QTL analysis associated with oleoresin content in intraspecific RIL population of chilli (*Capsicum annuum* L.)

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Abstract

Quantitative trait loci (QTLs) for oleoresin content were mapped in intraspecific advance recombinant inbred line (RIL) populations of chilli derived from cross between two contrasting parents 'California Wonder' and 'LCA235'. Oleoresin content of each RILs were estimated for 2 years (F_8 and F_9) along with parents. Three classes of molecular markers; simple sequence repeats (SSR), sequenced characterized amplified region (SCAR) and random amplified polymorphic DNA (RAPD) were used to generate linkage maps. A total of two QTLs for oleoresin content were mapped on two linkage group (LG). QTL *Qole.iivr-2.1* and QTL *Qole.iivr-3.3* contributed a minimum and maximum phenotypic variation of 8.74 and 32.4%, respectively over the years. The results of this investigation may be useful in improving the nutritional quality of pepper. The genomic regions of stable QTLs identified may serve as potential target regions for fine mapping and development of molecular markers for manipulation of yield and morphological traits in pepper.

Key words: Carotenoids, pepper, Simple Sequence Repeats, Quantitative Trait Loci

Introduction

Chilli, belonging to the genus *Capsicum*, are widely grown for their fruits valued for colour, flavour, spice, vegetable, nutrition made avaialble through several food items (Kumar *et al.,* 2011). One of the most important quality attribute of pepper is its oleoresin. Pepper oleoresins contains complex mixture of essential oils, waxes, pigments (mainly capsanthin, capsorubin, zeaxanthin, cryptoxanthin and lutein) and capsaicinoids and are commonly used as a pungent flavour in food, natural plant colour, and pharmaceutical ingredient (Hui and Barta, 2006; Sanatombi and Sharma, 2008). In food processing industries, especially in meat industry, concentrated oleoresin is added to the processed meat to impart attractive colour (Kumar *et al.*, 2006). In beverage industries, oleoresins are used to improve colour and flavors. Oleoresin is considered to be among the best substitute of synthetic colour used in food and cosmetic industries (Kumar *et al.,* 2006). Earlier study demonstrated that mature red colour of the fruits is dominant over yellow and is controlled by a single gene (*Y*) and later it was suggested that the mature fruit colour is controlled by three independent pairs of genes (*cl, c2* and *y*). The presence of dominant alleles at these three loci result in red mature fruits, while the presence of recessive alleles at three loci results in white mature fruits (Popovsky and Paran, 2000). The predominant pigments of the fruits *i.e.* capsanthin and capsorubin are synthesized by the enzyme capsanthin-capsorubin synthase (CCS). Intronless cDNA clone of CCS has been isolated and the expression studies indicated that CCS is induced during chloroplast differentiation at the time of fruit ripening and it is not expressed in leaves or green immature fruits (Bouvier *et al.,* 1994; Houlne *et al.,* 1994; Hugueney *et al.,* 1996). The absence of capsanthin and capsorubin in yellow fruits correlates with the lack of expression of CCS enzyme in yellow fruits (Bouvier *et al.,* 1994; Houlne *et al.,* 1994). For the identification of red and yellow-fruited genotypes a co-dominant DNA marker at seedling stage has been reported (Popovsky and Paran, 2000). A map was

constructed for the cross of 1154 x PI 152225 to identify QTLs controlling pigment content. The map comprised of 200 AFLP, RFLP and COSII (http:// solgenomics.net) markers. The AFLP markers were chosen as a subset of approximately 450 markers distributed throughout the genome. This map consists of 11 major linkage groups covering 1,374 cM (Mazourek *et al.,* 2009). The use of known RFLP and COSII markers allowed anchoring the linkage groups to the pepper chromosomes. Chromosomes 1 and 8 were converted into a single linkage group due to the reciprocal translocation differentiating *C. annuum* and *C. chinense* (Livingstone *et al.,* 1999). Two major pigment control QTLs that control chlorophyll content were identified *i.e. pc8.1* and *pc10.1* in a cross between a dark green *C. annuum* pepper and a light-green *C. chinense* pepper. The aim of present study was to extend our understanding of genetic control of capsaicinoid and carotinoid biosynthesis in *Capsicum annuum* by determining the QTLs for these quality traits.

Materials and methods

Plant materials: Two contrasting parents 'California Wonder' (large fruited, sweet pepper cultivar, having low oleoresin content) and 'LCA235' (small fruited hot pepper cultivar with high oleoresin content) were crossed in the year 2000. From a single self-fertilized F_1 plant, 225 F_2 seeds were advanced up to the F_8 generation following the single seed descent method. Eventually 74 recombinant inbred lines in the F_8 and F_9 generations were obtained and used in this study. RILs were evaluated for oleoresin content for two consecutive years during the cropping season of 2008-2009 and 2009-2010 at the experimental farm of the Indian Institute of Vegetable Research (IIVR), Varanasi, India. Thirty-days-old seedlings were transplanted on raised beds (4.5×1.0 m) in a randomized complete block design with three replications. Each plot had 20 plants and arranged at a distance of 60×45 cm (row-to-row x plant-to-plant). Standard agronomic and plant protection practices were followed. Ten randomly selected plants

in each replication were tagged. Oleoresin percentage from red ripe fruits of all RILs along with parents were estimated as suggested by Mathew *et al.* (1971) during the cropping season of 2008-09 and 2009-10. Oleoresin was extracted from the powder of dried chilli fruits by continuous shaking in acetone for 4-6 h on a mechanical shaker at room temperature and after that the extract was allowed to reflux on a clevenger apparatus for 4-6 hrs. The viscous liquid derived after percolation was further evaporated to near dryness.

Marker analyses: Genomic DNA was extracted from the parents ('California Wonder' and 'LCA235') and the RILs as described by Kim *et al.* (1997) with minor modifications. Leaf samples (1g each) were collected from 30-days-old transplanted plants. Leaf samples (1g each) were also taken from each tagged plant and bulked separately for each RIL. The leaf samples were ground in liquid nitrogen, and the resulting powder was transferred to 15 mL polypropylene tubes containing 5 mL DNA extraction buffer. Two mL of 6% of polyvinylpyrrolidone (PVP) was added to each sample and the mixture was incubated on ice for 5 min. Thereafter, 2% sodium dodecyl sulphate (SDS) was added and incubated at 65 °C for 30 min. The tubes were centrifuged for 15 min at 10,000 g and approximately 6 mL supernatant from each sample was transferred to a new tube. An equal volume of phenol:chlorophorm:isoamyl alcohol (P:C:I; 25:24:1) was added and tubes were gently shaken. The tubes were centrifuged at 6,000 g for 15 min and the supernatant was transferred to new tubes; 400 µL potassium acetate (3M) was added in each tube and DNA was precipitated with an equal volume of ice-cold isopropanol. The DNA was washed with 70% ethanol and dissolved in 500 µL distilled water after drying the DNA pellets. Polymerase chain reaction (PCR) of simple sequence markers (SSRs), sequence characterized amplified region (SCAR) and random amplified polymorphic DNA (RAPD) markers were performed as described by Lee *et al.* (2004). Amplification was performed in a 25 µL mixture containing 25-50 ng of genomic DNA, 250 µM of primer, 0.2 unit of Taq polymerase (5u/mL) and 10xPCR buffer. After initial denaturation at 94 °C for 4 min, 35 cycles were performed as: 94 °C for 1 min, 50-65 °C (depending on the primers) for 2 min, 72 °C for 2 min. Final extension was performed at 72 °C for 10 min and samples were held at 4 °C until taken out from the PCR machine. The PCR products were separated on 3.4% agarose gel and visualized in the presence of ethidium bromide (5 µg in 100 mL) using a gel documentation system (Alpha Imager and in-built software, Alpha Innotech, USA). Map construction and QTL detection the parents 'California Wonder' and 'LCA235' were screened with 290 microsatellite primers, 9 SCAR markers and 30 RAPD primers. All the polymorphic markers were used to genotype each of the 74 individuals of the RIL. Mapmaker v2.0 (Lander *et al.,* 1987) was used to create a framework linkage map with 39 SSRs, 1 SCAR and 5RAPD markers for QTL detection. The linkage map was constructed using the Likelihood of Odd Ratio (LOD) of >3 and recombination fraction of <0.4. Kosambi mapping function was used for map distance calculation. The commands 'Order' and 'Rip' were used to assign the order of markers on the map. The 'try' and 'compare' commands were used to include additional markers on the map. QTL Cartographer v2.5 (Wang *et al.* 2007) was used to find the QTLs performing composite interval mapping (CIM). The parameters settings for CIM were model 6, forward and backward stepwise regression with threshold of P <0.05 to select cofactors, window size 10 and

2 cM walking speed along chromosomes. A LOD threshold of 2.0 (equivalent to a P value of 0.05) for declaring a QTL as significant was based on 1000 permutations to control Type-I error. Adjusted mean of all traits were calculated before pooling the data from both the years. The QTL×QTL and QTL×Environment interactions were calculated using the program QTL Mapper 1.60 (Wang *et al.,* 2009). LOD curves for different traits are illustrated in Fig. 2. The names of detected QTLs were assigned according to the International Rules of Genetic Nomenclature (http://wheat.pw.usda.gov/ggpages/wgc/98/Intro.htm). For example, the name *QOle.iivr2.1* consists of a 'Q' followed by OLE (Oleoresin), institution designation, and linkage map assignment with QTL position number on the linkage map.

Data analyses: Analysis of variance (ANOVA) and correlation for all traits for 2 years was performed using SPSS 16. Heritability of each trait in the population was estimated using following formula (Nyquist, 1991):

$$h^2 = \frac{\sigma^2 g}{\sigma^2 g + (\sigma^2 ge/e) + (\sigma^2 g/re)}$$

where, h^2 represents the heritability, $\sigma^2 g$ is genetic variance, σ^2_{ge} is genotype × environment variance, σ^2 is error variance, r is number of replications and e is the number of environments. Phenotypic correlation coefficients were calculated using SPSS 16.

Results

Fully mature fruits of seventy-four RILs along with the parents evaluated for oleoresin content showed continuous variation (Fig.1). Sweet pepper cultivar 'California Wonder' showed low mean values while LCA 235 had higher mean values for oleoresin percentage therefore significant differences were found between the parents for this trait values across the years. Test of normality using Shapiro-Wilk test revealed that data from the RILs fit a normal distribution for oleoresin trait and showed moderate heritability *i.e.* 55 % (Table. 2). 'LCA235' had higher mean values for oleoresin % while 'California Wonder' had lower oleoresin % mean values across the year (Table. 1). In QTL analysis a total
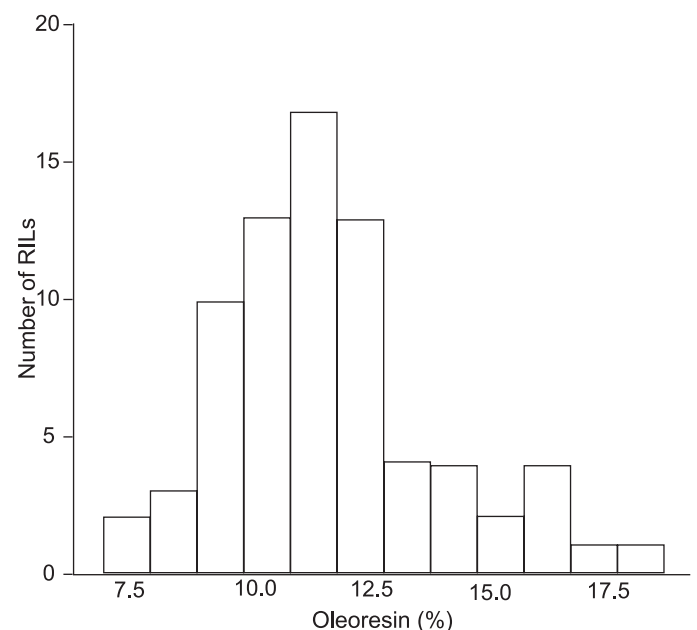

Fig. 1. Frequency distribution for the oleoresin content analysed in the RIL population, Parental mean values are designated as C.W. (California Wonder) and LCA-235

Table 1. Oleoresin in parents and RI lines in 2009 and 2010

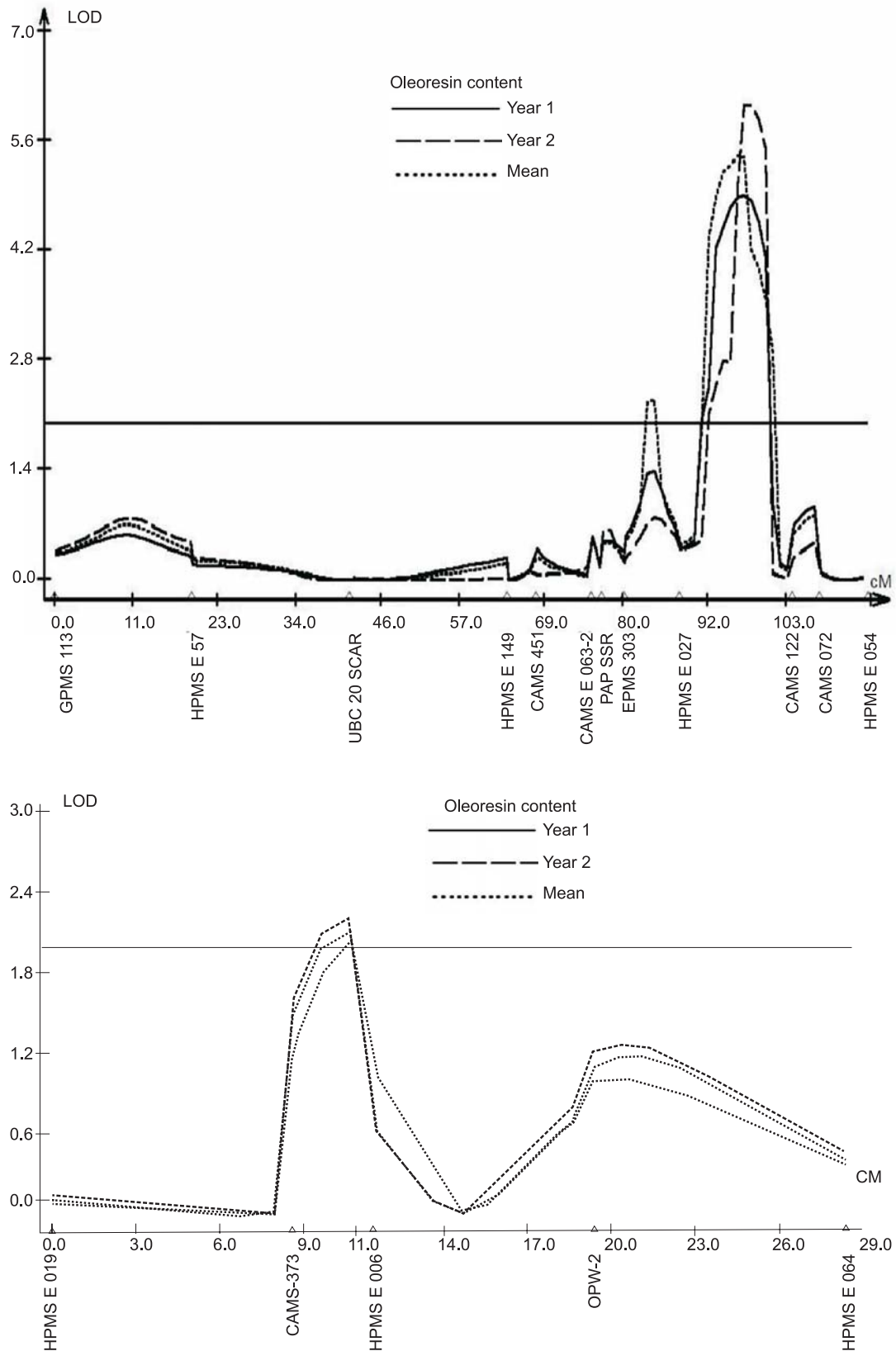| Traits	RIL range		'LCA 235'		'California Wonder'		RILs Mean ± SE	
	2009	2010	2009	2010	2009	2010	2009	2010
Oleoresin	7.1-16	7.9-15.6	14.63±0.17	15.2±0.17	7.37±0.03	8.64±0.22	11.3±0.2	11.8±0.5

Fig. 2. LOD curves for oleoresin content.

Table 2. Analysis of variance, degrees of freedom (df), mean sum of squares (MS), and heritability in the narrow sense

Source of variation	d.f.	Oleoresin %
Year	1	24.46
Year x Replication	4	20.32
Genotype	73	51.21
Year x Genotype	73	23.22
Residual	292	22.60
Total	443	
Heritability (%)		55

of 290 SSR, 30 RAPD and 9 SCAR markers were applied for the initial parental screening, 45 markers (13.68%) were found to be polymorphic and were used to genotype the RIL population. Among the three types of markers, RAPD showed maximum rate of polymorphism (16.7%) followed by SSR (13.4%) while the SCAR markers showed least polymorphism between the parents (11.1%). A total of 39 SSR, 5 RAPD and 1 SCAR markers were mapped generating 9 linkage groups. Among the 45 polymorphic markers, 36 markers were found linked in 9 linkage groups (LG) and 9 markers were unlinked. The number of mapped loci per linkage group ranged from 2 (LG 6, 7, 8, 9) to 12 (LG 3). Two QTLs were detected for oleoresin content on LG 2 and 3 (Fig. 2). QTLs were detected in both the years as well as in the mean over years. The percentage of phenotypic variation explained by the QTL *Qole.iivr-2.1* was 8.45% in 1st year, 8.05% in 2nd year and 8.74% in mean over the year (Table. 3). The QTL *Qole. iivr-3.3* contributed a maximum phenotypic variation of 32.3%, 36.3% and 32.4% in 1st year, 2nd year and in mean over the years, respectively. QTL *Qole.iivr-2.1* and *Qole.iivr-3.3* was found to be located between marker interval *CAMS-373-HpmsE006* (3.0cM) on LG 2 and *HPMSE-027-CAMS-122* (15.9cM) on LG 3, respectively (Table. 3). It was also found that both these QTLs *Qole.iivr-2.1* and *Qole.iivr-3.3* were derived from parent 'LCA-235' (Fig. 2).

Discussion

In the present scenario pepper oleoresin is extensively used to colour food products. Some reports has been published to understand the genetics of determination of fruit color in mature fruits of chilli (Popovsky and Paran, 2000; Lang *et al.,* 2004; Brand *et al.,* 2011). Several QTL mapping studies have also been reported for lycopene content in tomato (Tanksley *et al.,*1998; Fulton *et al.,* 2000). However, mapping for oleoresin content in pepper is now understudy. Efforts were made to find the markers linked to oleoresin traits. Oleoresin percentage values of RILs showed continuous distribution indicating quantitative inheritance of the trait (Fig. 1). Across two year we have identified 2 QTLs for oleoresin trait on linkage group 2, 3. Both the QTLs were contributed by parent 'LCA-235' which is a hot pepper cultivar having high oleoresin content developed at Agricultural Research Station (RARS), Guntur. The linkage map using 45 markers revealed a length of 205.54 cM with average interval size of

approximately 4.6 cM. Although the map coverage in our study was much lower than previously reported (Livingstone *et al.,* 1999; Brand *et al.,* 2011), the marker interval is fair enough, which means markers are not scattered. This clearly indicates that although we had a small set of intraspecific RILs, they were a good set of recombinants, unlike RILs derived from interspecific crosses. It is known that the use of fewer markers in QTL detection may not reduce QTL detection power (Wang *et al.,* 2007), but a reduced population size is crucial (Wang *et al.,* 1999). Nevertheless, there are several practical considerations on population size (Barchi *et al.,* 2009). These considerations should hold true in vegetables crops like peppers, in which one can have potential open pollinated cultivars or potential inbred lines for hybrid combinations of diverse market fruit types from the same fixed population (Poulos, 1994). Marker polymorphism always depends upon genetic relation between the two parents. In this study we found a low polymorphism rate of 13.7 % in our mapping population indicating narrow genetic base of two *C. annuum* parents belongs to the same species. Due to the use of fewer polymorphic markers, the possibility that some minor QTLs remain undetected cannot be ruled out. However, we were able to identify major QTLs explaining a large part of phenotypic variance 8.74% and 32.4% for oleoresin content. Recently, two major QTLs that control chlorophyll content were identified, *pc8.1* and *pc10.1*. The major QTL *pc8.1*, also affected carotenoid content in the ripe fruit. However, additional analyses in subsequent generations did not reveal a consistent effect of this QTL on carotenoid content in ripe fruit.

These findings suggest that certain QTLs may delineate genomic regions associated with correlated traits. Without fine mapping approaches, however, it is difficult to determine whether these QTLs exhibit pleiotropic effects or tightly linked. Fine mapping of QTLs in the RILs, or use of wild species introgression lines, such as those developed by Barchi *et al.* (2009) may aid in the resolution of these QTLs. Nevertheless, identification of multiple QTLs associated with a particular trait or suite of traits may provide a reference point for further investigation of important quality traits. In conclusion, the RIL population was effective in identifying QTLs controlling quality traits in chilli. This population should become a useful tool for dissection of QTLs and should contribute to our understanding of quantitative genetics in pepper.

Acknowledgments

Table 3. Effects of quantitative trait loci (QTL) on Oleoresin detected by composite interval mapping (CIM)

Trait/QTL (Oleoresin)	Interval (cM)	L G	Year 1			Year 2			Mean over years		
			LOD	R²	Add	LOD	R²	Add	LOD	R²	Add
Qole.iivr-2.1	CAMS-373-HpmsE006 (3.0cM)	2	2.06	8.45	0.53	2.12	8.05	0.51	2.22	8.74	0.54
Qole.iivr-3.3	HPMSE-027-CAMS-122 (15.9cM)	3	4.89	32.3	0.82	5.41	36.3	0.79	6.07	32.4	0.83

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