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A genome-scale integrated approach aids in genetic dissection of complex flowering time trait in chickpea

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Abstract A combinatorial approach of candidate gene-based association analysis and genome-wide association study (GWAS) integrated with QTL mapping, differential gene expression profiling and molecular haplotyping was deployed in the present study for quantitative dissection of complex flowering time trait in chickpea. Candidate gene-based association mapping in a flowering time association panel (92 diverse *desi* and *kabuli* accessions) was performed by employing the genotyping information of 5724 SNPs discovered from 82 known flowering chickpea gene orthologs of *Arabidopsis* and legumes as well as 832 gene-encoding transcripts that are differentially expressed during flower development in chickpea. GWAS using both genome-wide GBS- and candidate gene-based genotyping data of 30,129 SNPs in a structured population of 92 sequenced accessions (with 200–250 kb LD decay) detected eight maximum effect genomic SNP loci (genes) associated (34 % combined PVE) with flowering time. Six flowering time-associated major genomic loci harbouring five robust QTLs mapped on a high-resolution intra-specific genetic linkage map were validated (11.6–27.3 % PVE at 5.4–11.7 LOD) further by traditional QTL mapping. The flower-specific expression, including differential up- and down-regulation (>three folds) of eight flowering time-associated genes (including six genes validated by QTL mapping) especially in early flowering than late flowering contrasting chickpea accessions/mapping individuals during flower development was evident. The gene haplotype-based LD mapping discovered diverse novel natural allelic variants and haplotypes in eight genes with high trait association potential (41 % combined PVE) for flowering time differentiation in cultivated and wild chickpea. Taken together, eight potential known/candidate flowering time-regulating genes [*efl1* (early flowering 1), *FLD* (Flowering locus D), *GI* (GIGANTEA), *Myb* (Myeloblastosis), *SFH3* (SEC14-like 3), *bZIP* (basic-leucine zipper), *bHLH* (basic helix-loop-helix) and *SBP* (SQUAMOSA promoter binding protein)], including novel markers, QTLs, alleles and haplotypes delineated by aforesaid genome-wide integrated approach have potential for marker-assisted genetic improvement and unravelling the domestication pattern of flowering time in chickpea.

Keywords Chickpea · Flowering time · GBS · GWAS · QTL · SNP

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Introduction

Chickpea (*Cicer arietinum* L.) is an annual self-pollinated and diploid crop species with a genome size of ~ 740 Mbp whose domestication can be traced to regions of South

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Eastern Turkey and adjacent Syria (Fertile Crescent) around 10,000 years ago (Kumar and Abbo 2001; Abbo et al. 2003; Berger et al. 2005; Burger et al. 2008; Toker 2009; Jain et al. 2013; Kujur et al. 2013; Varshney et al. 2013; Saxena et al. 2014a). Chickpea is broadly categorized into two cultivar types- *desi* (purple flower and small seed size with yellow brown to light brown coloured seed coat) and *kabuli* (white flower and large seed size with beige coloured seed coat) in accordance with their distinctive plant architectures and diverse gene pools-derived domestication patterns. Globally, chickpea is the third most important food legume complementing cereals and considered a staple protein source for human diet (Kumar and Abbo 2001; Kumar et al. 2011; Gaur et al. 2012; Varshney et al. 2013). To meet the ever increasing dietary demand of fast growing population, the chickpea production needs to be enhanced. However, several abiotic stress factors, including drought impart a serious threat to the production of chickpea. Chickpea being a rainfed crop is more prone to drought (Kumar et al. 1996). Therefore, it is imperative to develop chickpea cultivars with early flowering, growth vigor and maturity to escape the end-of-season drought stress affecting their plant types (Kumar and Abbo 2001). The number of days to flowering is a key yield contributing trait that determines the rate of pod setting and thus seed/pod yield in chickpea under rainfed cropping patterns facing terminal drought conditions, specifically in the semi-arid tropical regions (Turner et al. 2007; Aryamanesh et al. 2010; Kashiwagi et al. 2013). The flowering time, a defining feature of vegetative-to-reproductive transition, is highly attuned with changing climates and diverse environmental factors in chickpea. This is also accompanied with significant changes in a wide range of other developmental traits, including maturity time and pod/seed yield and therefore, complex genetic interaction of flowering time with other developmental processes in chickpea is anticipated.

Vernalization is an important key response of flowering time that has been considered the most crucial evolutionary bottleneck during chickpea domestication leading towards evolution of presently cultivated vernalization insensitive *desi* and *kabuli* cultivars especially from the vernalization sensitive wild progenitor *Cicer reticulatum* (Summerfield et al. 1989; Abbo et al. 2003; Berger et al. 2005; Burger et al. 2008; Toker 2009; Abbo et al. 2014). This implicates that flowering time is a major adaptive domestication trait targeted in selective breeding of chickpea. Collectively, flowering time in chickpea is a major component of its adaptability in diverse agro-climatic conditions beside playing a pivotal role in defining its productivity, especially under short season environments encountering terminal drought and heat stress (Subbarao et al. 1995; Kumar and Abbo 2001; Hegde 2010). It is thus desirable to genetically dissect the flowering time in order to fine-tune chickpea cultivars grown under rainfed farming system and uncover the domestication pattern of this trait. The cues obtained from these studies will be useful to develop early flowering/maturing chickpea cultivars by which the overall agronomic practices, including fertilizer and labour cost and time involved in raising their plants in field as well as the effect of various abiotic and biotic stresses, including end-of season terminal drought can be minimized in chickpea (Lichtenzveig et al. 2006; Turner et al. 2007; Zaman-Allah et al. 2011). This will subsequently serve as a beneficial strategy to achieve the prime objectives of current genomics and breeding research with an aim to develop high-yielding stress tolerant chickpea cultivars.

The flowering time is a complex quantitative trait governed by multiple genes. In model crop plants like *Arabidopsis*, around 100 potential genes controlling flowering time have been identified and their complex regulatory interactions/networks underlying the target trait are being deciphered (Andres and Coupland 2012; Song et al. 2013). In legumes, a diverse array of gene (gene families) homologs that are known to control flowering time pathways in *A. thaliana* have been catalogued. A few selected genes (~20) of these, have been functionally characterized and utilized for understanding the genetic control of flowering time-related traits particularly in pea and soybean (Weller et al. 2009; Kim et al. 2012; Watanabe et al. 2012; Weller and Martínez 2015). Considering the well-documented functional roles of *FT* (*Flowering Locus T*) genes in integration of signals from environments with that of photoperiod detection sites during vegetative to reproductive phase transition at the time of flowering, these genes are of much interest for pea, *Medicago*, *Lotus* and soybean (Kong et al. 2010; Laurie et al. 2011; Pin and Nilsson 2012; Yamashino et al. 2013; Zhai et al. 2014).

Till date, very limited attentions have been paid towards understanding of the genetic inheritance pattern and dissection of the genetic architecture of this complex flowering time trait in chickpea (Or et al. 1999; Kumar and Van Rheenen 2000; Anbessa et al. 2006; Cobos et al. 2007; Pierre et al. 2008; Aryamanesh et al. 2010; Pierre et al. 2011; Zhang et al. 2013). The classical genetic analyses, for instance, documented different major gene loci [*efl1* (ICCV 2), *efl2*/*ppd* (photoperiod) (ICC 5810), *efl3* (BGD 132) and *efl4* (ICC 16641 and ICC 16644)] with varied allelic segregation patterns, which have got distinct impact on flowering time adaptation in diverse chickpea accessions (Or et al. 1999; Hegde 2010; Gaur et al. 2014; Weller and Martínez 2015). Molecular mapping of a very small number (about 10 QTLs) of low-resolution QTLs (quantitative trait loci) governing flowering time on multiple chromosomes and their colocalization with diverse known flowering time gene homologs [like *Efl1*, *Efl2*, *LFY* (LEAFY) and FT gene families] have been documented in chickpea

(Cho et al. 2002; Lichtenzveig et al. 2006; Anbessa et al. 2006; Cobos et al. 2007, 2009; Radhika et al. 2007; Aryamanesh et al. 2010; Hossain et al. 2010; Gowda et al. 2011; Rehman et al. 2011; Cho et al. 2012; Hiremath et al. 2012; Vadez et al. 2012; Jamalabadi et al. 2013; Varshney et al. 2014). Unfortunately, until yet none of these identified major QTLs controlling flowering time have been utilized for genomics-assisted crop improvement in chickpea.

The available draft genome sequences of *desi* (ICC 4958) and *kabuli* (CDC Frontier) chickpea (Jain et al. 2013; Varshney et al. 2013) have accelerated the genome and transcriptome resequencing of diverse *desi*, *kabuli* and wild accessions at a genome-wide scale. This has subsequently led to the identification and large-scale genotyping of numerous sequence-based informative markers via multiple cost-effective array- and NGS (next generation sequencing)-based high-throughput genotyping assays, including GBS (genotyping-by-sequencing) (Gaur et al. 2012; Hiremath et al. 2012; Jhanwar et al. 2012; Agarwal et al. 2012; Roorkiwal et al. 2013; Deokar et al. 2014; Stephens et al. 2014; Jaganathan et al. 2015; Kujur et al. 2015). Recent efforts have also been made towards deciphering the complex and interacting transcriptional gene regulatory networks controlling flower developmental process in a *desi* chickpea accession (ICC 4958) through global transcriptomics approach (Singh et al. 2013). This NGS-based whole genome transcriptome sequencing study has identified a large number (~1500) of preferential and tissue/stage (flower bud/flower)-specific known/candidate gene-encoding transcripts that are differentially expressed (up- and down-regulated) distinctly from the vegetative tissues during flower development in chickpea. In addition to these genomic resources, diverse genetic resources, including natural (core/minicore germplasm lines) and mapping populations (advanced generation back-cross and recombinant inbred lines) exhibiting a wide range of phenotypic diversity for flowering time are available in chickpea (Upadhyaya et al. 2001, 2008; Gaur et al. 2014). It would be thus interesting to adopt an integrative genomic approach involving SNP (single nucleotide polymorphism) marker-mediated candidate gene-based and genome-wide association mapping, QTL mapping, differential expression profiling, gene haplotype-specific LD (linkage disequilibrium) mapping for rapid delineation of potential candidate genes regulating flowering time at a whole genome level in chickpea. In recent years, such integrative approach has been successfully implemented to identify potential major candidate genes harbouring robust QTLs regulating seed size/100-seed weight and pod number/plant in chickpea (Kujur et al. 2013, 2014; Saxena et al. 2014b; Bajaj et al. 2015a). Interestingly, the efficacy of QTL mapping/map-based cloning and trait association analysis in combination to scan the potential candidate genes/QTLs governing complex flowering time trait at a genome-wide scale is well documented in *Arabidopsis* and legumes (Liu et al. 2007; Pierre et al. 2008; Ehrenreich et al. 2009; Jung and Müller 2009; Brachi et al. 2010; Watanabe et al. 2011; Xia et al. 2012; Yeoh et al. 2013; Zhang et al. 2013). Collectively, the aforesaid strategy will assist us to dissect the molecular basis of complex gene regulatory networks underlying the flowering time quantitative trait and understand its evolutionary pattern during chickpea domestication.

In light of the above, the present study made an effort to integrate GWAS (genome-wide association study) and candidate gene (known/candidate gene-encoding transcripts differentially expressed during flower development in chickpea and chickpea gene orthologues governing known flower development pathway majorly in *Arabidopsis*, *Medicago*, soybean and pea)-based association analysis for identification of potential genomic loci (genes) associated with flowering time in chickpea. Further, we integrated the trait association analysis with traditional QTL mapping, transcript profiling and gene-based haplotyping/LD (linkage disequilibrium) mapping to scale-down the functionally relevant novel molecular tags (markers, genes, QTLs, alleles and haplotypes) regulating flowering time and decipher their haplotype-specific evolutionary pattern in cultivated and wild accessions during chickpea domestication.

Materials and methods

Mining and genotyping of genome-wide and gene-derived SNPs

For genome-wide mining and genotyping of SNPs, the genomic DNA isolated from 92 (39 *desi* and 53 *kabuli*) diverse chickpea accessions (flowering time association panel, Table S1) were used to constitute a 96-plex GBS library (three accessions as biological replicates and one non-template control) and sequenced (100-bp single end) employing Illumina HiSeq 2000 NGS platform as per the modified methods of Elshire et al. (2011) and Kujur et al. (2015). The de-multiplexing and mapping of high-quality FASTQ sequence reads onto the reference *kabuli* draft chickpea genome (Varshney et al. 2013) and detection of high-quality SNPs from 92 accessions were performed using Bowtie v2.1.0 (Langmead and Salzberg 2012) and reference-based GBS pipeline/genotyping approach of STACKS v1.0 (http://creskolab.uoregon.edu/stacks) (following Kujur et al. 2015). The sequence reads unmapped with the reference *kabuli* chickpea genome were analysed through de novo-based GBS pipeline/genotyping approach of STACKS. Subsequently, SNPs with their putative physical positions (bp) were discovered from the high-confidence unique stacks (reassembled sequence reads)

generated among accessions following the detailed methods of Kujur et al. (2015). The reference genome GBS-based SNPs identified in different coding and non-coding sequence components of genes and genomes (chromosomes/pseudomolecules and scaffolds) were structurally and functionally annotated according to *kabuli* genome annotation (Varshney et al. 2013).

For large-scale mining and genotyping of gene-based SNPs in chickpea, a set of 1572 (including 290 flower tissue/stage-, 38 lineage- and 96 chickpea-specific transcripts) known/candidate gene-encoding transcripts that are differentially expressed during flower development in *desi* chickpea accession (ICC 4958) were obtained (Singh et al. 2013). In addition, a selected set of 150 known cloned genes involved in regulation of flower development (known flower development pathway) in *Arabidopsis thaliana*, *Glycine max*, *Medicago truncatula* and *Pisum sativum* (Ehrenreich et al. 2009; Jung and Müller 2009; Brachi et al. 2010; Jung et al. 2012; Andres and Coupland 2012; Weller and Martínez 2015) were acquired. The transcripts, including coding sequence (CDS) of these known flowering genes were BLAST searched against the CDS of *kabuli* genes to find out the best possible gene orthologs in chickpea. The CDS and 2000-bp URRs (upstream regulatory regions), exons, introns and 1000-bp DRRs (downstream regulatory regions) of these identified true chickpea gene orthologs (*E*-value: 0 and bit score ≥500) were targeted to design the multiple overlapping forward and reverse primers with expected amplification product size of 400–600 bp per primer. The genomic DNA of 12 chickpea accessions (selected from 92 accessions used for GBS assay, Table S1) were PCR amplified with the gene-based primers and the amplified PCR products were sequenced through automated 96 capillary ABI 3730xl DNA Analyzer (Applied Biosystems, USA). The high-quality gene sequences were aligned and compared to detect SNPs among accessions following Kujur et al. (2013) and Saxena et al. (2014a). The Illumina GoldenGate assay was utilized for large-scale validation and high-throughput genotyping of these mined SNPs in the genomic DNA of 92 accessions (flowering time association panel, Table S1) following Bajaj et al. (2015a).

Phenotyping for flowering time

For phenotyping, 92 *desi* and *kabuli* chickpea accessions belonging to a flowering time association panel were grown in the field according to RCBD (randomised complete block design) for two consecutive years (2011 and 2012) with at least two replications during crop growing season at two diverse geographical locations (Patancheru, Hyderabad: latitude 17°3′N/longitude 77°2′E and New Delhi: 28°4′N/77°2′E) of India. Moreover, greenhouse trail was conducted to evaluate the flowering time response of these natural chickpea accessions under both long (16 h light/8 h dark)- and short (10 h light/14 h dark) -day conditions at 22 ± 2 °C. The days to 50 % flowering time (DF) of each accession (10–15 representative plants from each accession) was measured individually by counting the number of days from sowing (first irrigation) to the stage when 50 % of their plants have begun to flower. The frequency distribution, coefficient of variation (CV), broad-sense heritability (H^2) and analysis of variance (ANOVA) of DF trait among accessions were estimated using SPSSv17.0 as per Saxena et al. (2014b).

Flowering time association mapping

The construction of unrooted neighbour-joining (NJ)-based phylogenetic tree, principal component analysis (PCA), and determination of population genetic structure and genome-wide/population-specific LD patterns and LD decay among 92 chickpea accessions (flowering time association panel) were performed as per Kujur et al. (2015). To perform DF trait association mapping, the genome-wide and candidate gene-based SNP genotyping information were correlated with the DF phenotyping, population structure ancestry coefficient (Q matrix), kinship matrix (K) and PCA (P) data of 92 cultivated *desi* and *kabuli* accessions (Kujur et al. 2013, 2014; Kumar et al. 2015). In addition, GWAS was conducted by use of mixed model (P + K, K and Q + K)-based P3D/EMMAX (mixed model association expedited) and CMLM (compressed mixed linear model) approaches of GAPIT as per Kumar et al. (2015). The quantile–quantile plot was compared individually with the relative distribution of observed and expected -$\log_{10}(P)$ value for each SNP marker-trait association to ensure their accuracy and robustness. The adjusted *P* value threshold of significance was corrected for multiple comparisons in accordance with false discovery rate (FDR cut-off ≤0.05) (Benjamini and Hochberg 1995). We integrated all four model-based outputs of TASSEL and GAPIT to identify potential SNP loci in the target genomic (gene) regions exhibiting significant association with DF trait at highest R^2 (degree of SNP marker-trait association) and lowest FDR adjusted *P* values (threshold $P < 1 \times 10^{-7}$).

QTL mapping

An intra-specific 190 F_6 mapping population (ICC 16374 × ICC 762) was utilized for validating the genomic loci associated with DF trait by QTL mapping. The chickpea accessions ICC 16374 (originated from Malawi) and ICC 762 (originated from India) are early (38 days) and late (65) flowering *desi* traditional cultivars/landraces. The multi-location/years replicated field phenotyping for

DF was performed in each mapping individual (10–12 representative plants from each individual) following the aforementioned methods. The homogeneity of mapping population across two seasons and their genetic inheritance patterns, including CV, broad-sense heritability (H^2) of DF trait was determined according to Bajaj et al. (2015a). A selected set of parental polymorphic 384 GBS-based SNPs and 32 previously reported SSR markers (Winter et al. 1999, 2000; Cho et al. 2002; Lichtenzveig et al. 2006; Anbessa et al. 2006; Cobos et al. 2007, 2009; Radhika et al. 2007; Aryamanesh et al. 2010; Hossain et al. 2010; Gowda et al. 2011; Rehman et al. 2011; Thudi et al. 2011; Cho et al. 2012; Hiremath et al. 2012; Vadez et al. 2012; Jamalabadi et al. 2013; Varshney et al. 2014) (as anchors) physically/genetically mapped on eight LGs (linkage groups)/chromosomes were genotyped using Illumina GoldenGate assay (Bajaj et al. 2015a) and fluorescent dye-labelled automated fragment analyzer (Kujur et al. 2013), respectively. The construction of high-density intra-specific genetic linkage map and QTL mapping [composite interval mapping (CIM)] at significant LOD (logarithm of odds threshold >5.0) was performed (as per Saxena et al. 2014b) to identify and map the major genomic loci harbouring the robust QTLs controlling DF trait in chickpea. The additive/pleiotropic effect and confidence interval (CI) of each significant major flowering time QTL were determined as per Bajaj et al. (2015a).

Differential expression profiling

To determine the regulatory pattern of genes associated (validated by association and QTL mapping) with flowering time, the differential expression profiling of these genes was performed using the semi-quantitative and quantitative RT-PCR assays. The RNA was isolated from vegetative young leaf and shoot apical meristem (SAM) tissues, and four flower development stages (FB1 and FB2: flower buds with 4 and 10 mm size, respectively and FL1 and FL2: young flowers with close petals and mature flowers with opened petals, defined as per Singh et al. 2013) of early (*kabuli* cv. ICC 12,968 with DF: 32.3 ± 0.81) and late (*desi* cv. ICCX-810800 with DF: 61.2 ± 1.2) flowering two contrasting chickpea accessions as well as mapping parents (ICC 16374 and ICC 762) and four homozygous mapping individuals. The isolated RNA was amplified with the gene-specific primers in RT-PCR assay following Bajaj et al. (2015b).

Molecular haplotyping

To perform gene-based marker haplotyping/LD mapping, the 2 kb URRs, exons, introns and 1 kb DRRs of flowering time-regulating potential genes (validated by association/QTL mapping and expression profiling) amplified from 92 cultivated *desi* and *kabuli* chickpea accessions and 10 wild accessions (two accessions each from *C. reticulatum*, *C. echinospermum*, *C. judaicum*, *C. bijugum* and *C. pinnatifidum*) (Table S1) were cloned and sequenced following Kujur et al. (2013, 2014) and Saxena et al. (2014b). The SNPs discovery from high-quality sequences, marker-based haplotypes constitution, LD patterns determination, and haplotype-based trait association mapping and evolutionary study of flowering time-associated genes were performed following Saxena et al. (2014b) and Bajaj et al. (2015b).

Results

Identification and annotation of genome-wide GBS- and candidate gene-based SNPs

A total of 246.5 million sequence reads with a mean of 2.5 million reads per accession (ranging from 2.1 to 8.9 million reads) were generated by GBS-based sequencing of 96-plex *Ape*KI libraries constructed from 92 diverse *desi* and *kabuli* chickpea accessions (constituted flowering time association panel). This produced 215.8 (87.5 %) million high-quality sequence reads (~18-fold sequence depth of coverage) that varied from 1.91 to 4.53 with a mean of 3.10 million reads per accession. On an average 84.7 % (ranging from 80.7 to 89.5 %) high-quality sequence reads of these, evenly distributed across 92 chickpea accessions, were mapped to unique physical locations on *kabuli* reference genome. The use of *kabuli* reference genome- and *de novo*-based GBS approaches discovered 24,405 high-quality SNPs (as defined by Kujur et al. 2015) from 92 chickpea accessions (Fig. 1A; Table S2). This includes 16,376 and 8029 reference genome- and de novo-based SNPs, respectively. The reference genome-based SNPs further comprised of 14,115 and 2261 SNPs that were physically mapped across eight chromosomes (average map density: 24.6 kb) and unanchored scaffolds of *kabuli* genome, respectively (Fig. 1A; Table S2). These 16,376 reference *kabuli* genome-based SNPs have been submitted to NCBI dbSNP (http://www.ncbi.nlm.nih.gov/SNP/snp_viewTable.cgi?handle=NIPGR) with SNP submission (ss) accession numbers 974751673–974768048. The minor allele frequency (MAF) of GBS-based SNPs (with <10 % missing data) varied from 2 to 24 % with an average of 15 %.

To discover candidate gene-derived SNPs, the PCR amplicons-based sequencing of 832 known/candidate gene-encoded by 1572 transcripts (differentially expressed during flower development in ICC 4958) and 82 known cloned flowering gene orthologues of chickpea (involved in regulation of flower development in *Arabidopsis* and legumes) was performed (Table S3). The comparison of these

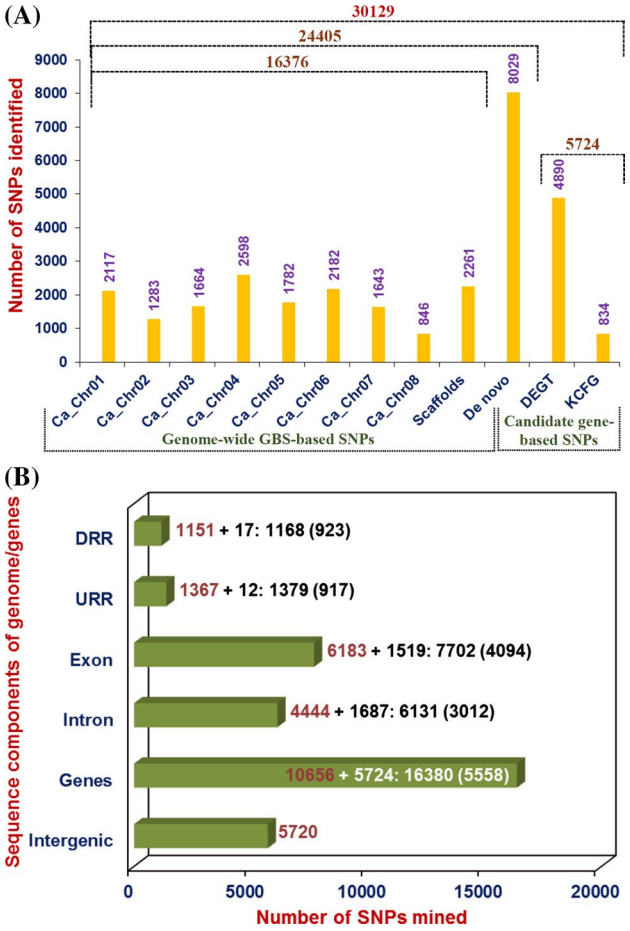

Fig. 1 **A** Relative distribution of 30,129 SNPs mined through reference *kabuli* genome (eight chromosomes and scaffolds)- and de novo GBS-based (24,405 SNPs) and candidate gene (DEGT and KCFG)-derived (5724 SNPs) SNP genotyping assays. *DEGT* differentially expressed gene-encoding transcripts and *KCFG* known cloned flowering genes. **B** Detailed annotation of SNPs in the diverse non-coding (intron, URR and DRR) and coding (synonymous and non-synonymous) sequence components of genes and intergenic regions of *kabuli* genome. The CDS (coding sequences), URR (upstream regulatory region) and DRR (downstream regulatory region) of genes were defined according to gene annotation information of reference *kabuli* genome (Varshney et al. 2013). The numbers with *red* and *black* colour fonts mentioned within *bars* indicate the structural annotation of genome-wide GBS- and candidate gene-based SNPs, respectively. Value mentioned in the *parenthesis* indicates the number of SNPs-containing genes

high-quality gene amplicon sequences among 12 representative *desi* and *kabuli* chickpea accessions and subsequent high-throughput genotyping of mined SNPs in a constituted flowering time association panel (92 *desi* and *kabuli* accessions) by Illumina GoldenGate assay successfully identified 5724 SNPs in 914 genes with an average frequency of 6.3 SNPs/gene (Fig. 1A). This includes 4890 and 834 SNPs mined from 832 differentially expressed genes-encoding transcripts (mean frequency: 5.9 SNPs/gene) and 82 known cloned flowering chickpea gene orthologues (10.2 SNPs/gene), respectively.

The structural annotation of 22,100 SNPs (including 16,376 *kabuli* reference genome- and 5724 gene-based SNPs) exhibited the presence of 16,380 and 5720 SNPs in 5558 genes and intergenic regions, respectively (Fig. 1B). Further, detailed annotation of SNPs in the genes depicted the presence of a maximum number of 7702 SNPs (6183 genome-wide and 1519 gene-derived SNPs in 4094 genes) in the exons (CDS). A minimum of 1168 SNPs (17 genome-wide and 1151 gene-based SNPs in 923 genes) were identified in the DRRs. The mined coding SNPs included 4128 synonymous and 3574 non-synonymous (missense and nonsense) SNPs in 2706 and 3093 genes, respectively. A maximum correspondence of 5558 SNPs-carrying genes with growth, development and metabolism-related proteins (46 %), followed by transcription factors (21.2 %) and signal transduction proteins (5 %) was evident.

Association mapping of flowering time

To perform GWAS and candidate gene-based association mapping, 30,129 informative SNPs (revealing polymorphism among 92 *desi* and *kabuli* chickpea accessions) identified based on genome-wide GBS- (24,405 SNPs) and candidate gene-based SNP genotyping (5724 SNPs) were utilized. The use of these 30,129 SNPs for neighbour-joining phylogenetic tree construction and determination of high-resolution population genetic structure and PCA, differentiated all 92 accessions from each other and clustered into two distinct populations; POP I (*kabuli*) and POP II (*desi*). The LD patterns in two population groups of 92 accessions using 19,839 SNPs (14,115 genome-wide and 5724 gene-based SNPs) physically mapped on eight chromosomes were determined. This exhibited a higher LD estimate [average frequency correlation among pair of alleles across a pair of SNP loci (r^2): 0.30–0.56] and extended LD decay (r^2 decreased half of its maximum value nearly at 250 kb physical distance on chromosomes) in *kabuli* as compared to *desi* population [LD estimate (r^2): 0.26–0.49 with ~200 kb LD decay)] (Fig. 2).

We observed a broader phenotypic variation (34–88 days, mean CV: 26 % and mean H^2: 80 %) along with normal frequency distribution for DF in 92 chickpea accessions grown at multiple geographical locations/years in field and green house (long- and short-day) conditions (Fig. 3A; Table S4). The use of CMLM model-based approach at FDR cut-off $\leq$0.05 in association analysis (Figure S1) overall detected eight genomic loci (gene-derived SNPs) revealing strong association with DF at a highly significant $P \leq 10^{-7}$ (Fig. 4A; Table 1). Three SNPs from these were identified from genome-wide GBS data, while five SNPs derived from known flowering genes

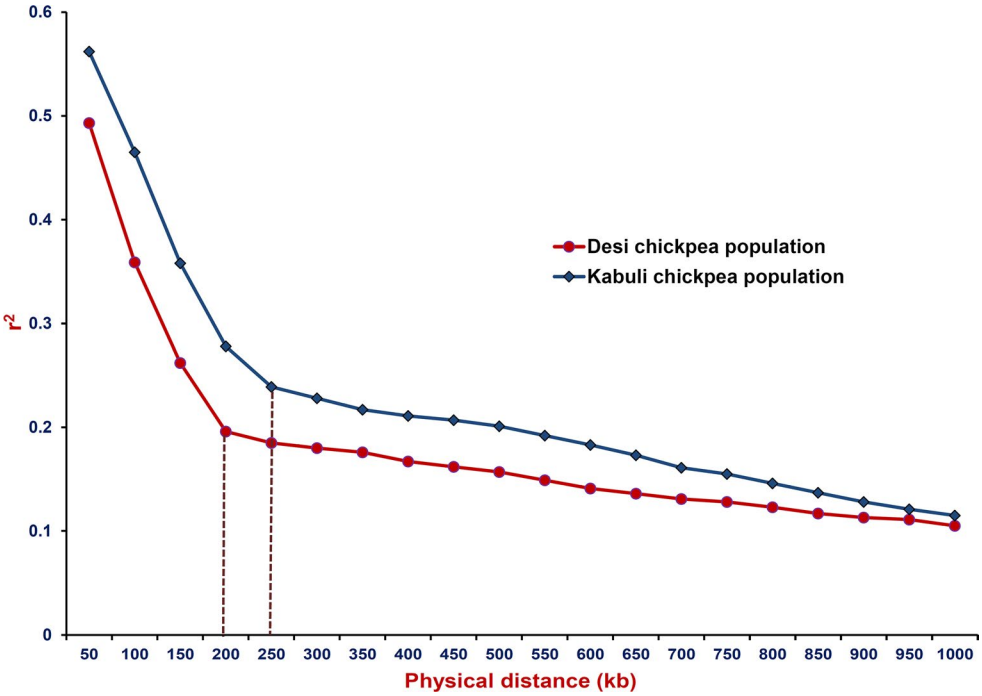

Fig. 2 LD decay (mean r^2) estimated in a flowering time association panel (92 chickpea accessions) belonging to *desi* and *kabuli* populations using 19,839 genome-wide and gene-based SNPs physically mapped on eight chromosomes. The plotted curved lines depicted the mean r^2 values among SNPs spaced with uniform 50 kb physical interval from 0 to 1000 kb

(three SNPs) and differentially expressed candidate gene-encoding transcripts (two SNPs). Seven and one DF-associated genomic loci were physically mapped on four chromosomes (3, 4, 5 and 6) and scaffold of *kabuli* genome, respectively (Fig. 4A; Table 1). A maximum of two DF-associated SNPs was derived from *kabuli* chromosomes 4 and 5. Five and three of eight DF-associated genomic SNP loci were represented from diverse coding (three non-synonymous SNPs) as well as non-coding [intronic (one SNP) and URR/DRR (two SNPs)] sequence components of eight genes, respectively.

The proportion of phenotypic variance for DF explained (R^2) by maximum effect eight SNP loci in eight known/candidate genes [*efl1* (early flowering 1), *FLD* (Flowering locus D), *GI* (GIGANTEA), *Myb* (Myeloblastosis), *SFH3* (SEC14-like 3), *bZIP* (basic-leucine zipper), *bHLH* (Helix-loop-helix) and *SBP* (SQUAMOSA promoter binding protein)] among 92 chickpea accessions varied from 8 to 17 % (Table 1). The percentage of DF phenotypic variance explained (R^2) by all significant eight SNP loci was 34 %. Three (including one non-synonymous coding SNPs) SNPs of these, in three known cloned flowering genes (*efl1*, *FLD* and *GI*) had significant association (P: 1.5×10^{-8} to 1.1×10^{-9} with R^2 of maximum effect and all significant SNP loci: 14–17 and 31 %, respectively) with DF in chickpea (Table 1). Strong association of two regulatory (URR/DRR) SNPs derived from two differentially expressed genes (*SFH3* and *Myb* protein-coding genes)-encoding transcripts (P: 2.0×10^{-8} to 1.0×10^{-9} with R^2 of maximum effect and all significant SNP loci: 8–13 and 28 %, respectively) and three (including two non-synonymous coding SNP) genome-wide SNPs identified in three genes (*bZIP*, *bHLH* and *SBP* transcription factors) (P: 1.5×10^{-8} to 1.2×10^{-9} with R^2 of maximum effect and all significant SNP loci: 7–13 and 25 %, respectively) was evident (Table 1). Interestingly, non-synonymous coding and regulatory SNPs detected in *efl1*, *FLD* and *GI* known flowering genes, *SFH3* candidate gene-encoding transcripts (differentially expressed during flower development) and *SBP* transcription factor gene (identified by GWAS) exhibited strong association ($P \leq 10^{-8}$ with $R^2 \geq 10$ %) with flowering time as compared to three other DF-associated SNPs identified in chickpea (Table 1).

Validation of flowering time-associated genes through QTL mapping and expression profiling

A high-density intra-specific genetic linkage map (ICC 16374 × ICC 762) was constructed by assigning 410 (including 378 SNP and 32 previously reported SSR markers) parental polymorphic markers across eight chickpea LGs (LG1 to LG8) (Table 2). Our constructed genetic map covered a total map length of 971.5 cM with a mean inter-marker distance of 2.37 cM. The LG5 (average inter-marker distance: 1.94 cM) and LG2 (2.94 cM) had most and least saturated genetic maps, respectively (Table 2). A significant variation (31–73 days with mean CV: 25 % and mean H^2: 80 %) of DF along with its bi-directional transgressive segregation-based normal frequency distribution in a developed mapping population grown at multiple geographical

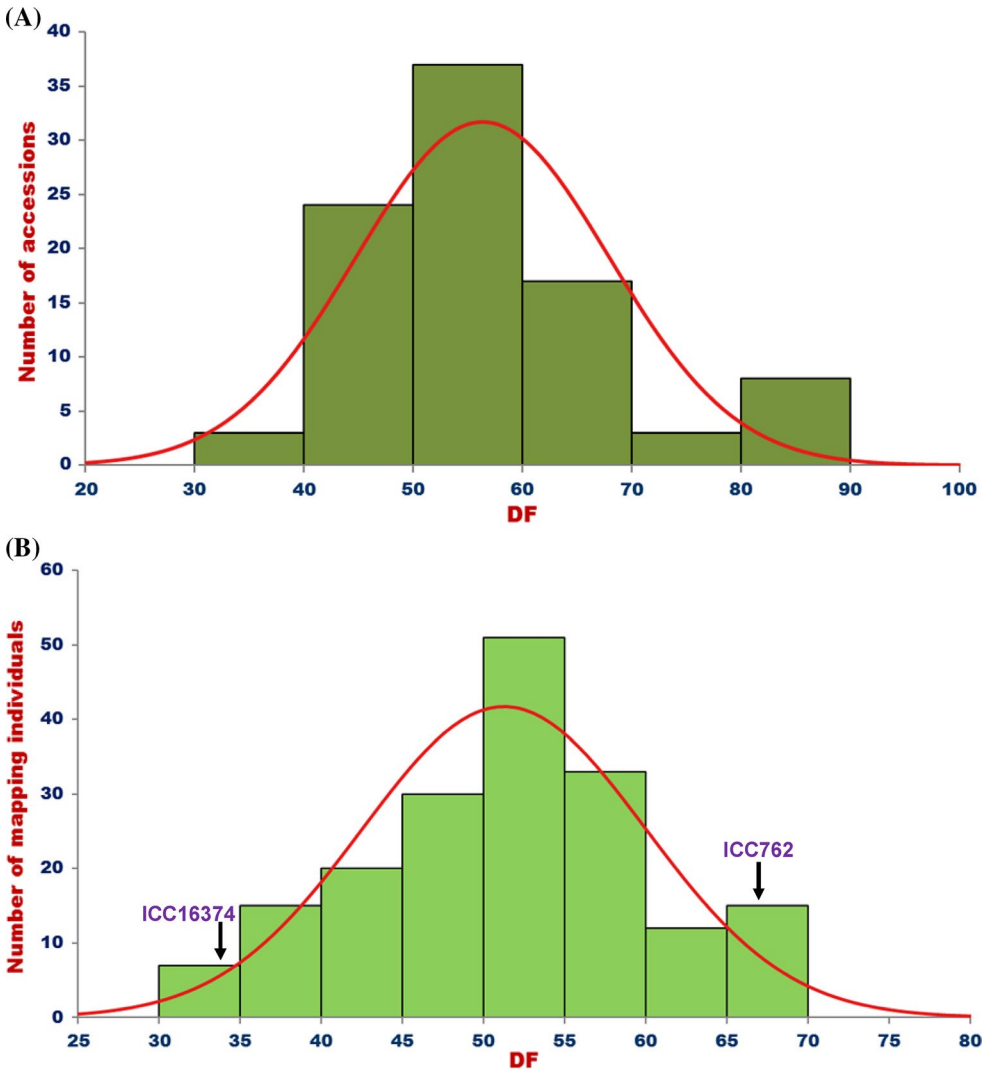

Fig. 3 Frequency distribution of average DF trait variation measured in 92 *desi* and *kabuli* chickpea accessions (flowering time association panel) **A** as well as in 190 individuals and parental accessions of a F_6 mapping population (ICC 16374 × ICC 762) **B**, grown at multiple geographical locations/years in field and green house (long- and short-day) conditions. This *distribution curve* depicted a goodness of fit to the normal distribution

locations/years in field and green house (long- and short-day) conditions was observed (Fig. 3B; Table S4).

For flowering time QTL mapping, the genotyping data of 410 SNP and SSR markers genetically mapped on eight LGs was correlated with multilocation/years DF field/green house phenotyping data of mapping individuals and parental accessions. Based on this analysis, five major (LOD: 5.4–11.7) genomic regions underlying robust QTLs (*CaqDF3.1*, *CaqDF3.2*, *CaqDF4.1*, *CaqDF4.2*, *CaqDF5.1*) (validated across two locations/years and multiple growth conditions) governing DF were identified and mapped on three (3, 4 and 5) *kabuli* chromosomes (Fig. 4B; Tables 1, S5). A maximum number (two QTLs) of DF QTLs was mapped on LG3 and LG4. The phenotypic variance explained (PVE) by individual DF QTL (R^2) ranged from 11.6 to 27.3 %. The combined PVE measured for all six major DF QTLs was 36.8 %. The additive gene effects (ranging 1.8–2.7) of all DF QTLs based on the effective contributions of ICC 16374 alleles for early flowering trait was observed. Interestingly, six non-synonymous and regulatory SNPs in the six known/candidate genes (*efl1*, *FLD*, *GI*, *SFH3*, *bZIP* and *SBP*) revealing tight linkage with all major five robust DF QTLs were identified, which also had high trait association potential for flowering time based on our association analysis (Fig. 4A, B; Tables 1, S5). In summary, we selected eight non-synonymous and regulatory SNP-containing eight known/candidate genes (*efl1*, *FLD*, *GI*, *SFH3*, *Myb, bZIP, bHLH* and *SBP*; validated by genetic association analysis), including six genes (*efl1*, *FLD*, *GI*, *SFH3*, *bZIP* and *SBP*; validated by QTL mapping) as target

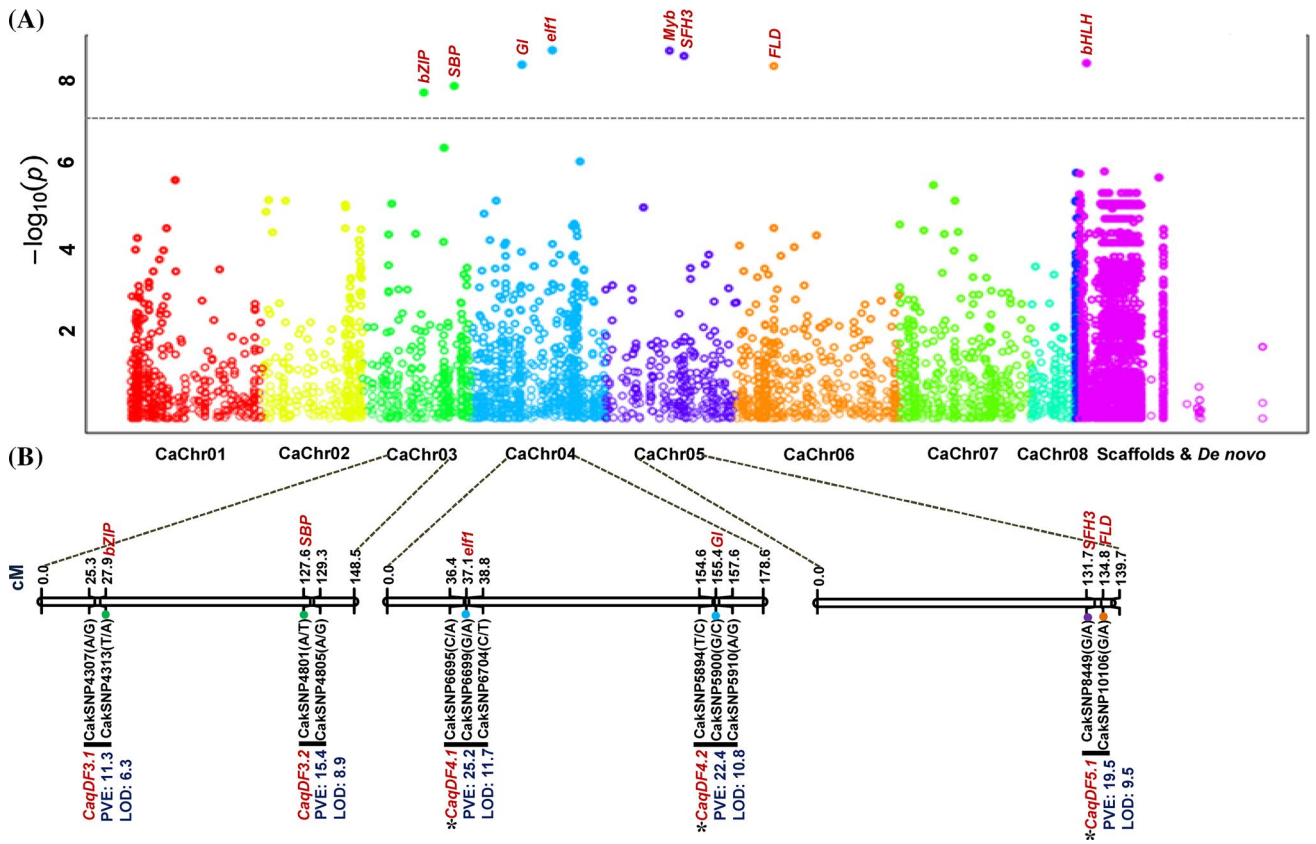

Fig. 4 **A** GWAS-based Manhatton plot revealing significant *P* values (estimated by CMLM model) associated with DF employing 30,129 genome-wide GBS- and candidate gene-based SNPs. The x-axis depicts the relative density of reference genome- and de novo-based SNPs physically mapped on eight chromosomes and scaffolds of *kabuli* genome. The y-axis represents the -$\log_{10}$ (*P*) value for significant association of SNP loci with DF. The SNPs exhibiting significant association with DF at cut-off *P* value $\leq 1 \times 10^{-6}$ are indicated with dotted lines. **B** Five major genomic regions underlying five significant QTLs (PVE 11.3–25.2 %) associated with DF identified and mapped on three LGs/chromosomes (with a significant LOD > 6.3, $P < 0.05$) using a 190 F_6 mapping population (ICC 16374 × ICC 762). The genetic distance (cM) and identity of the marker loci integrated on the LGs are indicated on the above and below of the chromosomes, respectively. Six genes harbouring five robust QTLs associated strongly with DF based on GWAS are indicated by colour coded circles. *CaqDF3.1* (*Cicer arietinum* QTL for days to 50 % flowering time on chromosome 3 number 1), *CaqDF3.2* (*Cicer arietinum* QTL for days to 50 % flowering time on chromosome 3 number 2), *CaqDF4.1* (*Cicer arietinum* QTL for days to 50 % flowering time on chromosome 4 number 1), *CaqDF4.2* (*Cicer arietinum* QTL for days to 50 % flowering time on chromosome 4 number 2) and *CaqDF5.1* (*Cicer arietinum* QTL for days to 50 % flowering time on chromosome 5 number 1). **CaqDF4.1*, *CaqDF4.2* and *CaqDF5.1* correspond to known QTLs reported previously for flowering time in chickpea

candidates for flowering time trait regulation (Table 1) by their further validation through differential expression profiling and molecular haplotyping in chickpea.

These SNP-containing eight flowering time-associated known/candidate genes were analysed for differential expression profiling to determine the regulatory patterns of the target genes for flowering time (Fig. 5; Table 1). The RNA was isolated from vegetative young leaf and shoot apical meristem (SAM) tissues, and four flower development stages (FB1, FB2, FB3 and FB4) of early (ICC 12968) and late (ICCX-810800) flowering two contrasting *desi* and *kabuli* chickpea accessions as well as mapping parents (ICC 16374 and ICC 762) and four homozygous mapping individuals. The isolated RNA was amplified with gene-specific primers using semi-quantitative and quantitative RT-PCR assays. All eight known cloned/candidate flowering genes showing strong association with flowering time (validated by association and/or QTL mapping) exhibited flower-specific [compared with vegetative tissues (young leaves and SAM)] expression, including their differential up- (>threefolds, $P \leq 0.001$ in *efl1*, *FLD*, *GI*, *bZIP*, *bHLH* and *SBP* genes) and down-(>3.5-folds, $P \leq 0.001$ in *SFH3* and *Myb* genes) regulation specifically in four flower development stages of early flowering *desi* and *kabuli* chickpea accessions/mapping individuals as compared to that of late flowering accessions/individuals (Fig. 5; Table 1). Interestingly, all the flower-specific genes differentially expressed during flower development

Table 1 Eight SNPs-containing genes regulating DF delineated by combining GWAS and candidate gene-based association mapping with QTL mapping, differential expression profiling and gene-based marker haplotyping/LD mapping in chickpea

SNP IDs	SNPs (EDF/LDF)	Chromosomes	Physical positions (bp)	*Kabuli* gene accession IDs	Annotation	Putative functions	Association potential of SNPs		SNPs (haplotypes) discovered	Association potential of haplotypes[#]	
							P value	R^2 (%)		*P* value	R^2 (%)
CakSNP4313[a*]	T/A	Ca_kabuli_chr3	29,844,509	Ca07292	Intron	*bZIP* (Basic-leucine zipper) TF[d]	2.0×10^{-8}	7	6 (2)	1.0×10^{-9}	15
CakSNP15256[a]	G/A	Ca_kabuli_scaffold300	2,213,710	Ca07844	CDS (non-synonymous) Glycine (GGA)-Arginine (AGA)	*bHLH* (Basic helix-loop-helix) TF[d]	1.5×10^{-8}	9	7 (3)	1.2×10^{-9}	19
CakSNP4801[a*]	A/T	Ca_kabuli_chr3	37,101,339	Ca01036	CDS (non-synonymous) Cysteine (TGT)-Serine (AGT)	*SBP* (SQUAMOSA promoter binding protein) TF[d]	1.2×10^{-9}	13	12 (4)	1.4×10^{-10}	21
CakSNP6699[b*]	G/A	Ca_kabuli_chr4	30,308,812	Ca14192	CDS (synonymous)	*efl1* (early flowering 1)[d]	1.1×10^{-9}	17	28 (5)	1.0×10^{-11}	32
CakSNP10106[b*]	G/A	Ca_kabuli_chr6	11,665,233	Ca25061	CDS (synonymous)	*FLD* (Flowering locus D)[d]	2.3×10^{-8}	15	6 (2)	1.7×10^{-9}	31
CakSNP5900[b*]	G/C	Ca_kabuli_chr4	11,277,574	Ca04359	CDS (non-synonymous) Valine (GTT)-Leucine (CTT)	*GI* (GIGANTEA)[d]	1.5×10^{-8}	14	25 (4)	1.2×10^{-9}	29
CakSNP8428[c]	A/T	Ca_kabuli_chr5	30,443,858	Ca04722	DRR	Myb (Myeloblastosis) TF[e]	2.0×10^{-8}	8	4 (2)	1.3×10^{-9}	16
CakSNP8449[c*]	G/A	Ca_kabuli_chr5	30,755,584	Ca04769	URR	*SFH3* (SEC 14-like 3)[e]	1.0×10^{-9}	13	6 (2)	1.0×10^{-10}	27

TF transcription factor

Flowering time-regulating genes identified through [a]GWAS and candidate gene ([b]known flowering chickpea gene orthologs and [c]gene-encoding transcripts differentially expressed during flower development in chickpea)-based association mapping

* Validated through QTL mapping

[#] Details of haplotypes constituted in the genes are provided in the Table S6

Genes differentially [d]up- and [e]down-regulated during flower development

Table 2 Markers mapped on eight LGs of an intra-specific chickpea genetic linkage map

Linkage groups (LGs)/chromosomes (Chr)	SNP + SSR markers mapped	Map length covered (cM)	Mean inter-marker distance (cM)
LG(Chr)01	32 + 4 = 36	104.4	2.90
LG(Chr)02	35 + 4 = 39	114.7	2.94
LG(Chr)03	59 + 4 = 63	148.5	2.36
LG(Chr)04	87 + 4 = 91	178.6	1.96
LG(Chr)05	68 + 4 = 72	139.7	1.94
LG(Chr)06	31 + 4 = 35	97.5	2.79
LG(Chr)07	37 + 4 = 41	102.7	2.50
LG(Chr)08	29 + 4 = 33	85.4	2.59
Total	378 + 32 = 410	971.5	2.37

contained SNPs in their up-/down-stream regulatory and coding regions (Fig. 5). Therefore, it would be interesting to constitute gene-specific haplotypes targeting/combining these regulatory and coding (non-synonymous) novel SNP allelic variants mined from different sequence components of the genes and evaluate trait association potential of these haplotypes with flowering time trait variation in chickpea natural population.

Haplotyping in flowering time-associated genes

For molecular haplotyping of flowering time-regulating gene (validated by association analysis, QTL mapping and expression profiling), the cloned amplicons covering the entire 2 kb URRs, exons, 1 kb DRRs and intronic regions of eight genes were sequenced and compared among 92 cultivated *desi* and *kabuli* and 10 wild chickpea accessions. This identified 94 SNPs, including 10 regulatory and 44 non-synonymous coding SNPs in these eight genes (Table 1; Table S6). The haplotype analysis in eight genes by utilizing the genotyping data of 94 SNPs among 102 accessions constituted a total of 24 haplotypes (ranged from 2 to 5 haplotypes) (Table 1, Table S6). The haplotype-based LD mapping [a higher degree of LD ($r^2 > 0.80$ with $P < 1.5 \times 10^{-6}$) resolution] and association analysis using gene-derived haplotypes inferred their strong association potential (P: 1.0×10^{-9} to 1.0×10^{-11} with R^2 of maximum effect and all significant SNP loci: 15–32 and 41 %, respectively) for flowering time trait variation (Table 1; Table S6). Interestingly, two major haplotypes identified in each of the eight genes that are affected either by functional non-synonymous coding and regulatory SNPs exhibited strong association potential for early (DF: 34–41 days) and late (DF: 77–85 days) flowering time differentiation, respectively.

This analysis detected novel natural regulatory and non-synonymous allelic variants and potential haplotypes in eight genes governing early and late flowering time differentiation, respectively in chickpea accessions. Interestingly, a significant sharing of all early and late flowering time-associated gene haplotypes among 92 cultivated *desi* and *kabuli* as well as 10 wild accessions representing primary (*C. reticulatum* and *C. echinospermum*) and secondary gene pools (*C. bijugum*, *C. judaicum* and *C. pinnatifidum*) was observed (Table S6). Nevertheless, different novel haplotypes (with diverse allelic recombination) exhibiting differential trait association potential for flowering time that are present exclusively either in cultivated or wild chickpea accessions were identified. The early flowering-associated gene haplotypes were mostly represented by the wild chickpea accessions (56 %) of primary gene pools rather than secondary gene pools (44 %). Among primary gene pools, the cultivated and wild accessions belonging to *kabuli* and *C. reticulatum*, respectively had maximum (61 %) early flowering-associated haplotypes (Table S6). Collectively, we ascertained a higher association potential of eight genes with flowering time trait variation by their combined validation in GWAS and candidate gene-based association analysis, differential expression profiling and SNP marker-based high-resolution gene-specific haplotyping/LD mapping as well through QTL mapping (validated six of eight DF-associated genes).

Discussion

The implication of integrated genomics-assisted breeding approach (combining QTL mapping/map-based cloning with association mapping) for rapid identification of potential candidate genes/QTLs governing flowering time is well demonstrated in *A. thaliana* and legumes (Liu et al. 2007; Pierre et al. 2008; Ehrenreich et al. 2009; Jung and Müller 2009; Brachi et al. 2010; Watanabe et al. 2011; Xia et al. 2012; Yeoh et al. 2013; Zhang et al. 2013). The clues obtained from these studies in legumes have encouraged us to utilize this integrated genomic approach for quantitative

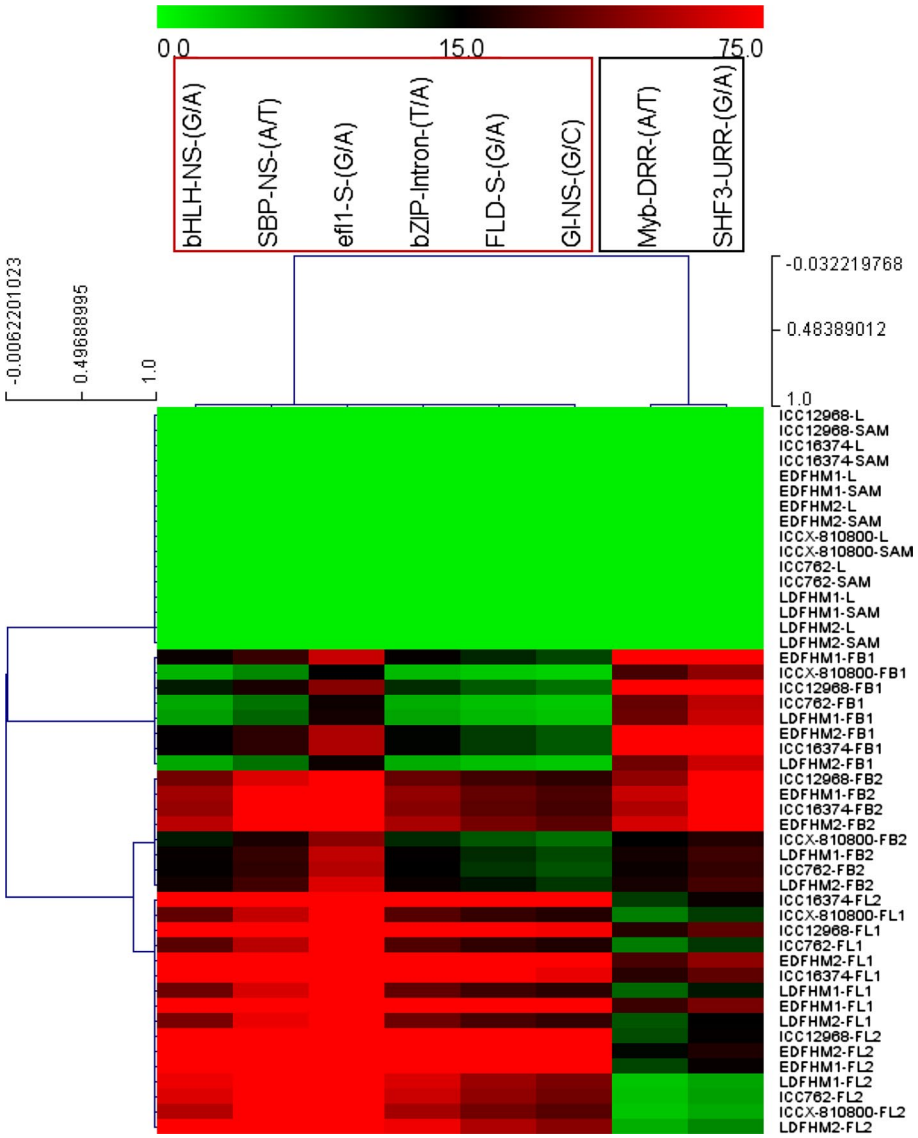

Fig. 5 Hierarchical cluster display illustrating the differential expression profiles of non-synonymous and regulatory SNPs-carrying eight genes (identified by association and genetic mapping) in diverse vegetative young leaf (L) and shoot apical meristem (SAM) as well as four flower development stages (FB1, FB2, FL1 and FL2) of early and late flowering two contrasting chickpea accessions (ICC 12968 and ICCX-810800), mapping parental accessions (ICC 16374 and ICC 762) and four representative homozygous mapping individuals. The average log signal expression values of genes in various tissues/stages are mentioned at the top with a *colour scale*; in which *green*, *black* and *red color* denote low, medium and high level of expression, respectively. The expression values across different tissues/development stages of accessions/mapping individuals were normalized using an endogenous control *elongation factor-1 alpha* in RT-PCR assay. The differential expression profiling of SNP-carrying genes in four flower development stages of early and late flowering accessions/mapping individuals was compared with their respective vegetative leaf and SAM tissues by considering the gene expression in these vegetative tissues as reference calibrator (assigned 1). The genes exhibiting flower-specific expression, including pronounced differential up- and down-regulation in early and late flowering accessions/mapping individuals during flower development are indicated with *red* and *black boxes*, respectively. The detail structural and functional annotation of SNPs-containing eight genes are provided in the Table 1. The tissues/stages and genes utilized for expression profiling are mentioned on the *right* and *top side* of expression map, respectively. EDFHM1 and EDFHM2: early days to 50 % flowering homozygous mapping individuals 1 and 2, respectively. LDFHM1 and LDFHM2: late days to 50 % flowering homozygous mapping individuals 1 and 2, respectively. Numbers of mentioned in the *vertical* and *horizontal bars* depict the range (minimum, optimum and maximum) of correlation coefficient varying among tissues/stages of diverse contrasting accessions/mapping individuals during flower development and across flowering time-associated genes, respectively. *NS* non-synonymous SNP, *S* synonymous SNP, *DRR* downstream regulatory region, *URR* upstream regulatory region

dissection of complex flowering time trait in chickpea. In current study, we have integrated genome-wide and candidate gene-based association mapping with QTL mapping, differential gene expression profiling and gene-based haplotyping/LD mapping to delineate candidate genes underlying the major QTLs regulating flowering time in chickpea. Initially, to perform genome-wide association study (GWAS), a high-throughput GBS assay that is well-established for simultaneous large-scale discovery and genotyping of SNPs at a genome-wide scale in chickpea (Deokar et al. 2014; Jaganathan et al. 2015; Kujur et al. 2015), was utilized. The use of this optimized GBS approach in a constituted flowering time chickpea association panel (92 *desi* and *kabuli* accessions) thus altogether identified 24,405 reference *kabuli* genome- and *de novo*-based SNPs for GWAS. More recently, the potential utility of GWAS and candidate gene-based association mapping in combination for efficient dissection of complex quantitative traits and identification of high-resolution trait-associated genes/QTLs in a larger genome of chickpea with narrow genetic base (400-500 kb of longer chromosomal LD decay) has been realized (Kujur et al. 2015). A diverse array of known cloned genes/QTLs controlling flower development pathway in *A. thaliana* and legumes (Weller et al. 2009; Kong et al. 2010; Laurie et al. 2011; Andres and Coupland 2012; Kim et al. 2012; Pin and Nilsson 2012; Watanabe et al. 2012; Song et al. 2013; Yamashino et al. 2013; Zhai et al. 2014; Weller and Martínez 2015) as well as numerous gene-encoded transcripts that are differentially expressed (exhibiting preferential and flower-specific expression) during flower development in chickpea (Singh et al. 2013) are well documented. Primarily, for candidate gene-based association mapping, we scanned diverse coding and non-coding (introns, URRs and DRRs) sequence components of 914 selected known/candidate flowering gene-orthologs of chickpea in 12 representative *desi* and *kabuli* accessions based on amplicon sequencing to mine potential genic SNP allelic variants. The subsequent large-scale validation and high-throughput genotyping of these genic SNPs in a flowering time chickpea association panel (92 *desi* and *kabuli* accessions) detected 5724 SNPs, which were further utilized for flowering time candidate gene-based association mapping. Henceforth, a total of 30,129 genome-wide and flowering gene-derived SNPs (non-synonymous and regulatory SNPs) discovered by us have profound potential to be utilized for multi-dimensional large-scale genotyping applications in chickpea. These SNPs, once validated and genotyped in large-scale natural and mapping populations, could be utilized for trait association mapping, construction of high-density integrated genetic and physical linkage maps and fine-mapping/map-based cloning to identify potential genes/QTLs controlling traits of agronomic importance in chickpea.

The use of 30,129 genome-wide and flowering gene-based SNPs scanned from a structured population (with 200–250 kb chromosomal LD decay) of chickpea in CMLM-based GWAS and candidate gene-based association mapping identified eight genomic loci associated with flowering time in chickpea. In line with Kujur et al. (2013), we observed a faster LD decay (~200 kb) and lower LD estimate (mean r^2: 0.35) in diverse *desi* population group than that of *kabuli* population. All the chickpea accessions included in the association panel for flowering time association mapping are self-pollinating and underwent selective sweeps along with sequential evolutionary bottlenecks during their domestication from wild common ancestors (Jain et al. 2013; Varshney et al. 2013; Kujur et al. 2013, 2015; Saxena et al. 2014a). Therefore, extensive LD estimate and extended chromosomal LD decay observed in two *desi* and *kabuli* chickpea populations of association panel is expected. Collectively, the GBS-SNP genotyping-based population-specific LD patterns documented in this study at a genome-wide scale implicates deep insight regarding the SNP marker density needed for efficient GWAS to scan potential genomic loci (gene-associated targets) governing important agronomic traits, including flowering time in a larger chickpea genome with narrow genetic base. The CMLM model-based approach deployed in this study for GWAS usually considers all compression levels of population structure, kinship and PCA algorithms for efficient estimation/detection of potential genomic loci associated with DF trait in chickpea. Therefore, this computationally efficient model-based strategy had potential for rapid genome-wide scanning of non-spurious SNP marker-trait association with maximal statistical power and high prediction accuracy (Lipka et al. 2012). Considering its added-advantages over other association model-based approaches as documented hitherto, the CMLM remains most widely adopted in GWAS for genetic dissection of complex traits in multiple crop plants (Stanton-Geddes et al. 2013; Saxena et al. 2014a, b; Kumar et al. 2015; Thudi et al. 2014). The flowering time trait-associated eight SNP loci being derived from diverse non-synonymous coding and regulatory sequence components of the eight known/candidate flowering genes are considered to be functionally relevant. Such non-synonymous and regulatory SNPs are known to be involved in regulation of diverse complex seed/grain size and weight quantitative traits in chickpea (Kujur et al. 2013, 2014; Saxena et al. 2014b; Bajaj et al. 2015a, b). Henceforth, the potential molecular tags (novel SNP allelic variants in genes) identified by integrating GWAS and candidate gene-based association mapping could essentially be employed for establishing quick marker-trait linkages and efficient identification/mapping of genes/QTLs governing flowering time trait in chickpea.

To ascertain the validity and robustness of the eight identified flowering time-associated candidate/known genes, the outcome of trait association mapping was further correlated with QTL mapping, differential gene expression profiling and gene-based molecular haplotyping/LD mapping. The molecular mapping of QTLs in an intra-specific high-density genetic linkage map (average inter-marker distance: 2.37 cM) constructed using a large advanced generation flowering time contrasting mapping population (ICC 16374 × ICC 762), validated six major genes harboring five robust QTLs (*CaqDF3.1*, *CaqDF3.2*, *CaqDF4.1*, *CaqDF4.2*, *CaqDF5.1*) controlling flowering time in chickpea. In order to evaluate the accuracy and novelty of eight genomic loci and five major QTLs regulating flowering time detected by association and QTL mapping, respectively, we compared outcomes of the present study with that of previous reports. For this, the markers linked/flanking the flowering time known QTLs/genes (reported earlier in QTL mapping studies), were selected for their validation in flowering time natural (92 diverse *desi* and *kabuli* chickpea accessions) and mapping population (ICC 16374 × ICC 762) constituted in our study. This revealed correspondence of four genomic SNP loci underlying three known QTLs (*CaqDF4.1*, *CaqDF4.*2 and *CaqDF5.1*) governing flowering time between past and our present study (Table S5) based on congruent flanking/linked marker physical/genetic positions on chromosomes (LGs) 4, 5 and 6. Therefore, four genomic loci and two QTLs controlling flowering time detected through high-resolution GWAS/candidate gene-based association and QTL mapping are novel, which possibly reflected their population-specific genetic inheritance pattern for flowering time trait regulation in chickpea. The flower-specific differential up/down-regulation of eight, including six (validated by QTL mapping) flowering time-associated genes specifically in flower developmental stages of early flowering contrasting *desi* and *kabuli* accessions and mapping parents/homozygous mapping individuals based on differential gene expression profiling was apparent. The effective integration of expression profiling further with SNP marker-based gene haplotyping delineated diverse natural allelic variants and major haplotypes in eight known/candidate genes (including six genes harboring five robust QTLs) exhibiting strong association with early and late flowering time differentiation in chickpea.

The flowering time is a complex quantitative trait and being regulated through a complex genetic network involving diverse array of genes in crop plants (Andres and Coupland 2012; Song et al. 2013). The floral signal florigens encoding *FT* (Flowering Locus T) genes (gene families) and their homologs are the key mobile flowering integrators that stimulate/promote floral transition in the shoot apex of multiple plant species, including *A. thaliana* (Abe et al. 2005; Wigge et al. 2005; Ito et al. 2012; Kumar et al. 2012; Liu et al. 2013; Nan et al. 2014). In the present study, by the use of a combinatorial strategy involving GWAS/candidate gene-based trait association mapping, QTL mapping, differential gene expression profiling and gene-based molecular haplotyping/LD mapping, we scaled down eight known/candidate genes (*efl1*, *FLD*, *GI*, *SFH3*, *Myb*, *bZIP*, *bHLH* and *SBP*), including six genes (*efl1*, *FLD*, *GI*, *SFH3*, *bZIP* and *SBP*) harboring five robust QTLs regulating flowering time in chickpea. Among these, diverse major known flowering development pathway and *FT* genes like *efl1*, *FLD* and *GI* harboring the QTLs associated with flowering time have been identified through molecular mapping of flowering time QTLs in different intra- and inter-specific mapping population-derived genetic linkage maps of chickpea (Cho et al. 2002; Lichtenzveig et al. 2006; Anbessa et al. 2006; Cobos et al. 2007, 2009; Radhika et al. 2007; Aryamanesh et al. 2010; Hossain et al. 2010; Gowda et al. 2011; Rehman et al. 2011; Cho et al. 2012; Hiremath et al. 2012; Vadez et al. 2012; Jamalabadi et al. 2013; Zhang et al. 2013; Varshney et al. 2014). A number of known *FT* genes, including photoperiod-independent *early flowering 1* (*efl1*) and photoperiod-dependent circadian-clock-related gene *GIGANTEA* (*GI*) have been isolated and functionally characterized to ascertain their role in flowering time regulation in legumes and *A. thaliana* (Hecht et al. 2007; Liu et al. 2008; Weller et al. 2009; Kong et al. 2010; Laurie et al. 2011; Watanabe et al. 2009; 2011; 2012; Andres and Coupland 2012; Kim et al. 2012; Pin and Nilsson 2012; Song et al. 2013; Yamashino et al. 2013; Zhai et al. 2014; Weller and Martínez 2015). The potential involvement of *bHLH* and *bZIP* transcription factors as well as *FLD* genes in controlling flowering time has been well deciphered in *A. thaliana* and legumes (Abe et al. 2005; Wigge et al. 2005; Searle et al. 2006; Udvardi et al. 2007; Li and Dubcovsky 2008; Yu et al. 2011; Ito et al. 2012; Kumar et al. 2012; Liu et al. 2013; Hu et al. 2014; Nan et al. 2014; Kawamoto et al. 2015). The transcriptional up-regulation, including elevated expression/accumulation of transcripts of these transcription factor genes in governing early flowering regardless of photoperiod is mediated through their preferential interactions (homo- and hetero-dimers)/binding with the *cis*-acting elements in the promoters of different genes like *CO* (*CONSTANS*), *AP1* (*APETALA1*) and *CRY2* (*CRYPTOCHROME*), which in turn activate the transcription of key flowering regulator *FT* genes. A potential *FLD* (*MtFD*) gene governing flowering time has been identified by utilizing an integrated approach of whole genome sequence-based association genetics and QTL mapping in *Medicago* (Stanton-Geddes et al. 2013). The finding of flowering time-associated similar *FLD* gene in chickpea inferred the potential utility of identifying various homologous genes contributing for natural allelic variations in

multiple legume/dicot species (*Arabidopsis thaliana*, *Glycine max*, *Medicago truncatula* and *Pisum sativum*) towards flowering time trait regulation. More interestingly, *bZIP* transcription factor forming complex with protein encoded by *FT* gene is known to play a central role in activation of genes governing floral transition from vegetative to reproductive development (Wang et al. 2009; Sussmilch et al. 2015; Weller and Martínez 2015). The *SBP* transcription factor gene is a direct upstream activator of floral meristem identity gene *AP1* and flower promoting MADS box genes are known to be involved in its constitutive expression for floral transition and regulation of early flowering in *A. thaliana* (Cardon et al. 1997; Guo et al. 2008; Yamaguchi et al. 2009; Wang et al. 2009). The *SFH3* gene is predominantly expressed in the flowers and usually involved in the transfer of phosphatidylinositol/phosphatidylcholine phospholipids during flower development for transcriptional regulation of flowering time in *A. thaliana* (Mo et al. 2007; Wang et al. 2014). The photoperiod-dependent circadian-clock-related gene *Myb* transcription factor plays a crucial role in flowering time regulation in the presence of light and temperature by directly repressing the expression of 'florigen'-encoding *FT* gene and gibberellin-encoding *GA20ox2* (*gibberellin 20 oxidase 2*) gene and activating *FLC* (*Flowering Locus C*) gene in *Arabidopsis* and legumes (Hecht et al. 2007; Dubos et al. 2010; Zhao et al. 2011; Liu et al. 2013; Yan et al. 2014). The aforementioned overview concerning flowering time-associated known/candidate genes identified by the present and past studies inferred that differential regulation of these eight known/candidate genes-encoding transcripts is vital for their effective interactions as well as transcriptional activation of *FT* genes in controlling flowering time in chickpea.

The gene-based haplotyping/LD mapping among 102 cultivated (*desi* and *kabuli*) and wild chickpea accessions identified numerous novel natural allelic variants (94) and haplotypes (24) in eight flowering time-associated genes (identified by an integrated genomic approach) with varying potential for flowering time trait regulation and evolution. Therefore, the flowering time with regard to differential vernalization response in cultivated *desi* and *kabuli* and wild chickpea accessions (evolved due to domestication-led bottlenecks, Abbo et al. 2003; Berger et al. 2003, 2005, Burger et al. 2008; Toker 2009; Meyer et al. 2012; Jain et al. 2013; Kujur et al. 2013; Varshney et al. 2013; Saxena et al. 2014a) possibly represents an important component of domestication trait in chickpea. The flowering time-regulating functionally relevant molecular tags (markers, genes, QTLs, alleles and haplotypes) identified in our study with an integrated genomic approach can be useful for quantitative dissection of complex flowering time trait and eventually marker-assisted genetic enhancement to develop early flowering cultivars of chickpea.

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