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ORIGINAL PAPER

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Genotypic variation and identification of QTLs for agronomic traits, using AFLP and SSR markers in RILs of sunflower (*Helianthus annuus* L.)

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Abstract A population of 77 recombinant inbred lines (RILs) were developed through single-seed descent from a cross between 'PAC-2' and 'RHA-266'. Seeds of the above-mentioned RILs and their parents were planted in the field in a randomised complete block design with two replications. Genetic control for some agronomical traits—sowing-to-flowering date, plant height, stem diameter (SD), head diameter (HD), grain weight per plant, 1,000-grain weight (TGW) and the percentage of oil in grains—were measured for RILs and their parents. Genetic variability was observed among 77 RILs for all traits studied. Transgressive segregation occurred for some traits, and the comparison between 10% of selected RILs with the best parent showed significant difference for SD and HD as well as for TGW. A set of 123 RILs from the same cross, including the 77 above-mentioned RILs and their two parents, were screened with 409 AFLP and SSR markers, and a linkage map was constructed based on 367 markers. Several QTLs associated with the studied traits were identified. The effects of each QTL are moderate, ranging from 7% to 37%, but a high percentage of phenotypic variance is explained when considering all the covariants (TR^2 mean around 80% in each trait). Although the detected regions need to be more precisely mapped, the information obtained should help in marker-assisted selection.

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Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important sources of vegetable oil in the world. Yield in sunflower, as in all other crops, depends especially on yield components, which are quantitative traits, presumably controlled by several genes, their effect being modified by environment (Fick and Miller 1997). Heritability for yield is relatively low compared to other agronomic traits (Fick 1978). Polygenic inheritance patterns are reported for sowing-to-flowering (STF) date in sunflower (Machacek 1979). Heritability for this trait ranges from 0.62 to 0.95 with dominant gene action (Jan 1986), but additive effects are also observed (Alvarez et al. 1992). As far as oil content in grain is concerned, heritability of the trait is rather high, estimated to be between 0.65 and 0.70 (Fick 1975). The development of QTL mapping analyses has provided an alternative approach to locating and subsequently manipulating genes related to different quantitative traits in plant. By the use of statistical analysis, the variation of a quantitative trait can be dissected into the effect of individual genome regions—the QTLs, linked to markers on a molecular-marker map (Paterson et al. 1988).

Sunflower, as one of the most important dicot crops, has been studied for construction of molecular genetic map. The first map of sunflower was reported by Gentzbittel et al. (1995) and Berry et al. (1995), using RFLP markers. Gentzbittel et al. (1999) presented an updated version of the above-mentioned map, using more RFLP markers. Two other maps were also published, using RFLP (Jan et al. 1998) and RFLP and AFLP (Gedil et al. 2001).

Species with a big genome like sunflower ($2n$=34) require techniques which provide a high number of markers. AFLP is considered to be an efficient marker technology due to its high multiple ratio (Pejic et al. 1998). This technique has been used in the establishment of several genetic maps, like rice (Mackill et al. 1996), maize (Castiglioni et al. 1999), ryegrass (Bert et al. 1999), tomato (Haanstra et al. 1999), melon (Wang et al. 1997), pine (Remington et al. 1999), eucalyptus (Marques et al.

1998), lettuce (Jeuken et al. 2001) and sunflower (Flores Berrios et al. 2000a; Rachid Al-Chaarani et al. 2002). SSRs, called microsatellites, are also used as molecular markers. Their polymorphisms have shown high efficiency and are used for genetic mapping, population and evolutionary studies, as well as for fingerprinting and pedigree analysis in different species (Plaschke et al. 1995; Rongwen et al. 1995; Guilford et al. 1997; Tang et al. 2002; Mokrani et al. 2002). SSR markers are now recognized as an efficient molecular marker for giving information at single locus. For sunflower, a molecular map based on SSRs was recently published, allowing their use in academic research programmes (Tang et al. 2002, 2003a).

Identification of chromosomal regions with effects on grain yield, oil percentage in grain and other agronomic traits would increase our understanding of the genetic control of the characters. Starting a programme of marker-assisted selection becomes possible after the identification of QTLs for traits of interest. QTLs controlling important traits such as in vitro regeneration parameters (Flores Berrios et al. 2000a–c), grain weight per plant (GWP), 1,000-grain weight (TGW), percentage of oil in grain (POG) and STF date (Mestries et al. 1998; Mokrani et al. 2002; Leon et al. 2003), resistance to downy mildew and black stem (Rachid Al-Chaarani et al. 2002), tolerance to *Sclerotinia sclerotiorum* (Mestries et al. 1998; Bert et al. 2002), as well as resistance to *Orobanche* Race E (Tang et al. 2003b) and physiological parameters (Hervé et al. 2001) are detected in sunflower.

Some studies have shown the advantage of RILs for detecting QTLs (Austin and Lee 1996). RILs are homozygous and can be propagated without further segregation. The RILs undergo multiple cycles of meiosis before homozygosity is reached; consequently, linked genes have a great probability of recombination (Burr and Burr 1991). This effect increases the power of testing differences between genotypic classes.

The objective of the investigation presented here was to evaluate the variability for some agronomical traits in RILs (F_8) of sunflower. We also consolidated a previously published genetic map (Flores Berrios et al. 2000a; Rachid Al-Chaarani et al. 2002) by some AFLP and microsatellite markers and carried out a QTL mapping analysis to characterize the genomic regions involved in different agronomic traits.

Materials and methods

Plant material and experimental conditions

A population of 77 RILs were developed through single-seed descent from a cross between 'PAC-2' and 'RHA-266'. The material was kindly provided by INRA (France). Seeds of the above-mentioned RILs and their two parents were planted in a randomised block design with two replications. Each replication consisted of three rows 4.6-m long, with 50 cm between rows and 30 cm between plants in rows, giving a total number of about 48 plants per plot. STF dates were recorded when 50% of the plants per plot were at anthesis. Plants were harvested at maturity, and plant height (PH), stem diameter (SD), head diameter (HD), GWP, TGW and the POG were measured for RILs and their parents in each replication. Genetic correlations between the traits were also determined.

Statistical analysis was carried out in order to determine the main effect of RILs for the studied traits. The mean of RILs and that of their parents were compared for all traits. The difference between the mean of the best RIL or the mean of of the selected RILs and the best parent was also determined for the studied traits.

Mapping population

A total of 123 RILs of the cross 'PAC-2 × RHA-266' including the 77 RILs used in the field experiment and their two parents were grown in greenhouse and used for AFLP and SSR analysis. The genomic DNA of the RILs and their parents was isolated according to method of extraction and purification protocols presented by Fulton et al. (1995). The AFLP procedure was conducted as reported by Mokrani et al. (2002).

As far as SSR procedure was concerned, about 300 pairs of SSR primers (primers syntheses by Life Technologies) were screened for polymorphism, among which 30 pairs presented polymorphism between the parents used in this study. The 'ORS' and 'IUB' SSR marker names are the same as they were originally published (Yu et al. 2002, 2003; Tang et al. 2002). The origin of 'SSL' and 'SSU' SSR markers were from GIE CARTISOL, which are public and can be requested. PCR amplifications were performed in a volume of 10 µl containing 2.5 µM each primer, 0.4 U *Taq* DNA polymerase (Life Technologies), 100 µM each dNTP, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.20 µl 1% W-1 (Life Technologies), 0.05 µl of $\alpha[^{33}P]$ATP from Amersham Pharmacia Biotech, ddH_2O and 25 ng template DNA, using a Gene Amp PCR System 9700 Thermocycler (PerkinElmer–Applied Biosystems). The PCR programme used varies: 95°C for 3 min followed by 30 cycles of 1 min at 94°C, 2 min at 55°C and 1 min 30 s at 72°C and at last, 10 min at 72°C. The reaction products were mixed with an equal volume of formamide dye (98% formamide, 10 mM EDTA, bromophenol blue and xylene cyanol). The PCR reaction was denaturated for 3 min at 90°C, and 5 µl of each sample was loaded on a 6% denaturing polyacrylamide gel in a 38×50-cm Sequi-Gen GT Nucleic Acid Electrophoresis Cell (Bio-Rad) for 2 h 30 min. Before running, the gel was pre-equilibrated and heated at 50°C by pre-run at 100 W for about 30 min. Then gel was vacuum-dried and exposed to Hyperfilm MP (Amersham Pharmacia Biotech) for 2–5 days.

The AFLP and microsatellite polymorphic bands were scored as present (1) or absent (0) on autoradiograms, whereas unreliable ambiguous bands were scored missing (−). The 123 RILs and their two parents were screened

with 409 marker loci (371 AFLPs and 38 SSRs), and a linkage map was constructed using MAPMAKER, version 3.0 (Lander et al. 1987), based on 367 markers. Twenty-one linkage groups were obtained with a recombination value of less than 0.30 and a LOD score of 4.0. The Kosambi (1944) mapping function was used to transform the recombination frequency to genetic distances.

QTL detection

The chromosomal locations of QTLs for the studied traits were resolved by composite interval mapping (CIM), using QTL Cartographer, version 1.13, model 6 (Basten et al. 1999). This model integrated two parameters for CIM: the number of markers which control the genetic background (n_m=15) and a window size (w=15) that will block out a region of the genome on either side of the markers flanking the test site. The inclusion of background markers makes the analysis more sensitive to the presence of a QTL in the target interval. At each interval the significance of the association is tested by the likelihood-ratio statistic (Haley and Knott 1992). Permutation tests (1,000 permutations) were done to establish experiment-wise significance thresholds.

Results and discussion

Quantitative variation

Analyses of variance for 77 RILs and their two parents 'PAC-2' and 'RHA-266' showed a highly significant genotype effect, the block effect being non-significant (Table 1). Genetic variability for all characters studied is presented in Table 2. The differences between parents were significant for STF date, GWP, TGW and the POG, whereas the differences were not significant for PH, SD and HD. The differences among RILs($\bar{X}_{LIRs}$) and their parents($\bar{X}_P$) were not significant, showing that RILs are representative of the whole possible combinations for the studied characters of the cross 'PAC-2 × RHA-266'.

The comparison between the best parent and the best RIL showed a significant difference for all the studied traits except STF date and POG (Table 2). This phenomenon might be due to the transgressive segregation, which occurs for quantitative traits when the alleles increasing and decreasing the trait are dispersed in the parental lines. The same phenomenon was observed when the best parent was compared with the mean of 10% of selected RILs for SD, HD and TGW.

Positive and significant correlations (Table 3) were observed between SD and HD, GWP and TGW. PH was positively correlated with STF date, STD and SDs and HDs, whereas POG was negatively correlated with TGW.

Linkage map and QTL analysis

The genetic map was constructed using 371 AFLP markers that were identified by using 27 primer combinations and 38 microsatellite markers that were identified by use of 30 primer combinations (Fig. 1). Out of 409 AFLP and SSR markers analysed, 367 were placed in 21 groups by the use of a minimum LOD score of 4.0 and a maximum recombination value of 0.30. The groups ranged from 20.9 cM to 329.9 cM in length and carried 4 to 52 markers. The total length of the map is 2,915.9 cM, which represents at least one marker for every 7.9 cM on average. Flores Berrios et al. (2000a) already constructed a genetic map of 2,833.7 cM, using 99 RILs and AFLP markers for the same cross (PAC-2 × RHA-266), which was improved by adding some more AFLP markers (Rachid Al-Chaarani et al. 2002). The present study was undertaken in order to increase the number of AFLP markers, to integrate SSRs markers and to add more RILs (123 instead of 99 RILs). Thus the new map should be considered as an improvement of the previously described one. The number of linkage groups in our new map (21) is higher than the number of chromosomes (17) in sunflower. This problem will be solved by increasing the number of markers in our future works. The length of our map (2,915.9 cM) is twice as long as the RFLP and SSR maps of sunflower presented by authors (1,400–1,500 cM). The reason is that we have used RILs, which are pure lines; other maps are constructed using F_2 or F_3 populations (Berry et al. 1995; Tang et al. 2002). Some other genetic maps of sunflower are also developed using different techniques: RFLP (Gentzbittel et al. 1995, 1999), RAPD (Riescberg et al. 1996), RFLP and AFLP (Gedil et al. 2001), AFLP and SSR (Mokrani et al. 2002) and SSR (Yu et al. 2002, 2003).

Table 1 Mean squares for sowing-to-flowering (*STF*) date, plant height (*PH*), stem diameter (*SD*), head diameter (*HD*), grain weight per plant (*GWP*), 1,000-grain weight (*TGW*) and percentage of oil in grain (*POG*) of sunflower RILs and their two parents

Source of variation	*df*	STF (days)	PH (cm)	SD (cm)	HD (cm)	GWP (g)	TGW (g)	POG
Total	157	18.74	347.31	0.28	20.19	161.96	314.55	13.77
Genotype	78	36.84*	604.43*	0.49*	36.97*	284.66*	585.98*	23.53*
Block	1	2.27 NS[a]	87.33 NS	0.16 NS	1.62 NS	33.38 NS	20.50 NS	0.33 NS
Residual	78	0.85	93.53	0.08	3.65	40.91	46.89	4.17

Significance level: *P=0.001
[a]*NS* Not significant

Table 2 Genetic gain and heritability for STF date, PH, SD, HD, GWP, TGW and POG in RILs of sunflower

Item	STF (days)	PH (cm)	SD (cm)	HD (cm)	GWP (g)	TGW (g)	POG
RHA266 (P1)	83.50	91.88	1.91	12.00	32.85	64.75	44.38
PAC2 (P2)	97.75	106.75	1.95	12.76	19.69	48.30	37.73
P1−P2	14.25*	14.87 NS	0.04 NS	0.76 NS	13.16*	16.45*	6.65*
$\bar{X}_P = (P1 + P2)/2$	90.63	99.32	1.93	12.38	26.27	56.53	41.06
$\bar{X}_{LIRs}$[a]	89.03	109.79	1.97	13.29	22.37	52.85	39.22
$\bar{X}_{LIRs} - \bar{X}_P$	1.60 NS	10.47 NS	0.04 NS	0.91 NS	3.90 NS	3.68 NS	1.84 NS
Best RIL (BRIL)	83.17	67.25	5.00	27.00	50.18	94.40	45.66
BP[b]	83.50	91.88	1.95	12.76	32.85	64.75	44.38
GG_1[c]=BRIL−BP	0.33 NS	−24.63*	3.05*	14.24*	17.33*	29.65*	1.28 NS
10% SF_8L[d]	83.94	−84.60	3.00	22.10	42.04	85.80	44.94
GG_2[e]=10% SF_8L−BP	0.44 NS	7.28 NS	1.05*	9.34*	9.19 NS	21.05*	0.56 NS

Significance level: *P=0.05
[a]$\bar{X}_{LIRs}$ Mean of all
[b]*BP* Best parent
[c]GG_1 Genetic gain when the best RIL compared with the best parent
[d]SF_8L 10% of the best recombinant F_8 lines
[e]GG_2 Genetic gain when the 10% of the selected RILs (10% SF_8L) compared with the best parent

Table 3 Simple correlation coefficients between STF date, PH, SD, HD, GWP, TGW and POG in RILs of sunflower

	STF	PH	SD	HD	GWP	TGW
PH	0.515***					
SD	0.065	0.290**				
HD	0.149	0.474***	0.652***			
GWP	0.034	0.406	0.429***	0.632***		
TGW	−0.200	0.170	0.348**	0.536***	0.545***	
POG	0.076	−0.102	−0.024	−0.057	0.176	−0.226*

Significance levels: *P=0.05, **P=0.01, ***P=0.001

The QTLs associated with the studied traits were identified, and the most important ones are presented in the Table 4. The QTLs were designated as follows: *stf* for sowing to flowering date, *ph* for plant height, *sd* for stem diameter, *hd* for head diameter, *gwp* for grain weight per plant, *tgw* for 1,000-grain weight and *pog* for percentage of oil in grains, followed by the corresponding number of linkage groups and the corresponding number of QTLs on the group. The map position and characteristics of QTLs detected are referred to Table 4.

The effects of each QTL are moderate (R^2 ranging from 0.07 to 0.37), but a high percentage of phenotypic variance is reached for them when considering all the covariants (TR^2 mean 80% in each cases), which should be rather over-estimated because of the high number of covariants included in the model (n_m=15). The transgressive phenotypes observed for some traits could be explained by the presence of QTLs of opposite sign in each parent. Three QTLs were detected for STF date in linkage groups 4, 9 and 14. The LOD score ranged from 7.53 to 10.45. The R^2 values explained by *stf*-4-1, *stf*-9-1 and *stf*-14-1 were 13, 20 and 19% respectively, whereas the total phenotypic variation plus covariants (TR^2) explained in the model was 79% and 83%. For the first QTL (*stf*-4-1), alleles having positive effects come from 'PAC-2' parent, whereas for the two others come from 'RHA-266". Recently, two QTLs presenting 89% of TR^2 for this trait were identified by Mokrani et al. (2002), but the lack of common markers make it difficult to compare the maps position of the QTLs. Six QTLs associated with growing degree days to flowering and photoperiod response are also reported (Leon et al. 2001). As far as PH is concerned, five QTLs are observed. The most important is *ph*-11-2, which is situated on linkage group 11 at 140.74 cM. The LOD score was 9.76, and individual effect of this QTL on the expression of the character (R^2) was 20%, whereas the TR^2 was 86%. For SD five QTLs are detected in linkage groups 4, 8, 14 and 15 (*sd*-4-1, *sd*-4-2, *sd*-8-1, *sd*-14-1 and *sd*-15-1). The individual effects of these QTLs range from 8% to 13%. The QTLs for this trait, explain about 82% of the TR^2, indicating that chromosomal location with a high effect was not identified in this study, whereas interaction between the QTLs was important. Alleles giving a positive effect for the QTL *sd*-14-1 come from 'PAC-2'. HD, which is an important yield component trait in sunflower, also presents several QTLs with low effects. However the TR^2 of these QTLs is around 85%, with LOD scores ranging from 4.02 to 7.27. Positive alleles for the trait come from both parents. Four QTLs were detected for GWP, on linkage groups 4, 6, 9 and 13. The R^2 values for these QTLs vary from 5% to 15%, whereas the maximum TR^2 explained was 89%. Alleles having positive effects for (*gwp*-6-1) come from 'PAC-2' and for the three other QTLs, they come from 'RHA-266'. Mokrani et al. (2002) have also identified two QTLs presenting a TR^2 of about 50%, using F_3 families. The results concerning TGW showed that through three detected QTLs, the one situated on linkage group 9 (*tgw*-9-1) has a high value for R^2 (37%), whereas TR^2 is 84%. The LOD score of this QTL is 13.51, and alleles with positive effects come from 'RHA-266'. Mestries et al.

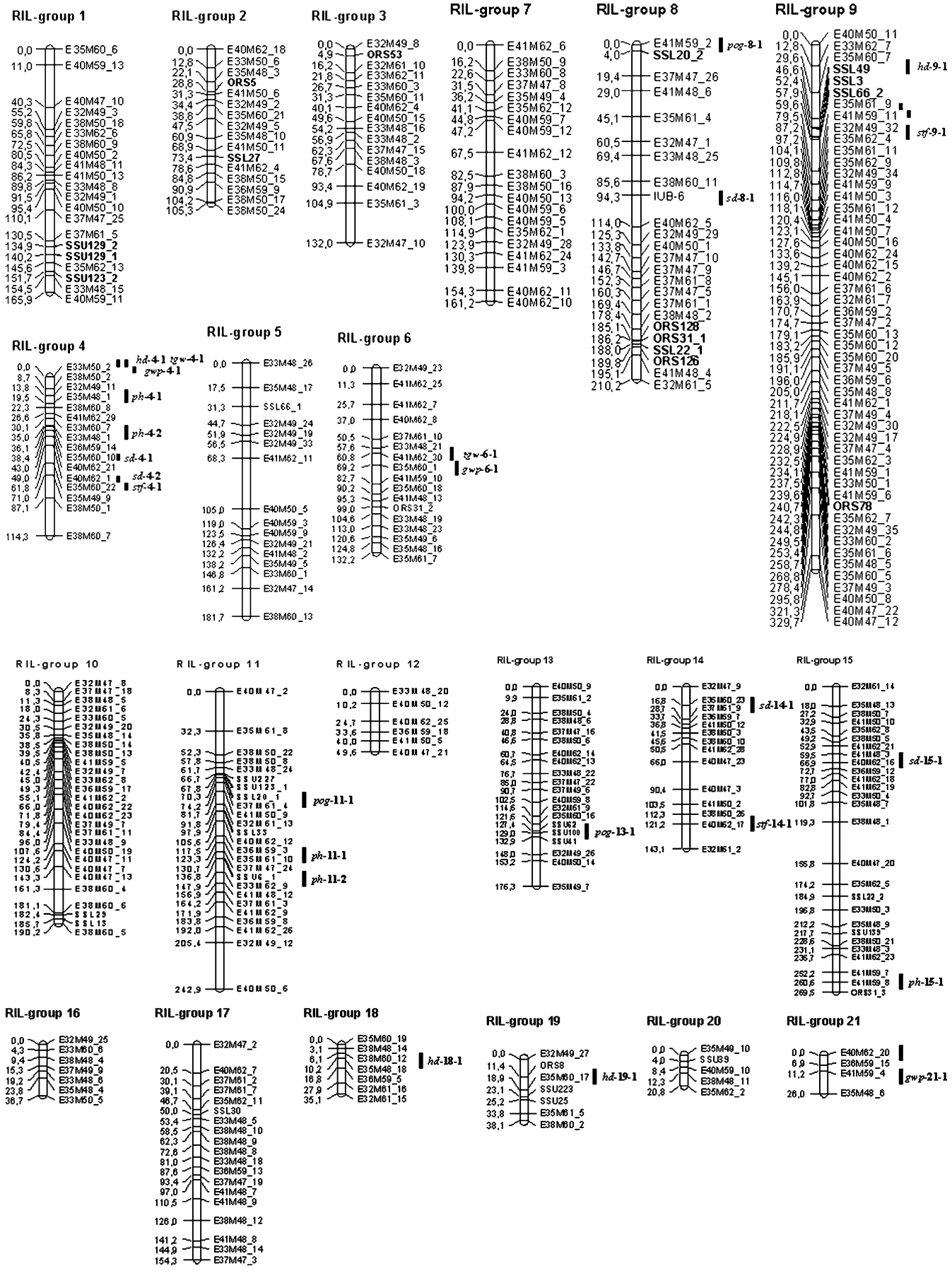

Fig. 1 Linkage map of sunflower (*Helianthus annuus* L.), constructed using 371 AFLP markers

Table 4 Map position and effect of QTLs detected in RILs for agronomical traits

Traits	QTL	Linkage group	Position (cM) [a]	LOD	Additive	R^{2b}	TR^{2c}
STF date	*stf*-4-1	4	60.96	7.53	−1.59	0.13	0.79
	stf-9-1	9	85.58	9.85	2.87	0.20	0.83
	stf-14-1	14	121.28	10.45	2.07	0.19	0.79
PH	*ph*-4-1	4	19.55	13.06	−6.99	0.13	0.90
	ph-4-2	4	32.12	10.45	−7.08	0.12	0.89
	ph-11-1	11	123.28	6.80	−8.34	0.13	0.91
	ph-11-2	11	140.74	9.76	−9.03	0.20	0.86
	ph-15-1	15	260.73	13.66	−7.06	0.13	0.90
SD	*sd*-4-1	4	38.42	6.23	−0.16	0.08	0.81
	sd-4-2	4	56.96	4.70	−0.15	0.09	0.82
	sd-8-1	8	94.26	6.72	−0.20	0.12	0.82
	sd-14-1	14	26.78	7.22	0.20	0.13	0.82
	sd-15-1	15	65.46	4.35	−0.22	0.08	0.78
HD	*hd*-4-1	4	0.01	6.07	−1.33	0.07	0.86
	hd-9-1	9	46.58	4.50	−1.67	0.05	0.86
	hd-18-1	18	6.10	5.06	1.11	0.05	0.86
	hd-19-1	19	18.93	7.27	1.40	0.08	0.85
GWP	*gwp*-4-1	4	2.01	9.62	−4.95	0.12	0.89
	gwp-6-1	6	66.79	12.32	5.92	0.15	0.89
	gwp-9-1	9	59.64	5.60	−3.75	0.05	0.87
	gwp-21-1	21	13.27	8.78	−4.30	0.11	0.89
TWG	*tgw*-4-1	4	0.01	4.82	−6.37	0.07	0.81
	tgw-6-1	6	59.59	6.30	8.15	0.09	0.83
	tgw-9-1	9	69.64	13.51	−11.45	0.37	0.84
POG	*pog*-8-1	8	2.01	8.46	1.33	0.13	0.83
	pog-11-1	11	70.29	4.33	−1.10	0.08	0.73
	pog-13-1	13	129.10	6.82	−1.33	0.10	0.81
	pog-21-1	21	0.01	4.04	−1.08	0.08	0.74

[a] From north of the linkage group

[b] Percentage of individual phenotypic variance explained, value determined by QTL Cartographer, version 1.13 (Basten et al. 1999)

[c] Percentage of phenotypic variance explained by the QTLs given all the covariants, determined by QTL Cartographer, version 1.13 (Basten et al. 1999)

(1998) had detected three QTLs for TGW, and Mokrani et al. (2002) reported only one QTL for this trait. The POG presented four QTLs with moderate phenotypic effects (R^2=8–13%). The epistasis effect is important in the expression of the QTL having the highest value (*pog*-8-1), and its effect on oil content can reach 83% of TR^2. Alleles with a positive effect come from 'RHA-266' for the above-mentioned QTL. Several QTLs have already been detected for grain oil content in sunflower (Mestries et al. 1998; Mokrani et al. 2002).

The results presented in Table 4 and Fig. 1 show that QTLs controlling different traits can be find some times close together: alleles having a positive effect should come from one or both parents. For example, QTLs responding to, SD (*sd*-4-2), HD (*hd*-4-1), GWP (*gwp*-4-1) and TGW (*tgw*-4-1) are all situated on linkage group 4. Genetic correlations between the traits (Table 3) confirm also these relations. For example, SD is correlated with HD, GWP and TGW. This phenomenon is also observed for linkage groups 6, 8, 9, 11 and 13.

The results presented here reveal regions related to different agronomic traits studies. The comparison of the present results with those reported previously is difficult, as different markers and nomenclatures are used in different experiments. Although these regions need to be more precisely mapped, the information obtained should help in marker-assisted selection.

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