A QTL linkage map of safflower for yield under drought stress at reproductive stage

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Abstract
This study reports QTL mapping for seed yield and its components in safflower genome under drought stress. The F_3 families derived from the cross Mex.22-191 (tolerant) × IL.111 (sensitive) were evaluated for agronomic traits in safflower. Drought tolerance was evaluated during 10% of the flowering stage. To identify QTLs underlying tolerance to drought, mapping quantitative trait loci (QTLs) was carried out by composite interval mapping function. A genetic linkage map (LG) assembled from SSR and ISSR markers, was mapped. A total of 145 DNA bands (SSR and ISSR markers) coalesced into 24 LGs which summed to 646 cM in the total map length. This analysis resulted in the identification of 18 QTLs related to seed yield and its components. Based on findings in this study, four major QTLs and three linkage groups (2, 4 and 6) played a crucial role in drought tolerance of safflower. The present linkage map may give a useful framework for mapping agronomic traits in safflower and the framework maps of *C. tinctorius* can serve as a foundation for future map integration, comparative genomics, QTL analysis and marker assisted breeding for drought tolerance.

***Key words**:* Drought stress, Linkage group, QTL analysis, Safflower.

INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is an oil seed crop that has moderate tolerance to drought (Dajue and Mundel, 1996) therefore, genes conferring resistance to abiotic stresses may be located in safflower. There is limited research around the world on safflower production under irrigated conditions. It has been revealed that it is a sensitive crop to water at the flowering stage (Quiroga *et al*., 2001; Bassil and Kaffka, 2002) and moderately tolerant to salinity. Iran is regarded as one of the diversity centers of safflower (Singh, 2007). In Iran water is a scarce resource due to the high variability of rainfall. The effects of water stress depend on the timing, duration and magnitude of the deficits (Pandey *et al*., 2001). Developing drought tolerant plants is vital to meeting increased demand for agricultural products (Parry 2005). Saini and Westgate (2000) pointed out that at all reproductive sub phases, safflower is sensitive to water deficit. It reduces seed and/or flower numbers per capitulum during early reproductive growth stages. Marita and Muldoon (1995) and Zarie *et al*. (2013) reported that safflower is sensitive to water deficit at the flowering stage. Breeding programs in safflower have been hampered by limited knowledge about genetic variability within C. *tinctorius* and the lack of genomic tools for trait breeding (Mayerhofer *et al*., 2010). Making progress for seed yield and its components under drought stress is difficult because of environmental effects and genotype × environment interactions (Bartels and Sunkar, 2005). The Quantitative Trait Loci (QTL) mapping approach (Collard *et al*., 2005) has been

successfully applied as a tool for genetic analysis of important traits and abiotic stress tolerance. On the other hand, identification of QTLs controlling important traits for screening adopted genotypes to drought stress is a major challenge for plant breeders. Molecular markers associated with quantitative trait loci (QTL) for drought tolerance traits could enhance progress in breeding for drought tolerance (Kirigwi *et al*., 2007). QTLs for drought tolerance has been detected in many gramineous plants such as rice (Wang *et al*., 2012), wheat (Kirigwi *et al*., 2007; Liqing *et al*., 2007) and sorghum (Wang *et al*., 2013) and also species of oil seeds including cotton (Saeed *et al*., 2011) and sunflower (Ebrahimi *et al*., 2009, Abdi *et al*., 2012) but literature review showed that there are no reports on this topic related to safflower. Only few studies have been reported on mapping the genes controlling high oleic acid (Hamdan *et al*., 2012) and male sterility (Hamdan *et al*., 2008). Chpman *et al*. (2007) developed universal markers to compare mapping and phylogenetic studies in Asteraceae. The first genetic linkage map in safflower has been prepared by Mayerhofer *et al*. (2010) that has established a foundation map for genetic studies in safflower. Pearl *et al*. (2014) mapped 61 QTLs underlying 24 domestication-related traits for the analysis of genetic architecture of safflower domestication and compared their differences to those from sunflower (*Helianthus annuus* L.). No research has been conducted to develop molecular markers linked to important traits suitable for drought tolerance in safflower breeding. Therefore, the present study is the first study to identify quantitative trait loci (QTL) controlling seed yield and its components under drought stress in safflower. The results of this study would help to identify genetic control of drought tolerance at the reproductive stage to improve drought tolerance of safflower by molecular aided selection (MAS) program to develop drought tolerant safflower cultivars.

MATERIAL AND METHODS

Field experiment and phenotypic evaluation

Sixty six plants from F_2 generation derived from the cross between Mex.22-191 (a Mexican drought tolerance line) and IL.111 (an Iranian semi drought-sensitive line) formed the mapping population. Phenotyping was done on F_2-derived F_3bulks of this population. Schon *et al*. (1993) pointed out that if the progeny means of F_3 lines are used instead of F_2 individuals for phenotyping, then only half of the dominance effects contribute to the genotypic mean of F_3 lines derived from heterozygous F_2 plants.

Sixty six $F_{2:3}$ families were grown under an irrigation regime in growing season of 2010-2011 as a complete randomized block design. There were two separate experiments for drought and normal conditions based on complete randomized block design by subsequent combined analysis. Thus, each $F_{2:3}$ families was represented by fifteen plants in each row within a plot by 4 rows under the similar environmental conditions at the research field of Shahid Bahonar University of Kerman, located in the South-Eastern part of Iran (56°58' E longitude and 30°15' latitude N, 2044 m altitude), with a hot and arid climate. Each experimental plot consisted of three rows with one meter long and 45 cm distance between rows. Fertilizer was applied before sowing (100 kg ha m^{-2} P_2O_5 and 25 kg ha m^{-2} Zn) and at stem elongation stage (50 kg ha m^{-2} N). The experimental plots were hand weeded as needed during the growing season. Drought stress was applied during 10% of flowering based on 50% of field capacity (FC). All other agronomic practices were conducted to reach optimum crop growth. Traits such as: plant height, number of branches per plant, number of capsules per plant, plant dry weight, seed yield per plant, 1000-seed weight, seeds number per plant and seeds number per capsule, were measured after drought stress (about 2 months after seed sowing). F1, F2 and F3 were evaluated at a time in the same trial.

Genotyping

F_2 plants were used for molecular assay. DNA was extracted from leaf tissues of six-week old plants from each individual of F_2 plants following the CTAB method (Doyle *et al*., 1987). The quantity and quality of the extracted DNA was assessed using a spectrophotometer and agarose gel electrophoresis, using λ DNA as standard. Totally, 96 SSR and 23 ISSR primers were used for parental survey, of which 12 SSR (Table 1) and 133 ISSR (Table 2) bands produced polymorphic patterns with clear bands. Polymorphic primers were applied on F_2 individuals to construct SSR and ISSR linkage map.

SSR analysis

The PCR reaction mixture (10 µL) for SSR analysis, contained 1× PCR buffer, 0.5 mM $MgCl_2$, 1 mM (dNTP), 1 µM (primer), 50 ng of template DNA, 0.25 Unit Taq DNA polymerase, 3.9 µL of distilled water. Amplification conditions included an initial denaturation for 4 min at 92 °C, followed by 40 cycles with initial denaturation for 1 min, followed by 30 seconds with different annealing temperature, 1 min at 72 °C for extension, with 5 min at 72 °C for ending with an extension period. Amplified products were resolved by electrophoresis on 6% polyacrylamide gels

Table 1. SSR primer sequences used for QTL mapping in safflower under drought stress.

SSR name	Direction	Primer sequence (5´to 3´)	GC (%)	Tm (°C)
CAT-4	F	CCTATGTACCAAGACCAAG	47.4	55
	R	CTCCTTCCGGCACTCAC	64.7	55
CAT-16	F	CGGGAGTGATGTAATGACCCA	52.4	64
	R	CAATCTTAGATTAATCACCACTG	34.8	64
CAT-17	F	GAACTGGTATGGTTCATATTCGA	39.1	65/5
	R	GAGTTTCAGTGAGTAGAACGAG	45.5	65/5
CAT-30	F	TAGCTGAGGCACTTTGGCTC	55	62
	R	AGGTAAGCATCAAACCATAC	55	62
CAT-38	F	GAGGAAGCTAGCTAATGAAATG	40.9	62
	R	ATGATGATATCCTTGCGGAATC	40.9	62
CAT-49	F	GCAAAGTGCATAATCTACTTAGCA	37.5	66
	R	GTGAATACTACAAGCGGAACTAC	43.5	66
CAT-65	F	AGAAGGTAAATCCATTGTGGAAG	39.1	64
	R	TGCAAGAGTCCCTCAAGAGTC	52.4	64
CAT-80	F	TGGATGGCCTCATTCTCCTTG	52.4	64
	R	GTTAATCATGGGCTTAGGCCA	47.6	64
CAT-92	F	CCACCGTAACCGAAGATGTG	55.0	62
	R	TCTAAAGGTAACCTTCGTAGTG	40.9	62
CAT-101	F	GACACTACCTAACGGTGGTG	55.0	61
	R	ACCACCTATAGGTAGTGTATG	42.9	61
CAT-102	F	GTTATATGGATGGGCTTGAC	45.0	58
	R	TCTCCCAAGAACACTATGGA	45.0	58
CAT-109	F	GATCTCATTTTATTAGTCCCGC	40.9	61
	R	GTTAGTGGAGGTTCACATAAG	42.9	61

in 1× TBE buffer with ethidium bromide incorporated in the gels and visualized under UV light.

ISSR analysis

The PCR reaction mixture (15 µL) for ISSR analysis, contained 1× PCR buffer (1.5 µL), 0.5 mM $MgCl_2$, 0.4 mM (dNTP), 0.4 µM (primer), 60 ng of template DNA, 0.25 unit Taq DNA polymerase, 8.4 µL of distilled water. DNA amplification was performed in an Eppendorf Master cycler according to Golkar *et al.* (2011) with minor modifications. The initial denaturation was set up at 94 °C for 4 min, and then DNA thermal cycler was programmed by 35 cycles of 1 min at 94 °C, annealing temperature for each primer prolonged for 2 min at 72 °C, and final extension prolonged at 72 °C for 7 min.

Statistical analysis

Analysis of experimental data, including calculation of heritability, mean and standard errors were performed using SAS (SAS Institute, 2004).

Linkage analysis

The genetic linkage map was constructed with the Map Manager 3.0 program (Manly and Olson 1999). Linkage groups (LGs) were identified with linkage criteria of LOD > 3. Map distances (cM) were then estimated using recombination distances and Kosambi's mapping function between ordered marker loci.

QTL analysis

QTL Cartographer 2.5 (Wang *et al*., 2010) was used to identify QTLs affecting drought tolerance on the basis of Composite Interval Mapping (CIM). Permutation thresholds (α = 0.05 and 0.1) for declaring QTL significance were estimated based on 1000 permutations (the type 1 error being 0.05) for each trait and named according to McCouch and CGSNL (McCouch, 2008). Finally, estimates of additive and dominance effects, the total variance explained by

Table 2. ISSR primer sequences that have been used for QTL mapping in safflower under drought stress.

No	Oligo Sequence	Tm (°C)
1	GAGAGAGAGAGAGAGARC	51
2	CACACACACACACACAG	52
3	TCTCTCTCTCTCTCTCG	51
4	CTCTCTCTCTCTCTCTRC	55
5	AGAGAGAGAGAGAGAGYT	54
6	ACACACACACACACACYG	54
7	TGTGTGTGTGTGTGTGRT	51
8	CACACACACACACACART	51
9	CTCTCTCTCTCTCTCTRG	54
10	DBDACACACACACACAC	51
11	BDBTCCTCCTCCTCCTCCTCCTCC	65
12	HVHTCCTCCTCCTCCTCCTCCTCC	65
13	CACACACACACACACAWT	47
14	CCACTCTCTCTCTCTCTCT	56
15	AGAGAGAGAGAGAGAGSC	56
16	GACAGACAGACAGACAGACA	55
17	GAAGAAGAAGAAGAAGAA	47
18	CACACACACACACACAGT	54
19	GTGTGTGTGTGTGTGTYC	55
20	TGTGTGTGTGTGTGTGG	52
21	ACACACACACACACACYG	55
22	AGAGAGAGAGAGAGAGT	50
23	CACACACACACACACART	52

Table 3. Phenotypic values of traits among parents and F_1, F_2 and F_3 generations under drought stress.

Character	P_1	P_2	F_1	F_2	F_3
Plant height	85.16± 9.23	144± 12.83	95±5.44	86.22±11.44	92.68±13.62
Branches/plant	1.33± 1.75	10.83± 4.35	8.50±1.35	5.77±2.68	8.08±2.87
Capsules/ plant	12.33± 7.94	27.16± 8.28	22.50±6.77	15±7.50	18.66±9.86
Dry weight/ plant	52.9± 10.3	98.66± 30.34	92.16±21.94	64.37±35.86	78.19±38.95
Seed yield /plant	13.16± 8.82	18.43± 8.96	20.76±6.49	12.88±7.49	16.54±10.26
1000-seed weight	43.10± 6.03	32.50±4.35	40.70±7.88	36.14±3.66	38.19±16.65
Seeds/ plant	313.33± 87.89	454.50±201.49	525.66±177.37	351.22±181	440.23±264.15
Seeds/ capsule	30.10± 11.55	19.32±3.36	23.24±2.69	24.35±6.20	24.92±9.50

P_1: drought tolerant line and P_2: semi-sensitive line

significant QTLs and coefficient of determination for identified QTLs were estimated using QTL Cartographer. The magnitude of the effect of each QTL was considered to be "large" if the percentage of total phenotypic variance (R^2) was greater than 20%, "small" if the R^2 was less than 10%, and "intermediate" if in between these values.

RESULTS

Phenotyping

Comparisons of means and standard errors of the mapping parents and different generations (F_1, F_2, and F3) are presented in Table 3. The F_1 mean was greater than the mean of both parents only for seed yield per plant, and the number of seeds per plant. This result implied that heterotic effects could be effective for the improvement of these traits. The means of F_2 generation for the studied traits were in the range of parental means, except for seed yield. The mean comparison showed that P_1 and P_2 had significant differences in most of the traits.

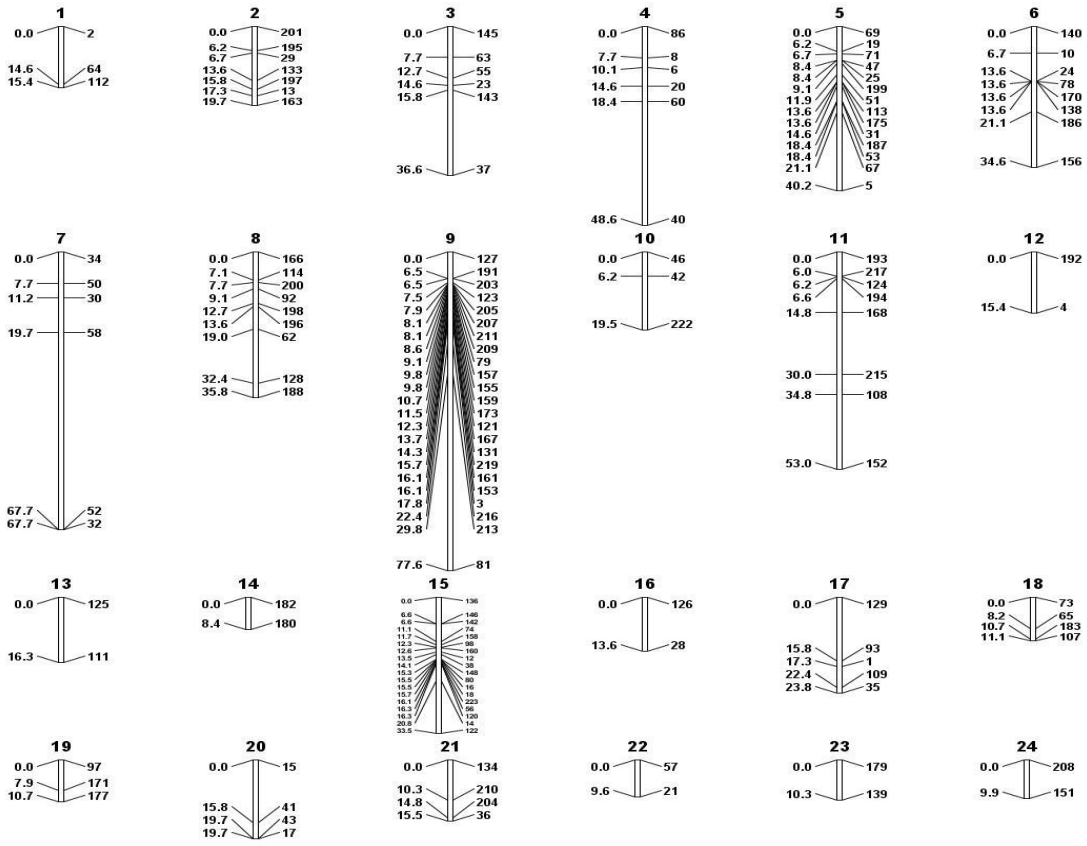

Figure 1. The genetic linkage map of IL.111 × Mex.22-191 of 24 linkage groups (LG) showing the location of putative QTLs for the agronomic traits in safflower drought stress.

Table 4. Estimation of broad-sense and narrow-sense heritability of under studied traits.

Trait	Broad-sense h^2 (%)	Narrow-sense h^2 (%)
Plant height	61	43
Branches /plant	84	27
Capsules/ plant	58	53
Dry weight /plant	99	49
Seed yield/ plant	73	66
1000-seed weight	97	35
Seeds/ plant	99	26
Seeds / capsule	94	90

Heritability of traits

The selection efficiency is related to the magnitude of heritability (Kearsey and Pooni, 1996). In this study, number of seeds per capsule, seed yield per plant and capsules/plant showed high narrow-sense heritability (Table 4) that the highest value belonged to the number of seeds/capsule (90%). Other studied traits had medium narrow-sense heritabilities. This implies that most of the genetic variances were due to dominance gene action (Table 4). Golkar *et al.* (2012) also reported a high value for broad-sense heritability of the number of seeds/capsule (99%) that was similar to our results. Kotecha and Zimmerman (1978) reported high broad-sense heritability (86%) for 100-seed weight in normal conditions. The high value for broad- sense heritability for the number of branches per plant (84%) in our study was not in line with the reports of Camas and Esendal (2006). The discrepancy in estimation of heritability for a trait is mostly because the heritability is not a property of a trait itself, but it is related to the population, environmental conditions, method of evaluation of genotype and parameter estimation (Falconer and Mackay, 1996).

Construction of the linkage map

Among 119 SSR and ISSR markers which produced 145 DNA bands on 66 f_2 individuals a total of 35 informative markers were obtained (Figure 1). A genetic linkage map comprising of 24 linkage groups

Table 5. The QTLs for different traits of safflower under drought stress.

Trait	McCuch name	Position	LOD	CIM (Additive effect)	CIM (Dominant effect)	R^2	Linkage group
Plant height	qPh6_1	14	2.7	2.3	316.62	0.17	6
	qPh6_2	30	3.09	66.2	-604.28	0.19	6
Branches /plant	qBpno4_1	16	2.80	-185.6	12045.7	0.17	4
	qBpno 4_2	44	2.50	768.66	-298.18	0.16	4
	qBpno6	20	2.38	3.66	-90.15	0.15	6
Capsules/ plant	qCpno2	1	2.68	7.21	-4179.2	0.17	2
Dry weight /plant	qDw2	16	3.46	34.33	-12738	0.21	2
	qDw4	16	2.47	-2316.5	150362	0.15	4
	qDw6	20	2.72	53.16	-1357.4	0.17	6
Seeds/ plant	qSpno2	16	5.88	273.25	-89211	0.33	2
	qSpno3	8	2.96	880.94	-204698	0.18	3
	qSpno4	16	3	-13917	905041	0.18	4
	qSpno7	6	2.66	265.007	-794.07	0.17	7
	qSpno9	40	2.91	-738.8	281170	0.18	9
	qSpno18	6	2.68	1468.64	254692	0.17	18
Seed yield/ plant	qSyp2	16	4.71	10.45	-3258.8	0.28	2
	qSyp9	40	3.17	-21.00	12215.8	0.19	9
1000-Seed weight	qThsw5	40	2.41	77.59	-2118.6	0.15	5

R^2: coefficient of determination for identified QTLs.

was constructed by the computer program Map Manager for IL.111 × Mex.22-191 population (Figure 1). The linkage map spanned 646.2 cM of the safflower genome with an average interval of 4.45 cM between two adjacent markers. The highest number of QTLs was denoted to the linkage groups of 2, 4 and 6 and the lowest number of QTLs was identified on linkage groups 3,5,7,9 and 18.

Identification of QTLs for traits attributed to drought tolerance

Totally, 18 QTLs were detected in F_2 population from IL.111 × Mex.22-191 cross. The detected QTLs using QTL Cartographer (Manly and Olson, 1999) are listed in Table 5.

Plant height

Two QTLs affecting plant height were identified on the linkage group of 6 located 14 and 30 cM above the LG6. The QTLs explained 17 and 19% of the total phenotypic variance with the additive effects of 2.308 and 66.26, respectively, which had the source of additive effect from Mex.22-191. These QTLs showed the load score of 2.7 and 3, respectively (Table 5).

Number of branches per plant

Three QTLs were detected for the number of branches per plant that were denoted to linkage groups of 4 and 6 with LOD scores >2. These QTLs explained 48% of total phenotypic variation (Table 5). Mex.22-191 carried positive alleles for these loci.

Number of capsules per plant

One QTL was recognized for the number of capsules per plant on LG2 with 17% of phenotypic variance. This trait increased by the Mex.22-191 allele on LG2.

Plant dry weight per plant

Three QTLs were detected on LGs of 2, 4 and 6, together explaining 54.7% of phenotypic variance. The additive effects of these QTLs were 34.33, -2316.5 and 53.16, respectively (Table 5).

Seed yield per plant

Two QTLs were detected for seed yield per plant on linkage groups 2 and 9 with significant LOD scores (>3) that totally explained 47% of total phenotypic variation (Table 5). The nearest marker to the QTL, 197 on the group 2, and 213 on the group 9 can be used for the selection of seed yield.

1000-Seed weight

One QTL was detected on group 5 for 1000-seed weight, accounting for 15.5% (LOD = 2.4) of the

phenotypic variance. A QTL with additive effect of 77.59 was simulated at 44 cM from the end of LG5.

Number of seeds per plant

The highest number of QTLs was mapped for the number of seeds per plant on groups 2, 3,4,7,9 and 18. The major QTL was mapped to group 2 and explained 33.7% (LOD = 5.8) of the phenotypic variance. The second major QTL was detected on group 4 with significant LOD scores (=3).

DISCUSSION

Comparisons of the means showed the mean of F_1 generation for seed yield per plant and the number of seeds per plant was greater than the mean of both parents. This result implied that heterotic effects could be effective for improving these traits. High percentages of broad-sense heritability (>70%) suggested that environmental effects constitute a minor portion of the total phenotypic variation of included traits. In this study, number of seeds per plant, dry weight per plant, and 1000-seed weight showed the highest broad-sense heritabilities and the number of seeds per capsule, seed yield per plant and the number of capsules per plant showed high narrow-sense heritabilities (Table 4), indicating that selection for these traits could be successful, because of the high proportion of the additive component in total genetic variance. Thus, marker assisted selection could be a suitable strategy for the traits.

Detection of different mapping population leads to the discovery of more QTLs, and comprehensive grasp of gene location in the chromosomes is the basis of genetic research for traits (Zhang *et al*., 2011). Many researchers reported that safflower is sensitive to drought stress at the reproductive stage (Saini and Westgate 2000; Marita and Muldoon, 1995; Zareie *et al*., 2013). The identification of marker loci linked to QTLs involved in drought tolerance is an important step in the genotypic evaluation of safflower germplasm. To our knowledge this is the first report on linkage map for safflower under drought stress at the reproductive stage. In the present map, the number of the linkage groups was equal to the number of arm chromosomal of safflower. In the past a variety of markers has been used in *Curthamus* for different purposes. The most commonly employed marker types are randomly amplified polymorphic DNA (RAPD) amplified fragment length polymorphism (AFLP) and inter simple sequence repeats (ISSR) (Mayerhofer *et al*., 2010). In the F_2 generation of a safflower cross Mex.22-191 × IL.111 (identified as being drought tolerant and sensitive lines, respectively), SSR and ISSR markers were used to study and map the genomic regions of some of the important agronomic traits. The total map distance recorded here is equal to 646.2 cM. The present linkage map may give a useful framework for mapping of agronomic traits in safflower and the framework maps of *C. tinctorius* can serve as a foundation for future map integration, comparative genomics, QTL analysis and marker assisted breeding. Our results indicated the existence of genes with major effects involved in the control of significant proportions of the phenotypic variation in some of the important agronomic traits such as the number of seeds per plant and seed yield on group 2, under drought stress. Identification of these major effect-QTLs could facilitate simultaneous transfer of tolerance components at reproductive stages of safflower using marker assisted selection. Based on findings in this study, four major QTLs and three linkage groups (2, 4 and 6) played a crucial role in drought tolerance of safflower, which could be applied in marker assisted selection and further investigation for drought tolerance. The present study is the report of QTL mapping for seed yield and its components in safflower genome under drought stress. New findings in the present study could be suitable to complete future studies in mapping genome projects and marker assisted selection makes more complicated breeding programmes feasible. Phenotypic selection for drought tolerance may not be difficult, but identified marker loci may be useful in multiple-trait selection where drought tolerance is one of many traits of interest.

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