.005)
CR							−0.06	0.12	0.19**	0.41***
							(0.385)	(0.099)	(0.006)	(0.000)

*, **, *** Significant at $P < 0.05$, $P < 0.01$ and $P < 0.0001$ probability level, respectively (P values are given in parenthesis).
The description of traits is given in table 1.

Table 6. Coefficient of correlation between the per cent contents and yields of specific TIA in leaves and roots in *Catharanthus roseus*.

Trait[a]	SLY	CLY	VLY	VBLY	SRY	ARY	CRY	SLRY	CLRY
SL	0.68***	0.16*	−0.005	0.10	−0.05	0.01	−0.04	0.12	0.04
	(0.000)	(0.022)	(0.948)	(0.155)	(0.457)	(0.864)	(0.555)	(0.081)	(0.604)
CL	0.28***	0.71***	0.30***	0.30***	0.06	−0.01	0.03	0.12	0.32***
	(0.000)	(0.000)	(0.000)	(0.000)	(0.395)	(0.911)	(0.698)	(0.087)	(0.000)
VL	0.24**	0.51***	0.75***	0.43***	0.27***	0.18**	0.21**	0.29***	0.37***
	(0.001)	(0.000)	(0.000)	(0.000)	(0.000)	(0.013)	(0.004)	(0.000)	(0.000)
VBL	0.32***	0.42***	0.36***	0.73***	0.18*	0.15*	0.15**	0.23**	0.29***
	(0.000)	(0.000)	(0.000)	(0.000)	(0.014)	(0.037)	(0.037)	(0.001)	(0.000)
SR	0.11	0.01	0.081	0.04	0.43***	0.19**	0.23**	0.40***	0.17*
	(0.123)	(0.865)	(0.257)	(0.546)	(0.000)	(0.007)	(0.001)	(0.000)	(0.015)
AR	0.14*	−0.001	0.07	0.08	0.19**	0.72***	0.38***	0.20**	0.28***
	(0.050)	(0.989)	(0.357)	(0.281)	(0.007)	(0.000)	(0.000)	(0.005)	(0.000)
CR	0.22**	0.10	0.18*	0.18*	0.44***	0.64***	0.74***	0.43***	0.59***
	(0.002)	(0.178)	(0.012)	(0.011)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)
SLY		0.62***	0.57***	0.61***	0.49***	0.42***	0.45***	0.67***	0.60***
		(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)
CLY			0.76***	0.73***	0.51***	0.34***	0.42***	0.59***	0.74***
			(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)
VLY				0.77***	0.51***	0.46***	0.53***	0.67***	0.72***
				(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)
VBLY					0.56***	0.44***	0.50***	0.63***	0.69***
					(0.000)	(0.000)	(0.000)	(0.000)	(0.000)
SRY						0.73***	0.87***	0.98***	0.87***
						(0.000)	(0.000)	(0.000)	(0.000)
ARY							0.84***	0.72***	0.77***
							(0.000)	(0.000)	(0.000)
CRY								0.86***	0.92***
								(0.000)	(0.000)
SLRY									0.89***
									(0.000)

*, **, *** Significant at $P < 0.05$, $P < 0.01$ and $P < 0.0001$ probability level, respectively (P values are given in parenthesis).
The description of traits is given in table 1.

Table 7. Coefficients of correlation between the yields of specific alkaloids from leaves and roots and per cent total alkaloid contents and total alkaloid yields in leaves, roots and whole plant in *Catharanthus roseus*.

Trait[a]	TAYL	TAYR	TAYW	TL	TR
SLY	0.64***	0.43***	0.61***	0.22**	−0.14*
	(0.000)	(0.000)	(0.000)	(0.002)	(0.048)
CLY	0.77***	0.44***	0.63***	0.36***	−0.22**
	(0.000)	(0.000)	(0.000)	(0.000)	(0.002)
VLY	0.86***	0.59***	0.75***	0.40***	−0.115
	(0.000)	(0.000)	(0.000)	(0.000)	(0.108)
VBLY	0.83***	0.52***	0.75***	0.39***	−0.174**
	(0.000)	(0.000)	(0.000)	(0.000)	(0.014)
SRY	0.69***	0.85***	0.77***	0.12	−0.08
	(0.000)	(0.000)	(0.000)	(0.097)	(0.262)
ARY	0.52***	0.70***	0.60***	0.07	0.01
	(0.000)	(0.000)	(0.000)	(0.350)	(0.904)
CRY	0.60***	0.82***	0.71***	0.05	−0.033
	(0.000)	(0.000)	(0.000)	(0.481)	(0.645)
SLRY	0.75***	0.83***	0.81***	0.16*	−0.10
	(0.000)	(0.000)	(0.000)	(0.029)	(0.146)
CLRY	0.77***	0.80***	0.80***	0.19***	−0.118
	(0.000)	(0.000)	(0.000)	(0.000)	(0.099)

*, **, *** Significant at $P < 0.05$, $P < 0.01$ and $P < 0.0001$ probability level, respectively (P values are given in parenthesis).
The description of traits is given in table 1.

Traits relating to content of alkaloids in organs

Total content of alkaloids in leaves (TAL): Six significant QTL were identified for the TAL trait. These QTL explained 7.40 to 17.31% of the phenotypic variation for the TAL. They were located on LG1 and LG2 and had LOD score ranging from 3.15 to 7.81. The *qTAL2* and *qTAL3*, both located on LG1, accounted for high percentage of phenotypic variation. These were the only *qTALs* identified in both years. Among the remaining *qTALs*, *qTAL1* located on LG1 was identified in the 2007 season and *qTAL4* located on LG1 and *qTAL5* and *qTAL6* located on LG2 were identified on the basis of combined analysis of 2007 and 2008 field trials. The SMA method detected all the *qTALs*, SIM detected all except *qTAL5* and CIM method also detected all the *qTALs* except *qTAL4*.

Total content of alkaloids in roots (TAR): Only one significant QTL was identified for the TAR trait. The *qTAR1*, identified by all the analytical methods, was located on LG4 and accounted for 16.04% of the phenotypic variability.

Total content of alkaloids in stems (TAS): For TAS, a total of four significant QTL were identified, one on LG2 and 3 on LG6. These QTL accounted for 14.14 to 33.63% of the phenotypic variation in TAS. Among the analytical methods, SMA, SIM and CIM, respectively, identified three (*qTAS1*, *qTAS3* and *qTAS4*), all four and two QTL (*qTAS2* and *qTAS3*).

HIL: Only one significant QTL located on LG1 got identified by all the analytical methods. *qHIL1* accounted for about 10% of the phenotypic variation for the trait.

HIS: Three significant QTL got identified, each on a different linkage group (*qHIS1* on LG1, *qHIS2* on LG2 and *qHIS3* on LG3). They accounted for 9.67 to 29.78% of the phenotypic variation for HIS. All the QTL got identified by SMA and SIM methods. The CIM method identified *qHIS3*.

HIR: In all, three QTL all located on LG1 got identified for this trait. The QTL accounted for 9.88 to 30.52% of the phenotypic variation for HIR. All the three QTL were identified by SMA and SIM methods and only two *qHIR1* and *qHIR2* by the CIM method.

TDW: Two QTLs located on different linkage groups were identified by all of the SMA, SIM and CIM procedures for the trait. The *qTDW1* located on LG1 and *qTDW2* located on LG2 respectively accounted for 9.55 and 6.21% of the phenotypic variation for the TDW trait.

Colocalization of QTL on linkage groups: Among LG1, LG2 and LG6, on which more than one QTL was located, QTL for different traits were located only on LG1 and LG2 (figure 1). Of the four QTL for TAS, three (*qTAS2*, *qTAS3* and *qTAS4*) were located in an interval of about 51 cM, between 10.0 and 61.3 cM positions, on LG6. Four QTL were located in three regions in LG2. Two of these QTL, for different traits, *qTDW2* and *qHIS2* were in a 26.2 cM region between 140.9 and 177.1 cM positions. The other two QTL on LG2, *qTAS1* and *qTAL6* were located in the regions lying between 50.1 to 77.2 cM and 175.9 to 196.7 cM positions, respectively. Ten QTL for five of the seven traits were located in six regions on LG1. The QTL *qHIL1*, *qTDW1*, *qHIS1* and *qTAL4* were located in the regions 15.3–40.4 cM, 105.8–121.7 cM, 171.1–175.7 cM and 253.1–285.6 cM, respectively. A cluster of QTL for the TAL trait (*qTAL1*, *qTAL2* and *qTAL3*) was located on LG1 in a 37.8 cM region from 22.7–60.5 cM. Another cluster comprising of all the three QTL for the HIR trait (*qHIR1*, *qHIR2* and *qHIR3*) was located in an interval on LG1 measuring 55.6 cM distance from 180.0 cM to 235.6 cM positions.

Parental contribution of favourable QTL alleles: The DNA markers located close to the identified QTL and their parental origins are given in the table 8. A total 137 DNA markers, 104 from 'Delhi Pink' and 33 from '*lli*' are listed against 20 QTL. When the markers located closest to the map positions of QTL were considered as the indices of donors, it was noted that the parental contributions were unequal, 'Delhi Pink' contributed 15 favourable QTL alleles and only five favourable QTL were contributed by '*lli*'.

Table 8. Summary of 20 quantitative trait loci identified for seven traits using QTL Cartographer software by applying the single marker regression analysis, simple interval mapping and composite interval mapping procedures on a recombinant inbred line mapping population of the cross *lli* × Delhi Pink in *Catharanthus roseus*. The trait expression data obtained from the trials in 2007 and 2008 seasons were used separately as well as jointly in the QTL analysis. The map locations of QTL in relation to the close by markers are also provided (also see figure 1). All the identified QTL were significant.

				QTL Cartographer								
				SMA ($P < 0.0001$)	SIM			CIM				
Trait[a]	Year	QTL	Linkage group	LOD peak position (cM)	LOD	LOD peak position (cM)	R^2 (%)[b]	LR threshold[c]	LOD	LOD peak position (cM)	R^2 (%)[b]	Marker/marker interval[d]
TAL	2007	*qTAL1*	1	22.7–39.9	3.36	35.8	9.86	13.40	3.44	35.8	8.88	CrES401 (DP), OPU12 (*lli*)
	2008		1	–[e]	–				–			–
	2007+ 2008		1	–	3.15	36.8	10.86		3.21	36.8	8.94	OPU12 (*lli*)
	2007	*qTAL2*	1	40.5–55.8	6.69	44.9	16.47	13.40	6.48	44.9	14.29	OPY1 (DP), CrSSR197 (DP), CrMR161 (DP), CrMR165a (DP)
	2008		1	43.2–56.1	5.73	48.9	13.26		6.18	50.5	12.45	CrSSR197 (DP), CrMR161 (DP), CrMR165a (DP)
	2007+ 2008		1	40.2–55.5	7.1	46.9	17.31		7.81	48.9	14.83	OPU13 (*lli*), OPY1 (DP), CrSSR197 (DP), CrMR161 (DP), CrMR165a (DP)
	2007	*qTAL3*	1	55.8–60.2	3.77	57.8	9.41	13.40	–			CrMR165a (DP), CrSSR56 (DP), CrES361 (DP)
	2008		1	56.1–60.2	5.05	58.8	11.42		5.74	58.8	11.28	CrSSR56 (DP), CrES361 (DP)
	2007+ 2008		1	55.8–60.5	5.31	58.8	11.99		6.27	58.8	11.74	CrMR165a (DP), CrSSR56 (DP), CrES361 (DP)
	2007	*qTAL4*	1	–	–			13.40	–			–
	2008		1	–	–				–			–
	2007+ 2008		1	253.1–285.6	3.18	264.6	7.40		–			OPR12 (DP), CrSSR183 (DP)
	2007	*qTAL5*	2	–	–			13.40	–			–
	2008		2	–	–				–			–
	2007+ 2008		2	175.9–196.7	-				4.64	185.9	10.36	OPK6 (*lli*), OPJ16 (DP), CrES301 (DP)
	2007	*qTAL6*	2	–	–			13.40	–			–
	2008		2	–	–				–			–
	2007+ 2008		2	196.7–225.8	–				3.97	210.4	7.30	OPM11 (DP), OPN6 (*lli*)
TAR	2007	*qTAR1*	4	239.8–256.7	5.53	250.0	12.68	13.39	7.12	249.7	16.04	OPC5 (*lli*), OPG17 (DP), CrSSR293 (DP)
	2008		4	–	–				–			–
	2007+ 2008		4	239.3–257.1	6.20	249.7	14.82		6.83	249.7	15.02	OPC5 (*lli*), OPG17 (DP), CrSSR293 (DP)
TAS	2007	*qTAS1*	2	50.1–76.9	6.45	60.0	22.44	13.63	–			CrSSR194 (DP), U849 (DP), CrSSR205 (DP)
	2008		2	50.4–76.9	6.45	60.0	22.44		–			CrSSR194 (DP), U849 (DP), CrSSR205 (DP)
	2007+ 2008		2	50.2–77.2	6.27	66	21.73		–			U849 (DP), CrSSR205 (DP)
	2007	*qTAS2*	6	–	6.52	9.0	18.75	13.63	7.37	10.0	17.78	OPF12 (DP)
	2008		6	–	6.52	9.0	18.75		7.37	10.0	17.78	OPF12 (DP)
	2007+ 2008		6	–	–				–			–
	2007	*qTAS3*	6	12.4–33.1	15.36	21.6	33.63	13.63	16.64	22.6	29.69	OPF12 (DP), OPF13 (DP), OPI7 (DP)
	2008		6	12.9–33.0	15.36	21.6	33.63		16.64	22.6	29.69	OPF12 (DP), OPF13 (DP), OPI7 (DP)
	2007+ 2008		6	12.5–33.2	15.36	21.6	33.63	13.63	16.64	22.6	29.69	OPF12 (DP), OPF13 (DP), OPI7 (DP)
	2007	*qTAS4*	6	39.7–57.0	4.63	48.5	14.15	13.63	–			CrES231 (*lli*)
	2008		6	39.9–56.6	4.63	48.5	14.15		–			CrES231 (*lli*)
	2007+ 2008		6	33.3–61.3	4.63	48.5	14.15		–			OPI7 (DP), CrES231 (*lli*), OPE15 (DP)

Table 8 (*contd*).

Trait[a]	Year	QTL	Linkage group	QTL Cartographer: SMA ($P < 0.0001$) LOD peak position (cM)	SIM LOD	SIM LOD peak position (cM)	SIM R^2 (%)[b]	CIM LR threshold[c]	CIM LOD	CIM LOD peak position (cM)	CIM R^2 (%)[b]	Marker/marker interval[d]
HIL	2007	*qHIL1*	1	–	–				–			–
	2008		1	15.3–35.3	–			12.93	4.05	18.8	8.40	OPT18 (*lli*), CrES401 (DP), OPU12 (*lli*)
	2007+ 2008		1	14.1–40.4	4.07	18.8	9.33		4.91	18.8	10.33	OPC12 (*lli*), OPT18 (*lli*), CrES401 (DP), OPU12 (*lli*), OPU13 (*lli*)
HIR	2007	*qHIR1*	1	180.3–193.0	7.90	180.9	19.28	12.98	11.28	192.8	29.92	CrSSR207 (DP), CrSSR154 (DP)
	2008		1	180.0–192.6	10.55	178.9	25.80		6.80	191.8	18.29	CrSSR207 (DP), CrSSR154 (DP)
	2007+ 2008		1	–	10.12	191.8	27.57		10.35	191.8	25.61	OPO20 (DP), CrES36 (DP), OPT1 (DP), CrSSR207 (DP), CrSSR154 (DP)
	2007	*qHIR2*	1	197.8–218.1	12.07	206.0	25.46	12.98	11.57	206.0	22.14	CrMR390a (DP), CrSSR108 (DP), CrES248 (*lli*), OPO6 (DP)
	2008		1	203.8–215.0	10.67	206.0	22.72		14.52	206.0	28.70	CrSSR108 (DP), CrES248 (*lli*), OPO6 (DP)
	2007+ 2008		1	198.4–218.3	15.00	206	30.52		16.65	206	30.07	CrSSR108 (DP), CrES248 (*lli*), OPO6 (DP), OPX4 (DP)
	2007	*qHIR3*	1	222.2–235.6	3.95	226.3	9.88	12.98	–			CrMR171a (DP), OPR16 (*lli*)
	2008		1	221.9–235.6	3.62	225.3	10.08		–			CrMR171a (DP), OPR16 (*lli*)
	2007+ 2008		1	218.9–234.8	5.06	225.3	12.17		–			CrMR171a (DP), OPR16 (*lli*)
HIS	2007	*qHIS1*	1	170.4–176.5	4.07	171.8	9.67	13.79	–			OPF8 (*lli*), OPO20 (DP), CrES36 (DP), OPT1 (DP)
	2008		1	170.4–175.6	–				–			OPF8 (*lli*), OPO20 (DP), CrES36 (DP), OPT1 (DP)
	2007+ 2008		1	171.1–175.7	4.80	171.8	11.19		–			OPO20 (DP), CrES36 (DP)
	2007	*qHIS2*	2	141.3–175.4	6.99	166.9	23.35	13.79	–			CrSSR265 (DP), OPA18 (DP)
	2008		2	141.6–172.5	6.12	165.9	18.27		–			CrSSR265 (DP), OPA18 (DP)
	2007+ 2008		2	140.9–177.1	8.50	165.9	24.6		–			CrSSR265 (DP), OPA18 (DP), OPK6 (*lli*)
	2007	*qHIS3*	3	26.7–77.6	10.94	51.3	23.28	13.79	4.83	51.3	8.54	CrSSR272 (DP), CrES27 (*lli*), OPH18 (DP)
	2008		3	35.4–77.1	9.72	51.3	21.12		4.91	51.3	8.77	CrSSR272 (DP), CrES27 (*lli*), OPH18 (DP)
	2007+ 2008		3	35.2–77.2	14.52	51.3	29.78		8.95	51.3	15.22	CrSSR272 (DP), CrES27 (*lli*)
TDW	2007	*qTDW1*	1	107.8–121.2	4.20	114.0	10.58	13.79	4.67	113.0	9.17	CrES392 (DP), OPS19 (DP), CrSSR138 (*lli*)
	2008		1	105.3–121.2	–				–			CrES392 (DP), OPS19 (DP), CrSSR138 (*lli*)
	2007+ 2008		1	105.8–121.7	3.96	113	9.09		4.93	113	9.55	CrSSR81 (DP), CrES392 (DP), OPS19 (DP), CrSSR138 (*lli*)
	2007	*qTDW2*	2	141.3–176.2	3.55	160.9	8.28	13.79	4.67	160.9	9.33	CrSSR265 (DP), OPA18 (DP), OPK6 (*lli*)
	2008		2	141.3–176.7	3.38	160.9	7.19		3.52	160.9	6.92	CrSSR265 (DP), OPA18 (DP), OPK6 (*lli*)
	2007+ 2008		2	141.2–175.5	3.87	160.9	9.01		3.23	160.9	6.21	CrSSR265 (DP), OPA18 (DP)

[a] TAL, TAS, TAR, total alkaloid content (%) in leaves, stems and root, respectively; The descriptions of HIL, HIS, HIR and TDW are same as in table 1.
[b] The amount of phenotypic variation explained by each identified QTL.
[c] Threshold determined by 1000 permutations.
[d] Among the markers in the interval, the underlined marker is closer to the QTL position.
[e] QTL was not detected by the concerned procedure.

Discussion

Evaluation of 30 traits related to the yield of TIA in the genetically distant germplasm 'Delhi Pink' and '*lli*' and 197 RIL derived from their cross, over two seasons, has revealed some properties of the variability expressed by different traits. Besides, marker assisted analysis of variation in the RIL population for seven traits has led to the detection of 20 QTL. The progress thus made in better understanding of the genetic basis of TIA related traits is discussed below.

Comparative properties of the TIA related traits and selection of traits for QTL analysis

Presently, the pharmaceutically important TIA from *C. roseus* are V, C, VB, S and A (van der Heijden *et al.* 2004; Hedhili *et al.* 2007; Pasquier and Kavallaris 2008; Wang *et al.* 2011a). Leaves of adult *C. roseus* plants are extracted to obtain V, C and VB in the main and S and A as by-products (Potier 1980; Kruczynsky and Hill 2001; Ishikawa *et al.* 2008; Tam *et al.* 2010). Economic production of S and A requires their extraction from plant roots and C is a by-product from the root extracts of *C. roseus* (van der Heijden *et al.* 2004; El-Sayed and Verpoorte 2007; Singh *et al.* 2008). Stems of *C. roseus* rich in S but largely devoid of V, C and VB (Singh *et al.* 2008) do not offer advantage over roots as a resource for S. The principal aim of studies directed towards development of medicinal cultivars of *C. roseus* is to isolate genotypes whose leaves and roots accumulate pharmaceutical alkaloids in high concentrations and in which the yield of TIA-rich organs is also high. Among the 30 traits evaluated in the RIL population, seven were concerned with biomass of leaves, stems and roots. The remaining 23 traits were about content and yield of all alkaloids (TIA) and individual pharmaceutical alkaloids from stems and leaves and/or roots. RIL population demonstrated significant genetic variability for all traits; and there were transgressive segregants in both directions.

The traits concerning contents of pharmaceutically important leaf TIA S (SL), C (CL), V (VL) and VB (VBL) were pairwise highly correlated. Similarly the root TIA content traits CR, SR and VR were highly correlated with each other. The organwise content traits for single pharmaceutical TIA were highly correlated with corresponding single TIA yield traits (for example SL and SLY, CL and CLY and CR and CRY). The single TIA yield traits (SLY, CLY, VLY, VBLY, SRY, ARY and CRY) were highly correlated with organwise total alkaloid-yield traits (TAYL and TAYR) and whole plant alkaloid-yield trait (TAYW). The traits TL and TAYL, TS and TAYS and TR and TAYR were also pairwise highly correlated. The expression of the traits TL, TS and TR appeared to determine the expression of all the TIA content and yield traits, directly or indirectly. The RIL population possessed segregants in which TL, TS and TR expression was respectively 1.5, 2.1 and 1.3 fold higher than the expression in the parent germplasm superior for the parent ('Delhi Pink' for TL and TR and '*lli*' for TS). For the above mentioned reasons the traits TL, TS and TR were selected for QTL analysis.

The organ-biomass traits LDW, SDW and RDW and whole plant biomass trait TDW were found to be pairwise highly correlated. The calculated values of harvest index traits HIL, HIS and HIR were based on the LDW, SDW, RDW and TDW values. The HIL, HIS and HIR traits were selected for QTL analysis because they reflected the architecture of the plant. The trait TDW was selected for QTL analysis because it provided a measure of the growth of entire plant.

Properties of the QTL detected for the selected traits

Only 20 QTL were detected for seven TIA yield related traits, 11 for the traits concerning concentration of TIA in organs (TL, TS and TR), two for the whole plant biomass trait (TDW) and seven for the harvest index traits (HIL, HIS and HIR). Each of the trait studied was highly complex and depended on many genes. TIA accumulation in organs is known to involve scores of genes concerning biosynthesis and transport of precursors and synthesis and deposition of TIA in specialized sink cells (McKnight *et al.* 1991; Bird and Facchini 2001; van der Heijden *et al.* 2004; Facchini and St-Pierre 2005; O'Connor and Maresh 2006; El-Sayed and Verpoorte 2007; Mahroug *et al.* 2007; Oudin *et al.* 2007; Ziegler and Facchini 2008; Costa *et al.* 2008; Guirimand *et al.* 2010a,b; Verma *et al.* 2011). The biomass traits are expected to be even more complex. Biomass of an organ can be visualized to involve genes governing uptake and transport of essential nutrients, metabolism and photosynthesis, cell division and differentiation and development of the organ. Although detection of QTL for a trait depends on differential contribution by parents of alleles of genes involved in the formative processes, yet number 20 is very small for seven traits. It is possible to suggest that some of the most decisive steps in the determination of the studied traits have been defined by the identified QTL.

The detected 20 QTL mapped on five of the eight LG of *C. roseus* ($2n = 2x = 16$) (Chaudhary *et al.* 2011). The QTL were located rather unevenly on the LG. Ten QTL relating to five traits were located on the LG1. On LG2, 5 QTL for four traits were located. There were three QTL for the same trait on LG6. Each of LG3 and LG4 had on them one QTL, for different traits. All three QTL for the HIR trait were clustered in a 56 cM interval on LG1. This LG had another region of 40 cM in which three QTL for the TAL trait were located. Another TAL QTL was also located on LG1, but distantly from the cluster. Three of the four QTL for TAS were located in a 50 cM interval on LG6. The clustering of QTL for the same trait is perhaps suggestive of common response to the environmental or internal stimuli and sharing of genetic control mechanisms. There was one example of a cluster of QTL of two different traits. The *qTDW2* and *qHIS2* were colocated at 160.9 cM (between 141.3 and 176.7 cM) position on LG-2.

This type of clustering most probably indicates involvement of a common function. This is likely high since both TDW and HIS traits relate to biomass, former to the whole plant biomass and latter to the fraction due to stems in the whole plant biomass.

Transgressive segregation was observed for all the seven traits for which QTL were detected and mapped. The favourable alleles for most of the QTL were contributed by the parent 'Delhi Pink'. The '*lli*' parent was noted to have contributed favourable alleles perhaps only for the QTL *qTAL1*, *qTAS4*, *qHIL1*, *qHIR2* and *qHIS3* or for five of the 20 QTL only.

Validity of the identified QTL in the markers assisted breeding for improvement of TIA yield

The screening of data pertaining to 197 RIL simultaneously for the expression levels for 16 relatively important traits (RDW, LDW, TDW, HIL, HIR, TL, TR, VL, VLY, CL, CLY, VBL, VBLY, CR, CRY and CLRY) revealed that there were at least six genotypes (D30, D59, D110, D111, D132 and D145) in which a large majority of 16 traits were at par or higher in expression level than the better parent. Among the six RIL, four RIL had lower TR expression than the better parent 'Delhi Pink' for this trait. One RIL was lower in expression than the better parent 'Delhi Pink' for the TDW trait and both the parents for the HIR trait. Analysis of the genotyping data showed that D30 was positive for 13 out of 13 QTL alleles detected as favourable for TL, TR, HIL, HIR and TDW traits. The feasibility of MAS for the genotypes possessing higher levels of all the traits concerned with the yield of V, C and VB and all the alkaloids from leaves and roots of *C. roseus* is well exemplified by the RIL D30. It appears that screening of *C. roseus* segregating population from the *lli* × Delhi Pink cross at young stage of growth for markers close to qTAL, qTAR, qHIL, qHIR, and qTDW QTL can lead to isolation of a smaller population enriched for favourable QTL alleles. The smaller population so obtained can then be screened for the expression of TL, TR and organ-wise biomass characters. This scheme is expected to isolate genotypes producing total and pharmaceutical alkaloids in high yields.

Concluding remarks

A $F_{2:7}$ transgressively segregating population of 197 RIL from a cross between two genetically distant lines was characterized for variation in 30 traits related to the yield of TIA in *C. roseus*. The population had been earlier genotyped to construct a genetic linkage map of 179 markers on eight LG. The analysis of variation for seven selected traits in the population with respect to already mapped DNA markers by SMA, SIM and CIM procedures allowed detection and mapping of 20 QTL on five of the eight LG. Four clusters of QTL affecting the same or different traits were detected on three LG. The selection of RIL superior for 16 traits, with the use of DNA markers linked to QTL for only five traits, was validated.

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