academicJournals

Vol. 13(6), pp. 754-761, 5 February, 2014
DOI: 10.5897/AJB2013.12325
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Qualitative trait loci analysis for seed yield and component traits in sunflower

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Accepted 8 January, 2014

The present investigation was carried out to identify the molecular markers associated with various characters in sunflower using recombinant inbred lines. Linkage analysis was carried out and five linkage groups were obtained with 19 simple sequence repeats (SSR) markers. Linkage map construction, single marker analysis (SMA) and composite interval mapping analysis were carried out with SSR primers and quantitative traits. In SMA, out of 50 SSR markers, a total of 29 SSR markers were found to be significantly linked to various traits. The adjusted R2 for the regression equation varies from 3.2 to 29.8%. Two traits namely, days to flowering and seed color recorded above 20% R2 value. Hull weight recorded above 10% R2 value. In inclusive composite interval mapping (ICIM), the quantitative trait loci (QTL) analysis resulted into two QTLs namely, seed and volume weight. QTL analyses were performed through inclusive composite interval mapping (ICIM). The QTL analysis revealed each one QTL for traits namely, stripes on seed margin, stripes between seed margin, 100-seed weight and seed yield. The LOD ranged from 1.5 to 1.9. The adjusted R^2 value ranged 10.6 (seed yield) to 65.0 (stripes between seed margin) percent. Among these QTL, QTL on stripes on seed margin and stripes between seed margin may be considered as potential as they recorded very high phenotypic variation accounted. As the distance between the flanking marker is more than 5 cm, fine mapping of this QTL region with more markers may be attempted to utilize these QTL in the marker assisted back cross programme.

Key words: Sunflower, Simple sequence repeats (SSR), quantitative trait loci (QTL), hundred seed weight, stripes on seed margin, stripes between seed margin, seed yield.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is grown mostly as a source of vegetable oil and proteins in the world. Sunflower oil yield is determined by the product of seed yield per unit area and the oil percentage in the grain. Therefore, consideration of seed yield and oil content are important when breeding for high oil yield. Yield in sunflower, as in all other crops, depends on many characters, especially yield components which are controlled by several genes. Molecular markers in applied breeding programs facilitate the appropriate choice of parents for crosses to map or tag the gene blocks associated with economically important traits often termed as Quantitative Trait Loci (QTL). In the present research programme, attempts were made to identify QTL for various yield and yield component traits in sunflower. In the course of plant improvement, plant breeders deal with several qualitative traits.

However, the most difficult problem is the manipulation of metric traits with complex inheritance. Many strategies are available which rely upon the statistical analysis of field data to evaluate what has occurred on the genotypic level, but these inferences are often not precise as to the

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Table 1. Characteristics of parental lines.

Character	TNHSF239-68-1-1-1 (female)	17B (male)
Seed stripes	No stripes on the surface of seeds	Stripes present on surface, stripes strongly expressed in the margins
Pollen colour	Yellow	White
Seed colour	Black	Brown color
100 seed weight (g)	3.7	5.2
Hull weight/100 seeds (g)	1.5	2.5
Hull (%)	25	35
Oil content (%)	Very high (40 to 42%)	Very low < 33%

number of genes involved and their mode of action. Tracking polygenes with genetic markers can be traced back to the early 1920's when Sax (1923) reported the association of quantitatively inherited seed size with monogenes controlling seed coat pigmentation and pattern in bean (Dudley, 1993; Paterson and Tanksley, 1997). Effort to construct high-density linkage maps of molecular genetic polymorphism (marker loci) is currently underway for sunflower (Gentzbittel et al., 1995; Jan et al., 1998; Tang et al., 2002). Simple sequence repeats (SSR), also called microsatellites, are widely used as molecular markers. Their polymorphism has shown high efficiency and they are used for genetic mapping, population and evolutionary studies, as well as for finger-printing and pedigree analyses.

Statistical associations between alleles at molecular marker loci and alleles at quantitative trait loci (QTL) can be used to select indirectly, but with potentially very high accuracy, for DNA segments containing favourable QTL allele. This process effectively increasing the heritability of economically important agronomic characters such as yield, plant status and its components, quality traits, resistance and environmental stresses (Dudley, 1993; Paterson and Tanksley, 1997). QTL can be followed in a segregating population with the help of molecular markers. The selection for QTL using genetic markers can be effective if a significant association is found between the quantitative trait and the genetic markers and using these associations to develop improved lines or populations. QTL regions obtained from one population can later be introgressed into other varieties, which may be more suited for specific environments (Dudley, 1993). These studies helped to bring forth the potential of exploiting non-adapted and wild germplasm using backcross QTL analysis for the enhancement of elite crop varieties (MAB: marker-assisted backcrossing). The quick discovery and transfer of these QTL from non-adapted to adapted germplasm ultimately opens the door for the expansion of the genetic base of sunflower (Vischi et al., 2001).

Within the broad field of genomics, the QTL approach can be further validated or supported by other areas such as transcriptional profiling, physical mapping, and other functional genomics technologies (Alibert et al., 2001).

MATERIALS AND METHODS

Plant materials

Two sunflower inbred lines namely, TNHSF239-68-1-1-1 and 17B with significant differences (Table 1) for various traits namely, stripe on seeds surface (both on margin and between margins), seed color, hull weight and oil content were selected as female and male parent respectively to develop the $F_{2:5}$ population. These parents were crossed during June to October, 2009. The F_1 plants were raised during January to April, 2010 and confirmed with polymorphic SSRs. The $F_{2:5}$ population was raised during June to October, 2011 and leaf samples were collected to extract DNA.

Pheotypic data

Recombinant inbred lines of 94 in $F_{2:5}$ generation was used in the present study. The experiment was laid out with two replications in randomized block design (RBD) with a plot size of 2.4 m^2 with spacing of 60 × 30 cm during June to October, 2011 at Oilseeds Farm, Department of Oilseeds, Tamil Nadu Agricultural University, Coimbatore. Data were recorded on five out of seven plants were selected randomly in each replications for recording morphological traits namely, days to 50% flowering, plant height (cm), head diameter (cm), pollen color (score), stripes on seed margin (score), stripes between seed margin (score), seed color (score), volume weight (g/100 ml), 100- seed weight (g), hull weight (g/100 seed), kernel weight (g/100 seed), hull (%), oil content (%), seed yield (g/plant) and oil yield (g/plant). The oil content of the seeds was estimated by using Pelicon Soxoplus apparatus and expressed in percentage. The mean data from each replication were subjected to statistical analysis as per the standard method. The mean data over replication were used as phenotypic data for QTL analysis.

DNA isolation, SSRs and PCR condition

Genomic DNA of individual progenies and parents were extracted by CTAB method (Doyle and Doyle, 1987) and the quality was checked by using 0.8 % (w/v) agarose gel electrophoresis. A total of 156 SSRs were to study the parental polymorphism. The polymerase chain reaction (PCR) mixtures (5 µl) contained 10 ng template DNA, 1 X *Taq* Polymerase buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM of forward and reverse SSR primers and 0.03 IU of Taq polymerase. Amplification was performed in 0.2 ml well PCR plates (96 wells/plate) in a thermal cycler (Applied Biosystems). The samples were initially incubated at 94.0°C for 3 min and then subjected to 20 times of the following cycle: 94.0°C for 30 s, 63.0°C for 30 s (-0.5°C reduction per cycle) and 72.0°C for 1 min. This was followed by another 20 cycle of 94.0°C for 15 s, 55.0°C for 30 s and 72.0°C for 1 min. Final extension was 72.0°C for 10 min. Amplified

products were analyzed using 6% non denaturing polyacrylamide gel at constant current of 350 V for about 4 h and silver stained (Benbouza et al., 2006).

Statistical analyses

Mean and variability analysis

Plant breeders are commonly facing with problems of handling segregating populations and selection procedures. Mean and variability are the important factors for selection. Mean serves as a basis for eliminating undesirable crosses. Variability helps to choose a potential cross since variability indicates the extent of recombination for initiating effective selection procedures. Selection for the improvement of quantitative traits can be effective only when segregating generations posses the potential variability. The probability of obtaining superior lines can be worked out in early generations through the estimates of first and second order degree of statistics (Jinks and Pooni, 1976). The various genetic parameters like variability, GCV, PCV, heritability and genetic advance as percent mean were calculated by adopting the formulae given by Johnson et al. (1955). The software used for this study is TNAU Stat.

Single marker analysis

Single-marker analysis (also 'single-point analysis') is the simplest method for detecting QTLs associated with single markers. The statistical methods used for single-marker analysis include t-tests, analysis of variance (ANOVA) and linear regression. Linear regression is most commonly used because the coefficient of determination (R^2) from the marker explains the phenotypic variation arising from the QTL linked to the marker. This method does not require a complete linkage map and can be performed with basic statistical software programs. Markers were subjected to single marker analysis, to identify the marker trait association using simple regression analysis. The phenotypic mean and marker data were considered as dependent and independent respectively. The significant threshold for association of marker to the trait was set at $P \leq 0.05$ for single marker analysis. The adjusted R^2 value was used as percent of variance explained by the marker on the particular trait of interest.

Linkage and QTL analysis

Genotyping and phenotyping data obtained from the mapping population was subjected to Linkage analysis using QTL IciMapping software version 3.2 (Wang et al., 2012). Linkage groups were established using a minimum LOD score of 3.0, ordering by RECORD, rippled by SARF criterion with a window size of 5. The resultant linkage map was used to estimate the QTL using ICIM through the QTL ICI mapping software version 3.2. The QTL were estimated using ICIM-ADD mapping method, with mapping parameters of 1 cM step and 0.001 probabilities in stepwise regression. The LOD threshold used was 1.5 as manual input.

RESULTS AND DISCUSSION

Analysis of variance for various characters

Analysis of variance showed significant differences for all the characters except plant height and head diameter (Table 2). It indicates the presence of significant variability in the experimental materials. Burli et al. (2001) reported significant differences among the parents and crosses for days to 50% flowering. A significant difference for seed yield was reported by Mohan and Seetharam (2005) and Loganathan et al. (2006). Similarly, significant differences for 100-seed weight and oil content were reported by Ashok et al. (2000).

Mean and variability analyses

In the present study, mean and variability parameters were estimated for various traits and presented in Table 3. Among the traits, pollen colour, stripes on seed margin, stripes between seed margin, seed colour, hull weight, kernel weight, seed yield and oil yield recorded high coefficient of variation. Traits namely, days to 50% flowering, plant height and volume weight recorded low coefficient of variation. Parameters namely, skewness and kurtosis helps the breeder to understand the nature of distribution of individuals in the population. Among the traits, days to 50% flowering, head diameter, seed colour and hull weight showed positively skewed distribution which indicates the more proportion of individuals in the lower values. In case of kurtosis, head diameter, hull weight, seed yield and oil yield had lepto kurtic nature. Traits namely, pollen colour, stripes between margin and seed colour had platy kurtic nature and hence selection could be effective for these traits. All other traits recorded normal distribution.

The mean, GCV, PCV, heritability (broad sense) and GA as percentage of mean worked out for 15 characters and presented in Table 4. These result indicated that sufficient level of variability were observed for most of the traits in this population. Heritability value alone may mislead during selection. Therefore, heritability and genetic advance together should be taken into consideration for selection (Johnson et al., 1955). The range of heritability (in broad sense) was from 1.65% (plant height) to 80.14% (pollen colour). High heritability and high genetic advance as percentage of mean were recorded for the traits pollen color, stripes on margin, stripes between margins, seed color, hull weight, kernel weight and 100-seed weight. High heritability and high genetic advance as percentage of mean indicates the presence of additive gene action. Directional selection for these traits would be more effective for desired genetic improvement.

Polymorphism survey on parents

In the present study, TNHSF239-68-1-1-1 and 17B have been chosen as parents. These parents have differential phenotypes for surface stripe (both on margin and between margins), oil content and hull weight. This

Table 2. Analysis of variance of F5 progenies for various characters.

Source of variation	Degrees of freedom	Days to flowering	Plant height (cm)	Head diameter (cm)	Pollen color	Stripes on margin	Stripes between margin	Seed color	Volume weight (g/100 ml)
Treatment	142	9.12 **	241.99	3.97	0.44 **	1.05 **	1.02 **	5.04 **	30.45 **
Error	142	3.93	234.13	3.52	0.04	0.24	0.27	1.68	6.87
Total	285								

Source of variation	Degrees of freedom	100-seed weight (g)	Hull weight (g/100 seed)	Kernel weight (g/100 seed)	Hulling percentage	Oil content (%)	Seed yield (g/plant)	Oil yield (g/plant)
Treatment	142	1.69 **	0.21 **	1.08 **	51.88 **	54.97 **	171.19 **	23.28 **
Error	142	0.41	0.07	0.32	27.58	15.78	88.86	12.53
Total	285							

**Significant at 1% level.

population was made in an attempt to identify marker linked to these traits. The parents were surveyed with 156 SSR primers to assess the parental polymorphism. Among the 156 SSR primers studied, 50 (36%) were polymorphic between parents. These polymorphic primers were utilized for profiling the $F_{2:5}$ progenies.

Construction of linkage map

Genotyping was carried out on 94 F_5 progenies of the cross TNHSF239-68-1-1-1 × 17B with 50 polymorphic SSR markers. Linkage analysis was performed using QTL IciMapping software version 3.2 (Wang et al., 2012). Linkage groups were established using a minimum LOD score of 3.0, ordering by RECORD, rippled by SARF criterion with a window size of 5. A total of 5 linkage groups were obtained with a total of 19 markers (Figure 1). The first linkage group has six markers namely, ORS307, ORS677, ORS847, ORS727, ORS1245 and ORS1040. The second linkage group has four markers namely, ORS552, ORS959, ORS605 and ORS371. The third linkage group has four markers namely, ORS595, ORS1144, ORS537 and ORS1237. The fourth linkage group has three markers namely, ORS996, ORS707 and ORS1012 and fifth linkage group has two markers namely, ORS1017 and ORS799. The total length covered by these 19 markers is 145.48 cm with an average length of 7.65 cm.

Single marker analysis

Among the 141 F_5 individuals, only 94 individuals were subjected to determine the association of marker to the respective phenotype. The markers were subjected to single factor regression analysis using the marker (as independent) and the respective phenotype (as dependent) as suggested by (Sax, 1923). The significant regression value b indicating that the particular marker is linked to trait. The R^2 value is considered as the percent of variability of the traits explained by the marker. Among the 50 SSR markers, a total of 29 SSR markers were found to be linked to various traits (Table 5). The number of associated marker varies from six SSRS (head diameter and seed color) to one SSRS (stripe on margin and stripes between margins). The adjusted R^2 for the regression equation varies from 3.2 to 29.8%. Two traits namely days to flowering (ORS509) and seed colour (ORS533) recorded above 20% R^2 value. Hull weight (ORS785) recorded 11.5% R^2 value. Similar results were reported by Anandhan et al. (2010). The markers associated with these traits are of potential use in marker assisted backcross programme.

QTL analysis

Inclusive composite interval mapping (ICIM)

Genotyping and phenotyping data obtained were analyzed for mapping QTL by using the method inclusive composite interval mapping (ICIM) through QTL ICI mapping software version 3.2.

Table 3. Mean and variability parameters for seed yield and component traits in sunflower RILs.

Character	Mean	CV (%)	Skewness		Kurtosis		Minimum	Maximum
Days to 50% flowering	55.38	3.84	0.86	**	0.99		51.00	62.50
Plant height (cm)	137.99	7.42	-0.32		-0.07		110.95	160.18
Head diameter (cm)	12.61	12.50	2.59	**	23.08	**	6.06	23.23
Pollen colour	1.40	33.13	0.39		-1.72	**	1.00	2.00
Stripes on seed margin	2.01	37.01	0.04		-0.95		1.00	4.00
Stripes between seed margin	1.88	38.04	0.17		-1.09	*	1.00	3.50
Seed colour	5.45	29.50	-0.56	*	-1.19	*	2.00	7.00
Volume weight (g/100 ml)	37.37	9.63	-0.14		0.00		28.00	46.00
!00-seed weight (g)	4.81	19.25	0.12		-0.63		2.89	7.01
Hull weight (g/100 seed)	1.55	20.50	0.85	**	1.69	**	1.01	2.86
Kernel weight (g/100 seed)	3.26	22.70	0.08		-0.84		1.80	4.94
Hull (%)	32.60	15.36	0.35		-0.07		19.68	46.90
Oil content (%)	34.94	15.35	-0.27		0.09		19.01	48.97
Seed yield (g/plant)	23.29	35.83	0.84		1.44	**	6.75	55.70
Oil yield (g/plant)	8.13	39.95	1.05		1.90	**	2.45	21.08

Table 4. Estimates of variability and genetic parameters of F_5 progenies for various characters.

Character	Mean	PCV (%)	GCV (%)	Heritability (%)	GA (%)
Days to 50% flowering (days)	55.45	4.61	2.91	39.79	0.93
Plant height (cm)	137.21	11.25	1.44	1.65	0.38
Head diameter (cm)	12.55	15.42	3.79	6.05	0.24
Pollen color (score)	1.44	34.58	30.96	80.14	102.59
Stripes on margin (Score)	1.93	41.76	32.96	62.29	52.38
Stripes between margins (score)	1.83	44.08	33.36	57.28	48.84
Seed color (score)	5.55	33.06	23.33	49.83	13.05
Volume weight (g/ 100 ml)	37.34	11.57	9.2	63.18	2.77
100-seed weight (g)	4.72	21.78	16.96	60.63	20.59
Hull weight (g/ 100 seed)	1.49	25.39	17.71	48.63	46.95
Kernel weight (g/100 seed)	3.24	25.80	19.05	54.53	25.63
Hull percentage	31.87	19.78	10.94	30.58	1.09
Oil content (%)	34.93	17.03	12.67	55.39	2.43
Seed yield (g/plant)	23.93	47.64	26.81	31.66	1.53
Oil yield (g/plant)	8.34	50.76	27.81	30.01	4.06

The QTL analysis revealed each one QTL for traits namely, stripes on seed margin, stripes between seed margin, 100-seed weight and seed yield. The LOD ranged from 1.5 to 1.9. The adjusted R^2 value ranged 10.6 (seed yield) to 65.0 (stripes between seed margin) percent (Table 6). Tang et al. (2006) identified 40 QTL for 100-seed weight, kerneland pericarp weight and kernel to pericarp weight ratio in 14 DNA marker intervals on 10 linkage groups using composite interval mapping. With the foregoing discussion, it can be concluded that QTL on stripes on seed margin and stripes between seed margin may be considered as potential as they recorded very high phenotypic variation accounted. As the distance between the flanking marker is more than 5 cm, fine

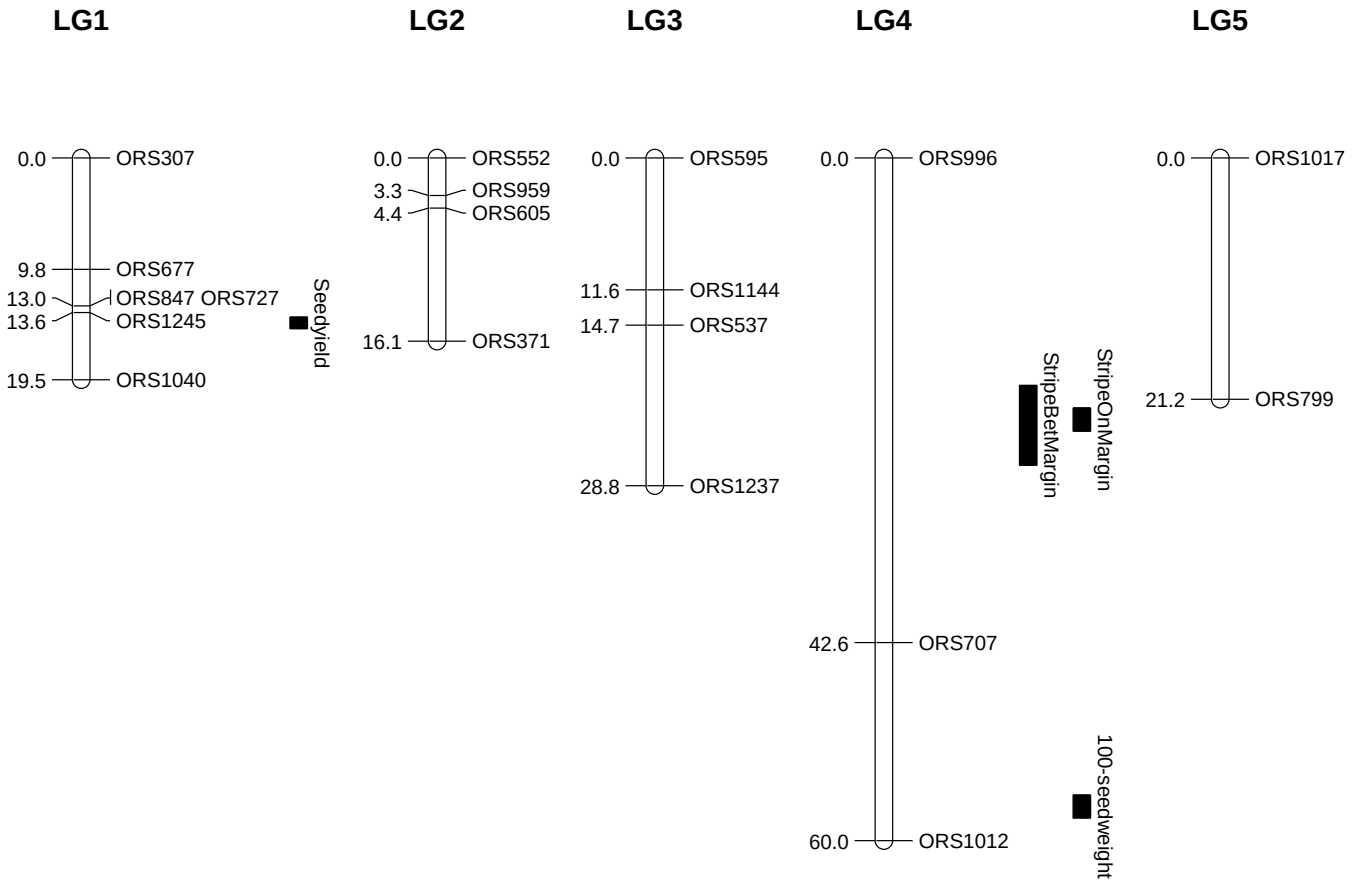

Figure 1. Genetic linkage map of sunflower and QTL position in the cross TNHSF239-68-1-1-1 × 17B.

Table 5. Single marker analysis for SSR primers and oil yield and yield components in the cross of TNHSF239-68-1-1-1 × 17B-1.

Character	Marker	Adjusted R^2 value (%)
Days to flowering	ORS502	7.8
	ORS509	21.8
	ORS533	4.8
	ORS799	8.5
Plant height	ORS613	4.1
	ORS878	3.4
	ORS1040	4.3
	ORS1144	4.3
Head diameter	ORS677	3.2
	ORS799	4.5
	ORS959	3.8
	ORS1040	3.7
	ORS1144	4.1
	ORS1245	4.2
Pollen color	ORS552	3.8
	ORS852	8.1
	ORS1237	3.8
Stripes on margin	ORS366	4.5
Stripes between margins	ORS1245	4.9

Table 5. Contd.

Seed color	ORS310	4.0
	ORS366	3.5
	ORS533	29.8
	ORS727	4.2
	ORS885	3.6
	ORS1037	10.0
Volume weight	ORS523	3.7
	ORS606	3.8
	ORS707	4.1
	ORS733	4.2
Hundred seed weight	ORS552	7.8
	ORS833	3.4
Hull weight	ORS502	4.0
	ORS552	5.5
	ORS785	11.5
	ORS885	4.9
	ORS1144	5.4
Kernel weight	ORS366	4.7
	ORS733	6.9
	ORS1017	3.6
	ORS1037	5.2
	ORS1065	4.8
Hull percentage	ORS833	4.6
	ORS1245	3.2
Oil content	ORS378	3.6
	ORS833	4.7
	ORS878	3.9
	ORS959	4.2
Seed Yield	ORS334	5.8
	ORS552	3.6
	ORS785	4.0
Oil yield	ORS334	3.8
	ORS503	3.2

Table 6. QTL analysis for various traits in sunflower.

Trait name	Chromosome	Position	Left marker	Right marker	LOD	PVE (%)	Add
Stripes on seed margin	4	23	ORS996	ORS707	1.5	54.6	-0.5
Stripes between seed margin	4	24	ORS996	ORS707	1.9	65.0	-0.6
100-seed weight (g)	4	57	ORS707	ORS1012	1.5	18.0	-0.4
Seed yield (g/plant)	1	14	ORS1245	ORS1040	1.52	10.6	2.77

mapping of this QTL region with more markers may be attempted to utilize these QTL in the marker assisted back cross programme.

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