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SHORT RESEARCH ARTICLE

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Validation of molecular markers linked to flowering time genes in chickpea (*Cicer arietinum* L.)

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Abstract

The objective of the study was to validate molecular markers linked to genomic regions controlling flowering time in a set of Recombinant Inbred Lines (RILs) population derived from Pusa 362 (Late) x BGD 132 (Early) and 21 diverse genotypes differing in time of flowering in chickpea (*Cicer arietinum* L.). The study of the polymorphic survey between parents using 27 linked markers of chickpea and *Arabidopsis* found 8 of them polymorphic. Bulked Segregant Analysis identified *TA64* and *TA142* as putatively linked to *efl3* locus governing early flowering in chickpea that can be utilized for marker assisted selection. The PCR amplification of 6 polymorphic markers in germplasm showed the presence of one or more alleles in many of them (up to 3), indicating the complex nature of flowering time in chickpea. The amplification pattern of *CaEfl3a-F1* marker linked to *Efl3* early flowering allele of *Arabidopsis* in 21 chickpea germplasm indicated the presence of some conserved genomic sequences controlling flowering time in the two species.

Keywords: Bulked Segregant Analysis, chickpea, early flowering, validation

India is the largest producer of chickpea (*Cicer arietinum* L.), with a share of about 66 % (11.38 million tons) of its global production (FAO 2018). Its cultivation in India can be broadly classified as short (peninsular India), medium (central India), and long (northern Indian plains and hills) duration environments. It is predominantly a rainfed crop cultivated under residual and receding soil moisture conditions during the post-rainy season. As a result, terminal drought is a major constraint to its production in over 80% of the area. Heat stress has also become an important constraint to its production at the reproductive stage due to changing climate. Breeding for early flowering and maturity is an important adaptive strategy to increase the productivity of chickpea and the stability of its production in environments characterized by terminal drought and heat stresses.

Four genes governing the inheritance of flowering time have been reported so far in chickpeas such as *efl-1* (Kumar and van Rheenen 2000; Gaur et al. 2015), *efl-2* (Or et al. 1999), *efl-3* (Hegde 2010), and *efl-4* (Gaur et al. 2015). Mallikarjuna et al. (2017) studied molecular mapping of quantitative trait loci (QTLs) controlling flowering time in chickpea using F_2 populations derived from four crosses involving early and late flowering parents and identified 10 genomic regions that are distributed across CaLG01, CaLG03, CaLG04, CaLG06 and CaLG08 of chickpea genetic map. They reported eighteen SSR markers that are linked to *efl-1, efl-2, efl-3, and efl-4* genes in chickpea. Stephen et al. (2017) reported that *efl-3* of *Arabidopsis* located on LG5 is orthologous to *the efl-1* gene located on the LG3 of chickpea. Cho et al. (2002) and Jamalabadi et al. (2013) found nine SSR markers linked to *Efl-3*. The quantitative trait loci (QTLs) controlling flowering time have also been identified on the LG1, LG2, LG3, LG4, and LG8 of the chickpea genetic map (Cho et al. 2002; Cobos et al. 2007; Lichtenzveig et al. 2006; Aryamanesh et al. 2010; Hossain et al. 2010; Jamalabadi et al. 2013). The presence of a large number of QTLs on different linkage groups and a wide range of variation for the time

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of flowering in the world collection shows that there may be many genes controlling this adaptive trait in chickpea (Hegde 2010). The diversity for a time of flowering that exists in different chickpea genotypes might be due to the presence of different alleles or a combination of alleles and their interaction. The validation of markers linked to already mapped QTLs for flowering time is required for further use in marker-assisted selection to develop early maturing high-yielding varieties better adapted to terminal drought and heat stress environments. This investigation was carried out to validate the molecular markers linked to different times of flowering genes in a Recombinant Inbred Lines (RILs) population and diverse germplasm differing in flowering time and to study the orthologous nature of flowering time genes between cultivated chickpea and *Arabidopsis*.

A population comprising of 250 RILs derived from a cross between Pusa 362 (a late flowering high yielding variety) and BGD 132 (an early flowering genetic stock carrying *efl-3* alleles of flowering time) and 21 diverse germplasm differing widely for time of flowering were utilized for the study. The RILs were developed from all the different F_2 plants advanced through plant to progeny row method up to F_7 by randomly selecting a single plant from each of the progenies in each generation and advancing them to the next generation. F_8 seeds of single plants harvested from each F_7 progeny were planted in the field in individual rows for phenotyping during *rabi* 2018-19 post-rainy season at the ICAR-Indian Agricultural Research Institute, New Delhi, in an augmented complete block design. The field was partitioned into five blocks, each comprising of 50 RILs and two parental lines. A single row of 3 m length with a spacing of 50 cm between rows and 10 cm between plants within a row was considered in each block. Thus, a developed RIL population was used for validation of molecular marker (s) associated with early flowering genes by Bulked Segregant Analysis (BSA). The RILs were phenotyped for flowering time recorded as the duration between the date of sowing and the date of first fully opened flower in each line. Based on the phenotyping data, 20 extreme RILs for time of flowering (ten extreme early and ten extreme late) were selected, along with the two parents involved in the cross. Twenty-one diverse germplasm lines (Table 1) were also phenotyped during *rabi* 2019-20 for validation of markers associated with four different flowering time genes. These genotypes were grown in a single row of 3 m length, each with a plant-plant spacing of 20 cm. The flowering time was recorded in each

Table 1. Different early and late flowering germplasm along with their allelic composition

Genotypes	Time of Flowering (days)	Amplification pattern (Chickpea markers)						Amplification pattern (*Arabidopsis* markers)		Probable allele Composition
		efl-1 (TR13)	*efl*-2			*efl*-3				
			GA6	TA122	TA30	TA142	TA64	CaEFL3a-F1	CaEFL3a-F3	
BG 1044	68	+	+	+	+	+	+	+	+	*efl-1, efl-2, efl-3*
BG 1054	66	-	+	+	-	+	+	+	+	*efl-2, efl-3*
BG 3073	69	-	+	-	+	+	+	+	-	*efl-2, efl-3*
BG 3078-1	68	-	-	+	-	+	+	-	-	*efl-2, efl-3*
BGD 70	70	-	+	-	-	-	-	-	-	*efl-2*
BGD 9722	69	-	+	-	-	-	-	-	-	*efl-2*
BGD 9965	66	-	+	+	+	+	-	-	-	*efl-2, efl-3*
BGD 9976	69	-	+	+	+	-	+	-	-	*efl-2, efl-3*
BGD 119	78	-	+	+	+	+	+	-	+	*efl-2, efl-3*
BGD 9617	59	-	+	+	+	-	-	-	+	*efl-2*
BGD 9812	59	-	+	-	-	-	+	-	+	*efl-2, efl-3*
ICCV 2	67	-	+	-	-	+	+	-	+	*efl-2, efl-3*
JGK 1	68	-	+	+	+	+	-	-	+	*efl-2, efl-3*
HARIGHANTAS	73	-	+	+	+	+	+	-	-	*efl-2, efl-3*
MEXICO LOCAL	74	-	+	+	+	-	-	-	-	*efl-2*
BGD 72	84	-	+	+	-	+	-	-	-	*efl-2, efl-3*
BGD 112	90	-	+	+	-	+	+	-	+	*efl-2, efl-3*
BG 372	84	-	+	-	+	+	+	-	+	*efl-2, efl-3*
BG 3062	78	-	+	-	+	-	-	-	-	*efl-2*
ICCV 5	86	-	+	-	-	+	-	-	-	*efl-2, efl-3*
BGD-128	85	-	+	+	+	-	-	-	+	*efl-2, efl-3*

+ = Amplified - = Not Amplified

of the germplasm as the number of days from sowing to the first flower appearance.

Leaf samples were collected from the two parents, ten extremely early and ten extremely late flowering RILs and 21 early and late flowering germplasm lines. The DNA isolation was performed as per the protocol given by Murray and Thompson (1980), popularly known as CTAB method with some modifications. The PCR amplification products were separated on 3% Agarose (HIMEDIA). The amplified products were separated on a horizontal electrophoresis system at 120 V for 3-4 h using a 1.0X TBE buffer. The gels were stained by Ethidium bromide (10 mg/ml) and visualized using Gel Documentation system (AlphaImager 2200, Alpha Innotech Corporation, and USA). Amplicons were scored as alleles for each marker loci. Scoring of the alleles was done manually, and their sizes (bp) were determined by comparing them with the 100 bp DNA ladder.

Parental polymorphism, bulked segregant analysis and marker analysis

The parental polymorphism was carried out between Pusa 362 and BGD 132 with 18 SSR markers of chickpea reported to be linked to flowering time alleles, *efl-1 to efl-4*, and nine markers linked to *Efl-3* flowering time allele of *Arabidopsis*. Among the 27 SSR markers used, 8 showed polymorphism between the two parents. The polymorphic markers obtained between the two parents were subjected to BSA as Michelmore et al. (1991) defined. The early and late-flowering bulks were separately constituted by combining the equal amount of DNA from 10 extreme early flowering and 10 extreme late flowering plants, respectively. Finally, both the bulks were tested along with parents using the polymorphic markers to recognize putatively linked markers to the gene of interest. The amplification of these polymorphic markers in early and late bulks showed that two of them, TA64 and TA142, co-segregated with the early flowering trait (Fig. 1) and hence were identified to be putatively linked to already mapped *Qefl3-2* locus in chickpea on LG03 (Mallikarjuna et al. 2017). Further, these two markers were amplified in the 10 extreme late flowering and 10 extreme early flowering plants of the RIL population individually to confirm their association with the concerned locus. It was observed that the evaluated RILs (Fig. 2) exhibited the expected DNA profile only for TA64 but not TA142. Therefore, only TA64 was found to be linked with the early flowering trait that can be used for marker-assisted selection to breed early varieties in chickpea.

Validation of markers

Validation of polymorphic markers in 21 diverse germplasm was carried out to understand their allelic composition for flowering time and to confirm the presence of any conserved sequences between the genomes of cultivated chickpea and *Arabidopsis* species. An amplification pattern was observed in all the selected genotypes. The amplification pattern of polymorphic markers (Table 1) clearly indicated the presence of more than one gene in most of the genotypes included in the study. Markers linked to *efl-1* gene amplified only in BG 1044 genotype, and therefore it may be carrying *an efl-1* allele for a time of flowering. Whereas markers linked to *efl-2* gene amplified in all 21 (early and late flowering) genotypes, including BG1044, suggesting the presence *efl-2* gene in them. Markers linked to *efl-3* gene showed amplification in early (BG 1044, BG 1054, BG 3073, BG 3078-1, BGD 9965, BGD 9976, BGD 119, BGD 9812, ICCV 2, JGK 1, Harighantas) as well late flowering genotypes (BGD 72, BGD 112, BG 372, and ICCV 5). Thus, the present study indicated the presence of *efl-1, efl-2* and *efl-3* genes in only one genotype, viz., BG 1044. However, previous studies based on the phenotypic expression of flowering time and Mendelian segregation pattern in F_2 and F_3 reported the presence of only *efl-1* in ICCV2 and *efl-2* in Harighantas (Hegde 2010; Gaur et al. 2015), indicating the complex nature

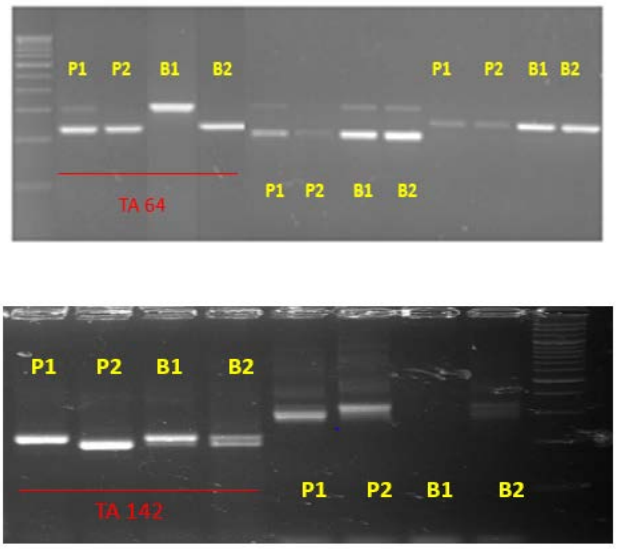

Fig. 1. BSA showing putatively linked marker

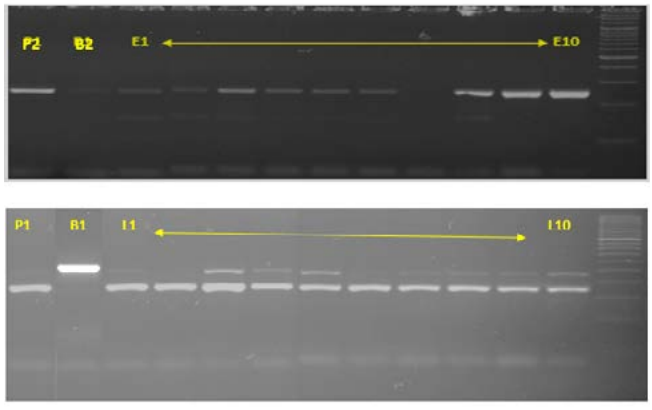

Fig. 2. Validation of marker TA64 linked to *Qefl3-2* loci of chickpea genotypes

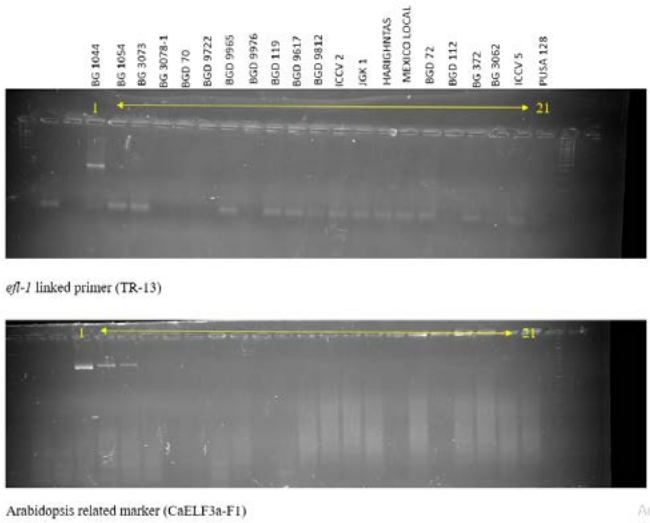

Fig. 3. Amplification pattern of *Efl-1* linked primer and Arabidopsis primer

of flowering time trait in chickpea. Hegde (2010) reported that several duplicate dominant genes governed the time of flowering in chickpea, and the large genotypic variation for time of flowering that exists in chickpea germplasm might be due to the presence of different alleles or combination of alleles and their interaction effect. The presence of *efl-2*, either alone or in combination with other alleles, in all the 21 genotypes studied indicates that *efl-2* allele may be contributing more to the total phenotypic variation present in the chickpea germplasm. Further research is needed on fine mapping of flowering time genes and development of near-isogenic lines for each gene for better understanding of the genetic basis of large variation for the trait and their effect on earliness and components of productivity in chickpea.

Stephen et al. (2017) found the orthologous nature of *Efl-1* flowering time gene of chickpea with the *Efl-3* gene of Arabidopsis. Therefore, validation of markers associated with these two alleles was carried out in 21 germplasm differing in flowering time to confirm the existence of any conserved genomic sequences between chickpea and Arabidopsis species for time of flowering. The amplification pattern of *efl-1* related marker *TR13* of chickpea showed a similar pattern with *Arabidopsis* related marker *CaEfl3a-F1* (Fig. 3). The analysis of the Chi-square statistic clearly indicated the presence of a strong correlation and no significant difference between the *Efl-1* linked primer and *Arabidopsis* primer. The chi-square value obtained from the observed and expected similarity of amplification pattern is 0.19047619048, which is much lesser than the table value of 3.841 at a 5% level of significance, suggesting the presence of some conserved sequences for a flowering time between genomes of two species. The markers validated in the present study are useful in the molecular characterization and identification of genotypes carrying specific allele(s) for flowering time in chickpea.

Authors' Contributions

Conceptualization of research (AN, VSH); Designing of the experiments (VSH, AN); Contribution of experimental materials (VSH); Execution of field/lab experiments and data collection (AN, VSH, BC, ST, RKS, SS); Analysis of data and interpretation (AN, VSH, BC, ST, PKJ, RK, RKS); Preparation of manuscript (AN, VSH).

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