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Association of functional markers with flowering time in lentil

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Abstract

In the present study, a diverse panel of 96 accessions of lentil germplasm was used to study flowering time over environments and to identify simple sequence repeat markers associated with flowering time through association mapping. The study showed high broad sense heritability estimate (h^2bs=0.93) for flowering time in lentil. Screening of 534 SSR markers resulted in an identification of 75 SSR polymorphic markers (13.9%) across studied genotypes. These markers amplified 266 loci and generated 697 alleles ranging from two to 16 alleles per locus. Model-based cluster analysis used for the determination of population structure resulted in the identification of two distinct subpopulations. Distribution of flowering time was ranged from 40 to 70 days in subpopulation I and from 54 to 69 days in subpopulation II and did not skew either late or early flowering time within a subpopulation. No admixture was observed within the subpopulations. Use of the most accepted maximum likelihood model (P3D mixed linear model with optimum compression) of MTA analysis showed significant association of 26 SSR markers with flowering time at <0.05 probability. The percent of phenotypic explained by each associated marker with flowering time ranged from 2.1 to 21.8% and identified QTLs for flowering time explaining high phenotypic variation across the environments (10.7-21.8%) or in a particular environment (10.2-21.4%). In the present study, 13 EST-SSR showed significant association with flowering time and explained large phenotypic variation (2.3-21.8%) compared to genomic SSR markers (2.1-10.2%). Hence, these markers can be used as functional markers in the lentil breeding program to develop short duration cultivars.

Keywords Lentil · Flowering time · Genetic diversity · Population structure · Association mapping · Functional markers

Introduction

Lentil (*Lens culinaris* L.) is a rainfed crop. It is cultivated in cool-season under residual soil moisture conditions. Generally, this crop encounters terminal moisture and heat stresses during the grain filling period. As a result, the crop experiences forced maturity and lower yield. Possibilities of forced maturity loom large in the coming years with rising atmospheric temperature due to climate change and the delayed sowing of lentil after harvesting of the rice in the Indian subcontinent. An estimate indicates that in India, about 11.7 million ha area now remains fallow after the harvesting of rice (Subbarao et al. 2001). However, a window of 100-110 days is still available for growing lentil after rice harvest in several countries including India. Therefore, development of early maturing cultivars is required for harvesting the highest yield potential within this short periods of time. Short duration cultivars will not only increase the lentil cultivation in rice fallow areas but also increase the productivity by reducing yield losses due to forced maturity. Therefore, in lentil, manipulation of flowering time can have an important role in the development of high yielding early maturing cultivars that are able to adapt to changing environmental conditions. Since the early 1990s, a single germplasm line Precoz has been widely used to improve earliness, seed size, and rust resistance to South Asia (Asghar et al. 2010; Erskine et al. 1998; Singh et al. 2006; Kumar et al. 2014). Recently, efforts have been made toward the identification of other germplasm sources to broaden the genetic base. In this direction, exotic and

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indigenous germplasm were screened to identify early flowering genotypes. Recently, 1525 accessions of cultivated species have been characterized on the basis of days to 50% flowering that flowered in 71-107 days (Kumar and Solanki 2014), while its range was from 27 to 141 days among 405 accessions of wild species (Singh et al. 2014).

Flowering time is highly influenced by temperature and photoperiod (Summerfield et al. 1985). Therefore, it is essentially required to study flowering time under different environments and also to know its genetics before initiating a breeding program for earliness. Genetic and molecular analysis revealed that several genes control natural variation for flowering time in crop plants (Jung and Muller 2009). However, both qualitative and quantitative inheritance of flowering time has been reported in lentil (Sarker et al. 1999; Emami 1996). One of the studies identified that a single recessive gene (*sn*) controls the early flowering in lentil (Sarker et al. 1999). The variants of early flowering at this locus could be useful for development of early flowering cultivars for water-limited environments and help to diversify the lentil gene pool in South Asia (Sarker and Erskine 2006). Other studies identified several quantitative trait loci (QTLs) using bi-parental mapping populations that control flowering time in lentil (Tahir et al. 1994; Fratini et al. 2007; Tullu et al. 2008). More recently, a major stable QTL across environments has been reported for flowering time in lentil (Kahriman et al. 2014). In these studies, markers are not very closely linked to QTLs for flowering time due to poor marker density. However, a high-resolution marker-trait association can be established through association mapping by using a diverse natural population because an association mapping panel is developed from several historical recombination events that are able to break apart linked markers. It also has the potential to pick up markers for genes that did not segregate in the bi-parental population. It has been used widely in several crop plants to study the association of molecular markers with agronomically important traits (Wang et al. 2012; Visioni et al. 2013; Mandel et al. 2013; Zhao et al. 2014).

In lentil, use of molecular markers has been accelerated due to the enrichment of genomic resources in the recent past (Kumar et al. 2015). More than 2000 simple sequence repeat (SSR) markers developed from lentil genome are now available in the public domain (Kaur et al. 2011). Besides, *de novo* assembly of short sequence reads of lentil has resulted in the development of an expressed gene catalog and molecular markers (Verma et al. 2013). Molecular markers including single nucleotide polymorphism (SNP), simple sequence repeat (SSR), inter simple sequence repeat (ISSR), and direct amplification of minisatellite DNA (DAMD) have been used in assessment of genetic diversity among genotypes of cultivated species, land races, and wild relatives (Reddy et al. 2010; Lombardi et al. 2014; Verma et al. 2014; Basheer-Salimia et al. 2015; Seyedimoradi and Talebi 2014; Jain et al. 2013; Dikshit et al. 2015; Khazaei et al. 2016). These molecular markers are useful genomic resources for studying marker-trait association in lentil. Flowering time is an important trait to lentil breeders for developing short duration lentil cultivars. A tightly linked molecular marker can be used for the precise manipulation of the genetic makeup of individuals along with other yield contributing traits in lentil. Therefore, in the present study, a diverse panel of lentil germplasm was used (i) to study flowering time over locations and years, and (ii) to identify simple sequence repeat markers associated with flowering time through association mapping.

Materials and methods

Plant material, experimental design, and recording of phenotypic data

In the present study, a diverse panel of 96 accessions was used to characterize flowering time in lentil and evaluated over two years (2014-15 and 2015-16) at two locations [Research Farm of Indian Institute of Pulses Research (IIPR), Kanpur and Regional Station of IIPR, Dharwad]. These two locations were selected on the basis of their differences in photoperiod and temperature during the flowering time for studying photo-thermo-sensitivity among the present genotypes. The present diverse panel included breeding lines, cultivars, landraces, and exotic lines that was constituted by covering the sufficient phenotypic diversity of flowering time available in the cultivated gene pool. The details of these accessions are given in Table 1. Experimental materials were evaluated in an augmented design in three-meter single row plots. Three genotypes namely Precoz, DPL 15, and DPL 62 were used as checks, which were repeated four times. Row to row distance was 25 cm and plant to plant distance was 5-7 cm. Standard agronomic practices were followed to raise a successful crop. Observations were recorded on days to flower initiation on five randomly selected plants. It is measured from date of seed sowing in the field to date of flower initiation in a particular genotype.

SSR markers and PCR amplification

Genomic SSR markers developed in lentil at ICRADA by Dr. A. Hamwieh (personal communication; Kumar et al. 2014) and EST-SSR markers developed by Kaur et al. (2011) were used in the present study. These markers represent both coding and non-coding regions of lentil genome. The primers were custom synthesized from Eurofins Genomics India, and

Table 1 Details about name of accession, type of material, pedigree, and source of lentil accessions used in the present study

Accession	Type of material	Pedigree	Source/origin	Country
IPL-121	Breeding line	88/142 × DPL 44	IIPR, Kanpur, India	India
IPL-133	Breeding line	88/142 × DPL 44	IIPR, Kanpur, India	India
IPL-203	Breeding line	DPL 35 × EC 157634/382	IIPR, Kanpur, India	India
Sehore-74-3	Cultivar	Local selection from Sehore, MP	JNKVV, Jabalpur, India	India
IPL-218	Breeding line	EC 208 037 × DPL 62	IIPR, Kanpur, India	India
IPL-219	Breeding line	ILL 7657 × DPL 61	IIPR, Kanpur, India	India
IPL-221	Breeding line	DPL44 × DPL 62 × DPL58	IIPR, Kanpur, India	India
IPL-325	Breeding line	ILL 101 × E 362 × DPL 62	IIPR, Kanpur, India	India
IPL-324	Breeding line	ILL 76 59 × DPL 58 × KL 178	IIPR, Kanpur, India	India
IPL-519	Breeding line	KL 178 × DPL 44	IIPR, Kanpur, India	India
IC-560291	Land race	Unknown	NBPGR, New Delhi	India
IC-560322	Land race	Unknown	NBPGR, New Delhi	India
IPLS-09-06	Breeding line	L 4603 × PRECOZ	IIPR, Kanpur, India	India
IC-560113	Land race	Unknown	NBPGR, New Delhi	India
IPLS-COLD	Selection	UNKNOWN	IIPR, Kanpur, India	India
IPLS-09-23	Breeding line	ILL8072 × ILL6037	IIPR, Kanpur, India	India
IPLS-09-32	Breeding line	ILL8072 × ILL6037	IIPR, Kanpur, India	India
IC-560117	Land race	Unknown	NBPGR, New Delhi, India	India
IPLS-09-03	Breeding line	L 4603 × PRECOZ	IIPR, Kanpur, India	India
IPLS-09-11	Breeding line	Selection from P91227	IIPR, Kanpur, India	India
IPLS-09-10	Breeding line	Selection from P91227	IIPR, Kanpur, India	India
IPLS-09-38	Breeding line	L 4603 × DPL 62	IIPR, Kanpur, India	India
IPLS-09-22	Breeding line	ILL8072 × ILL6037	IIPR, Kanpur, India	India
IC-560150	Land race	Unknown	NBPGR, New Delhi, India	India
IPLS-09-37	Breeding line	ILL7938 × ILL6037	IIPR, Kanpur, India	India
IPLS-09-36	Breeding line	ILL7938 × ILL6037	IIPR, Kanpur, India	India
IPLS-09-14	Breeding line	SELECTION FROM MASAN	IIPR, Kanpur, India	India
IPLS-09-33	Breeding line	ILL7938 × ILL6037	IIPR, Kanpur, India	India
ILS-09-01	Breeding line	L 4603 × PRECOZ	IIPR, Kanpur, India	India
IC-560111	Land race	Unknown	NBPGR, New Delhi, India	India
IPLS-09-27	Breeding line	ILL8072 × ILL6037	IIPR, Kanpur, India	India
IPLS-99/209	Breeding line	PL 639 × Precoz	IIPR, Kanpur, India	India
IPLS-09-34	Breeding line	ILL7938 × ILL6037	IIPR, Kanpur, India	India
IPLS-09-05	Breeding line	L 4603 × PRECOZ	IIPR, Kanpur, India	India
IPL-09-08	Breeding line	L 4603 × PRECOZ	IIPR, Kanpur, India	India
IPLS-09-17	Breeding line	Selection from P91227	IIPR, Kanpur, India	India
ILWLS-118	Selection from ILWL118	Wild (*L. orientalis*)	IIPR, Kanpur, India	India
ILWL-118	Wild	L. orientalis	ICADA, Syria, India	
L-4603	Breeding line	Precoz × L 3991	IARI, New Delhi, India	India
IPL-220	Cultivar	DPL 44 × DPL 62 × DPL 58	IIPR, Kanpur, India	India
DPL-58	Breeding line	PL 639 × PRECOZ	IIPR, Kanpur, India	India
PRECOZ	Exotic line	Argentina cultivar	ICARDA, Syria	Syria
PL-02	Cultivar	PL 4 × DPL 55	GBPUAT, Pant Nagar, India	India
ILL-7663	Exotic line	Unknown	ICADA, Syria, India	Syria
DPL-15	Cultivar	PL406 × L 4076	IIPR, Kanpur, India	India
IPL-81	Cultivar	K 75 × PL 639	IIPR, Kanpur, India	India
IPL-406	Cultivar	DPL 35 × EC 157634/382	IIPR, Kanpur, India	India
DPL-62	Cultivar	JL 1 × LG 171	IIPR, Kanpur, India	India
ILL-6002	Exotic line	ILL 4349 × ILL 4605	ICADA, Syria	Syria

Table 1 (continued)

Accession	Type of material	Pedigree	Source/origin	Country
IPL-316	Cultivar	SEHORE-74-3 X DPL 58	IIPR, Kanpur, India	India
IPL-526	Cultivar	DPL 62 × DPL 58	IIPR, Kanpur, India	India
JL-01	Cultivar	Local Selection from Sehore, MP	JNKVV, Jabalpur, India	India
DPL-59	Breeding line	K 75 × PRECOZ	IIPR, Kanpur, India	India
LL-864	Breeding line	LL 498×LH 84-8	PAU, Ludhiana, India	*ndia*
ILWL-248	Wild	*L. orientalis*	ICARDA, Syria	Syria
IPL-321	Breeding line	K 75 × DPL 62	IIPR, Kanpur, India	India
IPL-M-08	MUTANT	germplasm	IIPR, Kanpur, India	India
IPL-98/193	Breeding line	(SEHORE 74-3 × DPL44) × DPL 35	IIPR, Kanpur, India	India
JL-03	Cultivar	Local Selection from Sagar , Mp	JNKVV, Jabalpur, India	India
K-75	Cultivar	Local selection from Bundelkhand, UP	CSAUAT, Kanpur, India	India
KLS-218	Cultivars	KLS 133 × LG 362	CSAUAT, Kanpur, India	India
L-4076	Cultivar	PL 234 × Pl 639	IARI, New Delhi, India	India
L-4147	Cultivar	(L 3875 × P4) × PKVL1	IARI, New Delhi, India	India
HUL-57	Cultivar	Mutant of HUL 1	BHU, Varanasi, India	India
L-9-12	Cultivar	Local Selection from Punjab	PAU, Ludhiana, India	India
LH-84-8	Cultivar	L9-12 × JLS 2	CCS HAU, Hisar, India	India
LL-147	Cultivar	PL 284-67 × NP 21	PAU, Ludhiana, India	India
NDL 1	Cultivar	PL 406 × PRECOZ	NDUAT, Faizabad, India	India
PANT-L-234	Breeding line	Selection from P 230	GBPUAT, Pant Nagar, India	India
PANT-L-639	Cultivar	L 9-12 × TYPE 8	GBPUAT, Pant Nagar, India	India
PL-04	Cultivar	UPL 175 × (PL 184 × P 288)	GBPUAT, Pant Nagar, India	India
PL-05	Cultivar	L 4606 × LG 171	GBPUAT, Pant Nagar, India	India
RANJAN	Cultivar	Mutant of B77	Berhampur, West Bengal, India	India
SHALIMAAR MASOOR	Cultivar	Local selection from Jammu & Kashmir	SKUAST, Srinagar, India	India
T-36	Cultivar	Local selection from Badaun, UP	CSAUAT, Kanpur, India	India
VL-1	Cultivar	Local selection from UP hills	VPKAS, Almora, India	India
VL-103	Cultivar	Local selection from UP hills	VPKAS, Almora, India	India
VL-4	Cultivar	Local selection from UP hills	VPKAS, Almora, India	India
WBL-58	Cultivar	JLS 2 × T 36	Berhampur, West Bengal, India	India
WBL-77	Cultivar	Mutant of BR 25	RAU, Dholi, India	India
B-77	Cultivar	Local selection from Jorhat, Assam	Berhampur, West Bengal, India	India
BR-25-1	Cultivar	Local selection from Bihar	Dhioli, Bihar, India	India
EC-208362	Land race	Unknown	NBPGR, India	India
IPL-222	Breeding line	ILL 7657 × DPL 61	IIPR, Kanpur, India	India
IPL-223	Breeding line	ILL7663 × DPL 61	IIPR, Kanpur, India	India
IPL-224	Breeding line	DPL 44 × DPL 62 × DPL 58	IIPR, Kanpur, India	India
IPL-326	Breeding line	P 2016 × DPL 62 × DPL 58	IIPR, Kanpur, India	India
IPL-327	Breeding line	ILL 7659 × DPL 58 × KL 178	IIPR, Kanpur, India	India
IPL-328	Breeding line	VKS 16/21 × DPL 62	IIPR, Kanpur, India	India
IPL-529	Breeding line	ILL 5714 × KL 178 × DPL 62	IIPR, Kanpur, India	India
IPL-532	Breeding line	EC 208355 × DPL 62	IIPR, Kanpur, India	India
IP-534	Breeding line	KL 178 × DPL 62	IIPR, Kanpur, India	India
IPL-230	Breeding line	IPL 522 × DPL 62	IIPR, Kanpur, India	India
IPL-231	Breeding line	DPL 62 × JL 1	IIPR, Kanpur, India	India
IPL-10800	Breeding line	ILL 7657 × DPL 61	IIPR, Kanpur, India	India
IPL-11702	Breeding line	SEHORE 74-3 × JL 3	IIPR, Kanpur, India	India

Imperial Biosciences, USA. Initially, 534 SSR markers were used to identify polymorphic markers among the 20 accessions of lentil and then 75 SSR markers identified as polymorphic were used to genotype a total of 96 accessions having early, medium, and late flowering. The CTAB extraction protocol described earlier by Doyle and Doyle (1987) and Abdelnoor et al. (1995) were used to isolate the genomic DNA with certain changes (Kumar et al. 2014). The PCR reaction contains a total volume of 20 μl consisting of 50–100 ng genomic DNA, 1X PCR buffer with 1.5 mM MgCl2, 200 μM each of dNTPs (Bangalore Genei, Bengaluru), 0.5 U Taq DNA Polymerase (Bangalore Genei, Bengaluru), and 40 pmoles each of forward and reverse primers. The PCR amplification was performed in a G-STORM PCR System with a program for an initial denaturation of 94 °C for 4 min followed by 39 cycles of 94 °C for 1 min, annealing 50–55 °C for 1 min, elongation 72 °C for 1 min, and a final extension at 72 °C for 15 min.

Polyacrylamide gel electrophoresis (PAGE) and silver staining

The amplified products were run on 10% polyacrylamide gel along with 1 kb DNA ladder using high throughput vertical gel electrophoresis (CBS, USA). The bands were visualized by following silver staining (Benbouza et al. 2006). Each SSR marker amplified single to multiple bands. The multiple bands amplified in a genotype were considered separate loci and size variation in the amplified product of the targeted locus across the accessions was considered as allelic variants for that loci. Size of amplified product of each genotype was calculated online using fragment calculator (http://biotools.nubic.northwestern.edu/SizeCalc.html).

Statistical analysis

Mean and range of flowering time was analyzed using statistical tools (MS Excel 2007). Analysis of variance was performed by considering the recorded data over the years and locations as replication using R software (R Core Team 2013). A *T*-test was used to test the significance of a particular genotype in a specific environment for flowering time over the mean value of all studied environments. The mean of square due to error and genotypes was used for environmental variance (σ^2e) and phenotypic variance (σ^2p), respectively, to calculate the broad sense heritability (h^2 *bs*) using the following formula.

$$h^2\ bs = \left(\sigma^2\ \mathrm{p} - \sigma^2\ \mathrm{e} = \sigma^2\mathrm{g}\right)/\sigma^2\ \mathrm{p} \times 100$$

Population structure analysis

Model-based Bayesian clustering analysis was performed to infer the genetic structure and number of subgroups in the studied germplasm set using the STRUCTURE version 2.1 software (Pritchard et al. 2000a, b). This approach uses multi-locus genotypic data, such as SSRs without prior knowledge of their population affinities and assumes loci in Hardy-Weinberg equilibrium in order to assign individuals to clusters/groups (K). Admixture model with correlated allele frequencies was used to estimate each of the K clusters. For this, number of presumed subpopulations (K) was set from 1 to 10 for each accession along with the percentage of its genome derived from each cluster and used higher levels of length of the burning period (i.e. 1,000,000) and number of Markov chain Monte Carlo (MCMC) replication after burning (i.e., 1,000,000) for getting reliable subgroups. Further, each K was repeated ten times, i.e., iterations/replication in order to get consistent and reliable subgroups. DK method was used to determine the number of subpopulations (Evanno et al. 2005) using STRUCTURE HARVESTER available online (http://taylor0.biology.ucla.edu/structureHarvester/). Those markers had an allelic frequency less than 5%, which were excluded from the analysis (Wilson et al. 2004).

Marker-trait association analysis

The software program TASSEL version 2.1 (http://www.maizegenetics.net) was used to calculate the associations between individual markers and flowering time (over locations and years) following the mixed linear model (MLM, Q + K) approach. A structured association approach could correct false associations using the Q-matrix of population membership estimates. The marker data, Q values derived from the software STRUCTURE, and flowering data along with marker-based kinship matrix (K) obtained from TASSEL were used as covariates during MLM analysis. Further, among different options available within MLM, the widely adopted approach called "optimum levels of compression in combination with P3D" for variance component estimation was used for association analysis. The significance of marker-trait associations is determined at $P < 0.05$ and phenotypic variation explained (r^2) by each associated marker is stated (Li et al. 2012; Topal et al. 2004).

Results

Phenotypic variability and heritability

Analysis of variance (ANOVA) over four environments (two years and two locations) showed highly significant effects of year, locations, and genotypes on flowering time in lentil

(Table 2). Besides, genotypes also responded differently over locations and years as interactions of genotype with year and locations were observed highly significant in the present investigation ($P< 0.01$). The broad sense heritability was high (h^2bs=0.93) for this trait (Table 2).

In the present study, mean of 96 genotypes for flowering time was 47.8 ± 0.43 days in 2015 and 43.6 ± 0.24 days in 2016 at Dharwad location, while it was 75.1 ± 1.39 days in 2014-15 and 71.1 ± 1.50 days in 2015-16 at Kanpur location (Table 3). These results indicated that the long photoperiod (13 h) probably promoted early flowering at the Dharwad location. In spite of this, genotypic differences were significant at both locations. Among the present 96 accessions, four genotypes (i.e., ILWLS118, IPLS09-17, IPLS09-5, and IPLS 09-34) were identified significantly earlier in flowering ($P\leq 0.05$) over mean flowering time. However, each genotype did not differ significantly ($P<0.05$) from the mean flowering time calculated over four environments. These results indicated that these genotypes are not photo-thermo sensitive (Table 3).

Genetic diversity and population structure analysis

In the present study, out of 96 accessions, 20 accessions were first genotyped with 534 SSR markers in order to use only polymorphic markers for further genotyping of the entire set that led to saving time and money required to genotype 96 accessions of lentil. Only 75 SSR markers (13.9%) were found polymorphic. PIC and gene diversity of these markers ranged from 0.02 to 0.90. Polymorphic SSR markers generated 697 alleles from 266 loci with a range of two to 16 alleles per locus among the 96 accessions. Model based population structure analysis led to the identification of two distinct subpopulations among these accessions. Fifty-seven accessions were grouped in subpopulation I with a range of 40-72 days to flower initiation while the remaining 39 genotypes formed the second subpopulation with a range of 54 to 69 days to flower (Fig. 1). In addition to this, these subpopulations were broadly clustered according to their breeding history and subpopulation I contained breeding lines derived from exotic lines, while the subpopulation II contained most of the cultivars released in India and breeding lines involved these cultivars as one of the parents.

Table 2 Analysis of variation and heritability for days to flower among the 96 genotypes of lentil

Source	DF	MS	Heritability (h^2bs)
year	1	3295.9**	
location	1	83216.8**	
genotype	95	208.3**	0.93
genotype × location	95	241.5**	
genotype × year	95	1082.8**	
Error	287	13.2	

**Significant at $P\leq 0.01$

Marker-trait association analysis

In the present study, a total of 26 marker trait associations (MTAs) for the flowering time were identified over the two locations and two years using most accepted maximum likelihood model (P3D mixed linear model with optimum compression) of the MTA analysis. Our results showed a significant association of 13 EST-SSR and 13 genomic SSR markers with flowering time at p-value ($P<0.05$). These markers explained 2.1 to 21.8% of the total phenotypic variation (R^2) in flowering time (Table 4). Among 13 EST-SSR markers, PBLAC 0250 marker explained 21.8% of the total phenotypic variation (PV) in flowering time. While, another two EST-SSR markers, namely PBALC 0207 and PBALC 0242, showed a significant association at <0.04 and <0.0008 probabilities, respectively, across the locations and explained from 4.6 to 16.8% of the total phenotypic variation in flowering time. However, some MTAs with high PV were detected in a particular environment. For example, EST-SSR markers 'PBALC 0250' showed significant association with flowering time ($P<0.02$) and explained 19.5 to 21.8% of total phenotypic variation at Kanpur location (Table 4). Likewise, EST-SSR markers 'PBALC 0353' showed significant association with flowering time ($P<0.03$) and only explained 21.4% of the total phenotypic variation at the Dharwad location (Table 4). However, none of the 13 MTAs based on genomic SSR for flowering time were observed across all studied environments and these MTAs also explained the low amount of total phenotypic variation that ranged from 1.8 to 10.4% (Table 4). Only one genomic SSR marker ALD 40 showed highly significant ($P<0.0007$) association with flowering time and explained 10.2% of total phenotypic variation at the Dharward location in 2015 (Table 4).

Mining of the elite alleles

The phenotypic allele effect of each SSR that significantly associated with flowering time was shown in Suppl. Table S1. Among the markers associated with flowering time, EST-SSR marker PBALC 0207 was associated with flowering time across locations. Its allele PBALC0207-144 had the most positive phenotypic effect and ability to increase the flowering time by 7.28 and 9.26 days, whereas allele PBALC 0207-169 had the most negative phenotypic effect (−5.39 and -4.44 days) in 2014-15 and 2015-16, respectively at Kanpur location. These alleles (PBALC 0207-144 and PBALC 0207-169) also had a positive phenotypic effect (0.81 days) and negative phenotypic effect (-0.09 days) in 2014 at the Dharward location. For EST SSR marker PBALC 0250 that showed

Table 3 Mean, range, and s.e. over 96 accessions and details of the identified early flowering genotypes at different locations/ years

Item/accession name	Locations				
	Kanpur		Dharwad–off-season		mean ± s.e. over environments
	2014-15	2015-16	2014	2015	
Mean (over 96 accessions)	75.1 ± 1.39	71.1 ± 1.50	47.8 ± 0.43	43.6 ± 0.24	-
Range (over 96 accessions)	37- 104	38- 97	42- 57	37-53	-
LSD (P<0.05)	10.09		6.4		-
Flowering time (days) in selected early flowering genotypes					
ILWLS 118	37 (n.s.)	38 (n.s.)	42 (n.s.)	37 (n.s.)	38.5 ± 1.19
IPLS 09-17	39 (n.s.)	39 (n.s.)	47 (n.s.)	39 (n.s.)	41.0 ± 2.00
IPLS 09-05	44 (n.s.)	51 (n.s.)	43 (n.s.)	44 (n.s.)	45.5 ± 1.85
IPLS 09-34	43 (n.s.)	45 (n.s.)	43 (n.s.)	39 (n.s.)	42.5 ± 1.26
Environmental conditions during flowering time -					
Photoperiod (h)	10	10	13	13	-
Mean temperature (°C)	25.5	21.9	20.5	26.5	-

Note: n.s: non-significant at P<0.05 and 3 degree of freedom over mean value of four environments

significant association with flowering at Kanpur location, the allele PBALC 0250-200 had a positive phenotypic effect and the ability to increase the flowering time by 16.92 days, while another allele had the most negative effect (-11.38 days) in 2014-15. Similarly, these alleles also had the most positive (10.33 days) and negative (-9.85 days) phenotypic effects in 2015-16. In addition to this, other alleles (i.e., PBALC 0250-160 and PBALC 0250-150) also had separately almost similar positive (10.54 days) and negative phenotypic effects (-9.89 days) in this year (Suppl. Table S1). The allele PBALC 0353-97 had the most positive phenotypic effect and was able to increase the flowering time by 3.71 days, whereas two alleles,

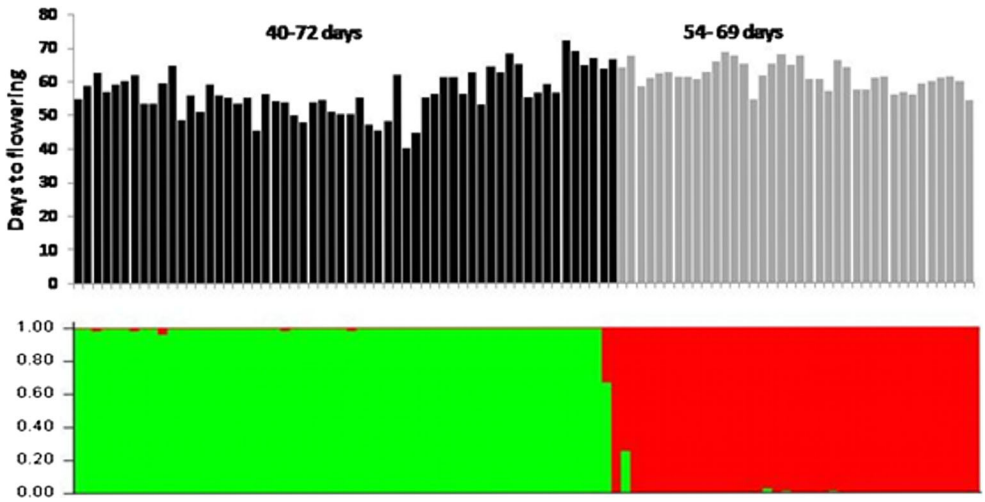

Fig. 1 Clustering of 96 accessions of lentil on the basis of flowering time (above) and model based population structure analysis using polymorphic SSR markers; **Sub-population I (1-57)**- 1) IPL-121; 2) IPL-133; 3) IPL-203; 4) SEHORE-74-3; 5) IPL-218; 6) IPL-219; 7) IPL-221; 8) IPL-325; 9) IPL-324; 10) IPL-519; 11) IC-560291; 12) IC-560322; 13) IPLS-09-06; 14) IC-560113; 15) IPLS-COLD; 16) IPLS-09-23; 17) IPLS-09-32; 18) IC-560117; 19) IPLS-09-03; 20) IPLS-09-11; 21) IPLS-09-10; 22) IPLS-09-38; 23) IPLS-09-22; 24) IC-560150; 25) IPLS-09-37; 26) IPLS-09-36; 27) IPLS-09-14; 28) IPLS-09-33; 29) ILS-09-01; 30) IC-560111; 31) IPLS-09-27; 32) IPLS-99/209; 33) IPLS-09-34; 34) IPLS-09-05; 35) IPL-09-08; 36) IPLS-09-17; 37) ILWLS-118; 38) ILWL-118; 39) L-4603; 40) IPL-220; 41) DPL-58; 42) PRECOZ; 43) PL-02; 44) ILL-7663; 45) DPL-15; 46) IPL-81; 47) IPL-406; 48) DPL-62; 49) ILL-6002; 50) IPL-316; 51) IPL-526; 52) JL-01; 53) DPL-59; 54) LL-864; 55) ILWL-248; 56) IPL-321; 57) IPL-M-08; **Sub-population II- (58-96)** –58) IPL-98/193; 59) JL-03; 60) K-75; 61) KLS-218; 62) L-4076; 63) L-4147; 64) HUL-57; 65) L-9-12; 66) LH-84-8; 67) LL-147; 68) NARENDRA MASSOOR-1; 69) PANT-L-234; 70) PANT-L-639; 71) PL-04; 72) PL-05; 73) RANJAN; 74) SHALIMAAR MASOOR; 75) T-36; 76) VL-1; 77) VL-103; 78) VL-4; 79) WBL-58; 80) WBL-77; 81) B-77; 82) BR-25-1; 83) EC-208362; 84) IPL-222; 85) IPL-223; 86) IPL-224; 87) IPL-326; 88) IPL-327; 89) IPL-328; 90) IPL-529; 91) IPL-532; 92) IP-534; 93) IPL-230; 94) IPL-231; 95) IPL-10800; 96) IPL-11702

Table 4. Marker-trait associations (MTAs) identified for flowering time in lentil

S. No.	Locus name	Marker name	Primer sequence (5'-3')	R^2 (P value)			
				Location—Kanpur		Location—Dharwad (off-season)	
				Year 2014-15	Year 2015-16	2014	2015
EST-SSR							
1	PBALC 0250	SSR3	F:TGCATTTACCATCATCTCTAAC R:TGATTGATTCGGTACTTTTTG	19.5 (0.02)	21.8 (0.02)	-	-
2	PBALC 0370	SSR9	F:AGAAATGGAGTCAGCTTGAA R:ACAAACAAACACCAACATTTC	4.5 (0.05)	9.1 (0.003)	-	-
3	PBALC 0353	SSR10	F:CCATAACAGACAAAACCCTA CT R:ATTCTCAAAGCCCATTTAGTT	-	-		21.4 (0.03)
4	PBALC 0333	SSR12	F:CACATCCAACAATACAGATGA R:AAGGCCACTGAAGCAGAG	-	-	8.3 (0.01)	-
5	PBALC 0664	SSR35	F:GATTGTATTGCATACGCTTGT R:CCAGAAAGGAAAAATCAAAA	-	-	-	4.3 (0.02)
6	PBALC 0554	SSR43	F:GAGACACCATAGATGGATTTG R:ACAACACACGCTACTTCACTT	-	3.1 (0.05)	-	-
7	PBALC 0631	SSR51	F:TTCAGAAAGTGAAGCATTAGG R:AGGAGGACAGTTATTTTCAGG	3.1 (0.01)		-	-
8	PBALC 0207	SSR63	F:ATGGAACACAAACCAATACAC R:TGTGGTGTCCTTTGTAGAAGT	10.7 (8.3 x 10^{-4})	11.1 (9.9 x 10^{-4})	16.4 (9.8 x 3.7 x 10^{-2})	-
9	PBALC 0229	SSR70	F:AAGGGCAACCACTGATATAAT R:TATAAGCTTGGTCATGGTAGC	6.4 (0.04)	-	-	-
10	PBALC 0242	SSR72	F:CTACAAGTTGCAATGTTTCCT R:CAAAGGCAGAATATGATGAAT	4.6 (0.04)		14.9 (0.02)	16.8 (3.7 x 10^{-2})
11	PBALC 209	SSR85	F;GGAGTTGGTTAGAAGGAAAGA R:CTAGATATCATCGATCCATCC	-	2.3 (0.02)		3.6 (0.03)
12	PBALC 370	SSR94	F:AGAAATGGAGTCAGCTTGAA R:ACAAACAAACACCAACATTTC	-	-	-	3.8 (0.04)
13	PBALC 0683	SSR244	F:TTGTGCTTATTGGAATGAAAG R:CAAACTATGGAAGTGGAAGAG	-	-	-	4.0 (0.02)
Genomic SSR							
14	ALD P2	SSR17	F:CGGCGGATGAAACTAAAG R:CATTTCCTTCACAAACCAAC	-	7.2 (0.04)	-	7.8 (7.6 x 10^{-2})
15	ALD 30	SSR58	F:CAAACAGTACAAGGAAAGGAG R:CTGACTGAGCTGCTTGAAC	3.9 (0.01)	-	-	-
16	ALD P8	SSR95	F:GCTCGCATTGGTGAAAC R:CATATATAGCAGACCGTG	-	2.3 (0.02)	7.9 (0.01)	
17	ALD 40	SSR102	F:GCGGCGAGCAAATAAAT R:GGAGAATAAGAGTGAAATG	-			10.2 (7.6 x 10^{-4})
18	ALD 40	SSR104	F:GCGGCGAGCAAATAAAT R:GGAGAATAAGAGTGAAATG	5.0 (2.1 x 10^{-2})	4.7 (0.01)	-	4.0 (0.02)
19	ALD 22	SSR126	F:CATCTGAGGAGTTGCTTGC R:GTTCACACGGCTGTAAGTC		2.3 (0.01)	-	-
20	ALD 31	SSR145	F:GGTCTATTTGCGTGCC R:GCAAGTCCTTATACCAAG	1.9 (0.02)	2.1 (0.01)	-	-
21	ALD P1 (117)	SSR157	F:GAGAGATACGTCAGAGTAG R:GATTGTGCTTCGGTGGTTC		1.8 (0.04)		4.5 (0.01)
22	ALD 16	SSR184	F:GACTCTCCAAGGATTCACTC R:GCACAGGTCGTCATTATTAC	2.4 (0.02)	3.1 (0.01)	-	-
23	ALD 28	SSR196	F:GGTAGTGGTGAGGAATGAC R:GCATCACTGCAACAGACC		3.1 (0.01)	-	-
24	ALD 29	SSR211	F:CATAGGTACCAAGGAGATG R:GCGAAGTCTCTGACAACAC	6.0 (0.02)		-	-
25	ALD 42	SSR222	F:CCGTAAGAATTAGGTGTC R:GGAAAATAGGGTGGAAAG	-	3.4 (0.01)	-	-
26	ALD P 5	SSR224	F:CAAACAGTACAAGGAAAGGAG R:CTGACTGAGCTGCTTGAAC	-	3.9 (0.03)	-	3.6 (0.04)

namely PBALC 0353-125 and PBALC 0353-107, were able to decrease the flowering time of 1.78 and 1.36 days, respectively, only at the Dharwad location in 2015.

Alignment of EST sequences to identify their role in controlling the flowering time

EST-SSR markers used in the present study have been developed from gene sequences of lentil, i.e., express sequence tags (ESTs). Hence, EST sequences of those SSR markers that explained >10% of the total phenotypic variance across environments or in a particular environment were aligned with the nucleotide database of NCBI in order to know their similarity with gene sequences available in the public domain. The results showed close similarity between lentil EST sequences and the genes of other legumes (Table 5).

Discussion

Flowering time (FT) contributes significantly to crop adaptation and grain yield (Kumar and Van Rheenen 2000; Anbessa et al. 2006). However, early flowering in lentil generally leads to poor biomass (Kumar et al. 2014). Therefore, lentil breeders target the development of short duration cultivars having high biomass. For this purpose, identification of diverse genetic resources for earliness, their use for hybridization, and selection of recombinant having high biomass along with earliness are the logical steps that a breeder takes. Flowering time is highly influenced by day length (photoperiod) and temperatures (Welch et al. 2005). Therefore, flowering in crop plants, including lentil responds differently at different latitudes under different climate regimes, seasons, sowing dates, and altitudes (Summerfield and Roberts 1988). In our study, analysis of variance showed significant effects of locations and years on flowering time because two locations used in this study differed for photoperiod and temperature at the time of flowering (Table 3). For example, high temperature (25.5°C and 26.5°C) resulted in early flowering at the Kanpur location in 2014-15 and Dharwad location in 2015-16, respectively, in comparison to 2013-14 at Kanpur (22.4°C) and Dharwad (20.5°C) among the four selected genotypes (see Table 3). This shows that warm conditions promote early flowering in lentil. Erskine et al. (1990) also reported that Indian germplasm is more sensitive to temperature and less sensitive to photoperiod compared to the genetic materials belonging to West Asia. Other studies reported that multiple signaling pathways, including the components of the photoperiod pathway, are involved in promoting flowering under warm conditions (Thines et al. 2014). The genes sensitive to high temperature might be a part of genes that are involved in the photoperiod pathways as reported earlier (Jung and Muller 2009). The photoperiod pathways have the two important genes, i.e., CONSTANS (CO) and FLOWERING LOCUS T (FT), which regulate flowering time (Imaizumi 2010). It has also been reported in *Arabidopsis* and cereals that these genes are controlled by several positive and negative regulators (Jung and Muller 2009). In previous studies, the allelic variation has been reported to influence flowering time among the genotypes of crop plants. For example, in rice, the CO ortholog *Hd1* has allelic variation, which controls much of the phenotypic variation in flowering time (Takahashi et al. 2009). In the present study, genotypes differ in flowering time under different environmental conditions. This could be the outcome of the photo-thermo sensitivity genes present in the background of these genotypes. However, non-significant differences in flowering time over the locations and years among the selected early flowering genotypes indicated their photo-thermo insensitivity.

Association mapping is widely used in crop plants to identify genomic regions associated with a trait of interest that would not be picked up by analysis of a bi-parental population. However, a high frequency of spurious associations is also detected through association mapping in those cases where population structure is not to be taken into consideration (Rosenberg and Nordborg 2006). Therefore, we used model-based analysis for determining population structure of studied genotypes and its information was used in association mapping analysis to reduce the chance of false positive association. In our study, the present genotypes were structured into two distinct subpopulations, and early, medium, and late flowering genotypes were distributed in each subpopulation. Moreover, genotypes in these subpopulations were broadly clustered according to their breeding history. One subpopulation contained breeding lines that derived from exotic lines, while another one comprised the Indians' cultivars and those breeding lines which have these cultivars as one of the parents in their breeding history. Overall, the nature of population

Table 5 Nucleotide sequence alignment results of the EST sequence of SSR markers associated with flowering time in the present study

Locus name	% identity with gene sequences	Protein encoded by gene
PBALC 0250	91%	50S ribosomal protein of *Medicago truncatula*
PBALC 0353	86%	snakin-2-like of *Cicer arietinum*
PBALC 0242	100%	actin isoform PEAc14-1 of *Pisum sativum*
PBALC 0207	51%	uncharacterized protein of *Cicer arietinum*

structure in the present study suggests that flowering time has no bias within a specific sub-population. It shows that the population is appropriate for association analysis and association of marker loci with flowering time recorded in the present study should be real and the chance of obtaining many false positives would be less (Pritchard et al. 2000a; Cardon and Palmer 2003; Gupta et al. 2005; Malysheva-Otto et al. 2006; Ostrowski et al. 2006; Jaiswal et al. 2012).

In the present study, 13.9% of SSR markers were polymorphic, while a high level of polymorphism (66% of genomic SSR and 47.5% of EST-SSR markers) has been observed in earlier studies (Kaur et al. 2011; Kumar et al. 2014). This low-level polymorphism of SSR markers probably resulted due to the use of elite breeding lines in the present study. Previously, a small population size having <100 individuals was used in many association mapping studies to identify QTLs in crop plants (Szalma et al. 2005; Salvi et al. 2007). However, a population of 100 individuals can be sufficient to identify the QTL(s) for a trait of interest, if we use breeding lines in association mapping (Bradbury et al. 2011). Also, use of diverse breeding lines makes association mapping more cost-effective because these are routinely recorded for phenotypic traits. Moreover, elite breeding materials are generally narrow in the genetic base. If QTLs for a trait are identified in the background of such materials, they can be used directly in marker-assisted selection (Kumar et al. 2017). Previously, molecular marker-trait association analysis was conducted for a number of economically important traits in the legumes as well other crops (Sun et al. 2011; Niu et al. 2013; Zhang et al. 2014; Cheng et al. 2015; Bajaj et al. 2016; Contreras Soto et al. 2017). In rice, association mapping detected 76 significant (P<0.5) trait—marker associations between agronomic traits and SSR markers, and among them 11 significant MTAs explained >10% of the total genetic variation (Zhang et al. 2014). Similarly, in barley, marker loci associated with plant height and stem diameter explained 4.59-17.48% of the total variation (Sun et al. 2011). In our study, association mapping detected 26 MTAs that are significantly associated with flowering time. Both genic and genomic SSR markers were found to be associated with flowering time. The percentage of total phenotypic variation explained by these MTAs ranged from 2.1 to 21.8%. Interestingly, EST-SSR markers showed highly significant association with flowering time along with high phenotypic variance. These markers can be closely associated with the genes that control flowering time in lentil and hence can be used as functional markers in a breeding program. Thus, our results show that a number of genes/QTLs are responsible for controlling flowering time in lentil. However, previously, a single major gene or QTL was reported for flowering time and a recessive allele of a major Mendelian locus *Sn* conferred an early flowering under non-inductive short-day conditions (Sarker et al. 1999; Kahriman et al. 2014). While other genetic studies reported that flowering time in lentil is controlled by two to four QTLs. These QTLs can be distributed on two or more than two linkage groups (Tahir et al. 1994; Fratini et al. 2007; Tullu et al. 2008). More recently, a number of QTLs having a main effect and interactions between QTLs have also been reported for days to 50% flowering in a bi-parental mapping population (Saha et al. 2013). However, association mapping has an ability to detect novel QTLs because it uses diverse germplasm that has allelic diversity. Therefore, marker locus associated with flowering time in the present study might be different from QTL/genes identified in earlier studies. One of the reasons for this is many QTLs are not segregated in the bi-parental population because only two alternative alleles are present at a limited number of loci for a trait in such kind of mapping population (Jaiswal et al. 2012). In our study, markers associated with the flowering time explained phenotypic variation from 2.1 to 21.8%, which is comparatively lower than the phenotypic variation observed by analysis of bi-parental populations. However, it is not surprising to observe lower phenotypic variation in association mapping due to several reasons, including low LD and low allele frequency (Yan et al. 2011). In earlier studies, a marker associated significantly with a trait of interest explained low phenotypic variation in association mapping studies (Roy et al. 2010; Pasam et al. 2012).

The longer reproductive duration provides enough time to synthesize greater sink size leading to higher grain yield in lentil (Erskine et al. 1990). In another study, a reproductive period of 35-40 days has been reported as enough to develop high yielding genotypes (Kumar and Srivastava 2014). Thus, combining the early flowering (<50 days) with 40 days of reproductive periods can help in the development of high yielding super early genotypes in lentil (i.e., maturing <100 days). In South Asian countries, these super early genotypes can lead to an expansion of lentil in rice fallow areas and increase the yield productivity of lentil by avoiding the terminal heat, which has become a major challenge under changing environments (Choudhary et al. 2016). As, in the present study, several alleles of the associated markers have contributed significantly to reducing flowering time. Moreover, the sequence of express sequence tags (ESTs) belonging to EST-SSR markers associated with flowering time showed high-level similarity with the gene sequences of closely related food legumes. The roles of these genes in controlling flowering time have also been reported in earlier studies (Ito et al. 2000; Roxrud et al. 2007; Zhang et al. 2009, 2013; Kandasamy et al. 2002; Šlajcherová et al. 2012). Therefore, in the present study, EST-SSR markers could be associated with those genes that are involved in controlling flowering time in lentil. These EST-SSR markers can be used as functional markers and their allelic variants, responsible for reducing flowering time, can provide an opportunity to use them in lentil breeding programs for precise introgression of early

flowering QTL in the background of high biomass genotypes to develop high yielding super early genotypes in lentil.

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Author contributions JK designed the work, analyzed and interpreted the results, and drafted the manuscript. RSB, SG, SD, and PG helped in collection and tabulation of genotypic and phenotypic data. NPS helped to edit the revised manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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