Construction of a linkage map with TRAP markers and identification of QTL for four morphological traits in sunflower (*Helianthus annuus* L.)

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ABSTRACT

A linkage map containing 202 TRAP (target region amplification polymorphism) and 24 SSR markers was constructed in an F_2 population derived from a cross between two sunflower breeding lines. This map contains 17 linkage groups spanning a total distance of 1597.5 cM. The QTL for plant height, leaf color, leaf shape and head shape were identified in the F_2 and F_3 generations. Totally 18 QTL were detected for these traits with individual QTL explaining 6.7-49.5% of phenotypic variation, suggesting the multiple gene status for these traits. Two QTL for plant height and two QTL for chlorophyll content were identified in both F_2 and F_3 generations, and one of them each explained more than 27.2% of the phenotypic variation. These QTL will be useful in molecular breeding.

Key words: chlorophyll content – head shape – leaf shape – plant height – QTL mapping – TRAP

INTRODUCTION

The cultivated sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops of the world. Sunflower oil accounts for approximately 10% of the total world consumption of plant-derived edible oil (Jan and Seiler, 2007). The advent and development of molecular markers and genetic maps assists in understanding the genetic basis of economically important traits and facilitates plant breeding via marker-assisted selection. To date, about a dozen linkage maps have been constructed using different molecular markers, including RFLP, RAPD, and SSR in sunflower, and most of the maps had 17 linkage groups (Berry et al., 1995; Gentzbittel et al., 1995; Gedil et al., 2001; Tang et al., 2002).

The TRAP marker technique, developed by Hu and Vick (2003), takes advantage of the annotated EST information to generate PCR-based markers near the target sequence. This molecular marker technique has demonstrated great potential in exploiting genome polymorphisms and molecular mapping (Hu et al., 2004). It has been successfully used in defining the linkage group ends (Hu, 2006), in mapping a nuclear male-sterile gene (Chen et al., 2006), and in mapping an apical branching gene (Rojas-Barros et al., personal communication) in sunflower. This marker technique has also been used for molecular mapping in other crops, such as mapping of disease resistance QTL in common bean (Miklas et al., 2006) and wheat (Liu et al., 2005).

Morphological traits like plant height, leaf color, leaf and head shape etc. are important traits in sunflower breeding. However, genetic studies on only one or two of these traits have been conducted in previous reports (Hervé et al., 2001; Mokrani et al., 2002; Burke et al., 2002; Bert et al., 2003; Al-Chaarani et al., 2004). Here we report the construction of a linkage map comprised primarily of TRAP markers and QTL mapping for the four morphological traits mentioned above in sunflower.

MATERIALS AND METHODS

Two sunflower inbred lines with significant differences in some morphological traits of interest, Lgl (light green leaf) and HA379, were selected for developing an F_2 population in this study. Lgl was introduced from Australia with light green leaf color, HA379 is a male maintainer line with reduced-height released by USDA-ARS (PI 561919) (Miller, 1993). One hundred and twenty F_2 individuals and their parents were planted in one-gallon plastic pots in the greenhouse in the winter of 2006, one plant per pot. Ninety-five $F_{2:3}$ families and their parents were planted in the experimental field in Fargo, ND, USA, during the growing season of 2007 following a field design of randomized complete block with two replicates, with 15 to 20 plants in a one-row plot.

Four traits, including leaf color (chlorophyll content or greenness degree), plant height, leaf shape, and head shape were investigated in the F_2 and F_3 generations. At the flowering stage, leaves from each plant of the F_2 population were sampled for measuring chlorophyll content following the procedures described by Chory et al. (1989). The sampling and measurements for each individual were conducted

twice and the average values were used for analysis. In the F_3 generation, the plants within a family segregated for chlorophyll content. Therefore, the greenness degree, a parameter highly related to leaf chlorophyll content, was measured for each plant with a handheld chlorophyll meter (SPAD-502, Minolta Camera Co. LTD, Japan) following the manufacturer's instructions. Measurements were performed on the fully expanded uppermost leaves with a minimum of three measurements taken per leaf, about 2 cm away from the leaf edge.

Plant height (cm) was measured from the soil surface to the head at the mature stage for all the plants in both generations. Leaf shape and head shape were visually scored on a scale of 1 (triangle leaf shape, flat head shape) through 4 (round leaf shape, most convex and misshapen head) for all the plants at the mature stage. The means of the data collected from individual plants of each $F_{2:3}$ family were used in the analysis. Total genomic DNA was isolated from about 50 mg (fresh weight) leaf tissue sampled from individual plants of the parental lines and the F_2 population using the Qiagen DNeasy® 96 Plant Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. The TRAP assays followed the updated procedures described by Hu (2006). Totally 27 fixed primers were designed against ESTs involved in chlorophyll synthesis, gibberellin synthesis, microRNA sequences and disease resistance, as well as seven arbitrary primers labeled with either IR (infrared) 700 or IR 800 dye (Hu and Vick 2003), and were used to generate TRAP markers for map construction (Table 1). The TRAP markers were designated by the combination of the code of the fixed primer involved, the code of the labeled arbitrary primer, and the fragment size in base pairs.

Table 1. ESTs used for fixed primer design and sequences of fixed and arbitrary primers.

Code	EST accession no.	Sequences 5'—3'
Fixed primer		
T99	QHB26P17.yg.ab1	GTT TTC CGT CAT ACT CGT TA
T100	QHB33I23.yg.ab1	GAA GGG GTC AAA AAT TTA AC
T101	QHB34F17.yg.ab1	TCC ACA CTT TTG AAG TCA TT
T102	QHG18P13.yg.ab1	AAG AGT TTG ACC AAT GTC AA
T103	QHK1O04.yg.ab1	GAT ACA GGT TAT GGC AGA AA
T104	QHK7L05.yg.ab1	TTA TGT CTA TGG CAC CAA CA
T105	QHL12I06.yg.ab1	GCT TAC CGT CAT CAA GAA AC
T109	EB700927	GTA TCC AAA CGA CAC GAG TT
T110	CV987281	CAT ACA AGG TGG TCG AAA TT
T111	EC683354	AGG AAA TGT CTA TTT GGC AA
T112	QHJ12G10.yg.ab1	ACC ACA CAA TCA TGA CTA GG
T114	QHJ4A19.yg.ab1	TAA TAG CAA AAG CTC CAA TG
T115	QHB12C18.yg.ab1	ATT CAC TAT ATC ACG AGC CA
T116	QHB29B22.yg.ab1	GCA TTA TAC TTT GGT GGA GA
T117	QHM10I05.yg.ab1	ATT TGT TTG TTT GTT TTT GG
T02	QHA10B18b.yg.ab1	GTT TGC CTT TAA GAA CCG
T05	QHA11D14F1.yg.ab1	ATA CCC ACC CGT CAC TAC
T07	QHA11I24a.yg.ab1	AGG CTT GGA TGT TGA TGC
T10	QHA12P24b.yg.ab1	CTC CAG TCT GAC CCG TTG
T30	QHB14G14b.yg.ab1	AAT CTC AAG GAC AAA AGG
T37	QHB22D05b.yg.ab1	GAAGCTTCACAGGGAGTT
T131	miR157b	GATCATTGTCCAGATTC
T134	miR159a	GATCCTTGGTTCTTTGG
T137	miR165a	GATCCGTCTATGCTTTT
T141	miR166f	GATCACCTAATTCTCTA
T146	miR170	GATCGGATGCTCCTTTC
T152	miR394a	GATCAAGGAATAGGTGA
Arbitrary primer		
R03	TRAP03(IR-700)	CGTAGCGCGTCAATTATG
R19	SA12(IR-700)	TTCTAGGTAATCCAACAACA
R20	SA14(IR-700)	TTACCTTGGTCATACAACATT
R21	SA4(IR-700)	TTCTTCTTCCCTGGACACAAA
R13	TRAP013(IR-800)	GCGCGATGATAAATTATC
R22	GA3(IR-800)	TCATCTCAAACCATCTACAC
R23	GA5(IR-800)	GGAACCAAACACATGAAGA

We selected a total of 223 mapped SSR markers from each of the 17 linkage groups (Yu et al., 2003) in the initial screening for polymorphisms between the two parents. Twenty-four polymorphic SSRs were used to genotype the whole F_2 population to align the linkage groups constructed in this study with the published sunflower SSR map. SSR assays were carried out following the procedures described by Tang et al. (2002).

The linkage maps were constructed using the computer program of Mapmaker/EXP 3.0 (Lander et al., 1987) (LOD>4.5). Interval QTL mapping was performed with both F_2 and F_3 data employing the software of Mapmaker/QTL1.1 (Lander and Botstein, 1989; Lincoln et al., 1993).

RESULTS

The phenotypic differences between the parents, as well as the variation in the populations are summarized in Table 2. Transgressive segregation was observed in one or both directions for all the traits investigated. The values of skewness and kurtosis for these traits are less than or close to 1.0 except for chl*a*/*b*, indicating that these traits (with the exception of chl*a*/*b*) fit a normal distribution (Table 2). HA379 had deep green leaf color with significantly higher values of chl*a*, chl*b*, chl*t*, and greenness degree than Lgl in both generations. On the other hand, the values of chl*a*/*b*, as well as leaf shape, head shape, and plant height in Lgl, were significantly higher than that of HA 379. The mean for plant height in the F_3 generation was much higher than that in the F_2 generation, and this was the case for the parents in both generations (Table 2). This could be explained by the differences in their growing conditions, i.e. the F_2 generation in the greenhouse and the F_3 generation in the field.

Table 2. The measurements of the traits in the F_2, $F_{2:3}$ populations and their parents.

Traits [1]	HA379[2]	Lgl [2]	Mean	$F_2/F_{2:3}$ Range	Skewness	Kurtosis
Leaf shape	0.0/0.3	$3.0^{**}/3.0^{**}$	2.0/2.2	(0.0-4.0)/(0.5-3.8)	0.3/-0.1	-1.0/-0.4
Head shape	0.0/0.0	$3.0^{**}/3.0^{**}$	2.5/2.0	(0.0-4.0)/(0.0-3.9)	-0.5/0.3	0.1/-0.8
Plant height	38.1/63.9	$102.2^{**}/161.1^{**}$	71.9/108.8	(13.3-162.6)/(58.3-183.7)	0.6/0.3	-0.1/-0.7
Chl*a*	1.8^{**}/-	0.5/-	1.4/-	(0.4-2.3)/-	-0.4/	-0.6/-
Chl*b*	0.5^{**}/-	0.1/-	0.4/-	(0.1-0.6)/-	-0.7/-	-0.6/-
Chl*t*	2.3^{**}/-	0.6/-	1.8/-	(0.5-2.7)/-	-0.5/	-0.7/-
Chl*a*/*b*	3.6/-	4.8^{**}/-	3.9/-	(3.3-6.5)/-	1.6/	2.8/-
GD	-/42.9^{**}	-/20.1	-/37.7	-/(26.0-46.7)	-/-0.3	-/-0.4

[1]GD is greenness degree, the values on the left side of the sign "/" are the data collected in the F_2 generation, and those on the right side were collected in the F_3 generation.
[2]**, the difference is significant at the 0.01 level between the two parents.

Four traits related to leaf color (chl*a*, chl*b*, chl*t* and greenness degree) were highly intercorrelated ($0.6<r<1.0$), especially for chl*a* and chl*t* ($r=1.0$). Head shape in the F_3 generation was positively and significantly correlated to leaf shape, chl*a*, chl*b*, and chl*t* in the F_2 generation ($0.21<r<0.25$) and to greenness degree in the F_3 generation (0.26). However, chl*a*/*b* was negatively correlated to chl*a*, chl*b*, chl*t*, and leaf shape in the F_2 generation ($-0.64<r<-0.27$), and to greenness degree in the F_3 generation ($r=-0.55$). Significant negative correlations were also identified between plant height and greenness degree ($-0.40<r<-0.36$). Moreover, correlations between the F_2 and F_3 generations for these traits were strong, ranging from 0.42 for leaf shape to 0.91 for plant height.*A total of 322 polymorphic bands/markers were generated from 54 pairs of TRAP primer combinations (one fixed primer + one arbitrary primer labeled by IR700 or IR800). Each primer combination amplified 1 to 16 markers with an average of 6.0 markers per combination. Of the 223 SSRs screened, only 22 (9.9%) were polymorphic between the two parents and resulted in the generation of 24 SSR markers.*

After a preliminary mapping test, 202 TRAP markers that were evenly distributed across the sunflower genome and 24 SSR markers were selected to construct the linkage map for QTL analysis. The linkage map had a total length of 1597.5 cM, and the average distance between adjacent markers was 7.1 cM (Fig. 1). Integration of the 24 previously mapped SSR markers to the TRAP map allowed us to align 12 of the linkage groups to the previously published SSR maps (Yu et al., 2003), with each linkage group containing 1 to 4 SSR markers. One TRAP marker (T10R21-280) had already been assigned to linkage group 12 according to another sunflower map (data unpublished). The 13 linkage groups identified in this study corresponded to linkage groups 1, 2, 3, 5, 7, 8, 10, 11, 12, 13, 14, 16 and 17, respectively, on the Yu et al. (2003) map.

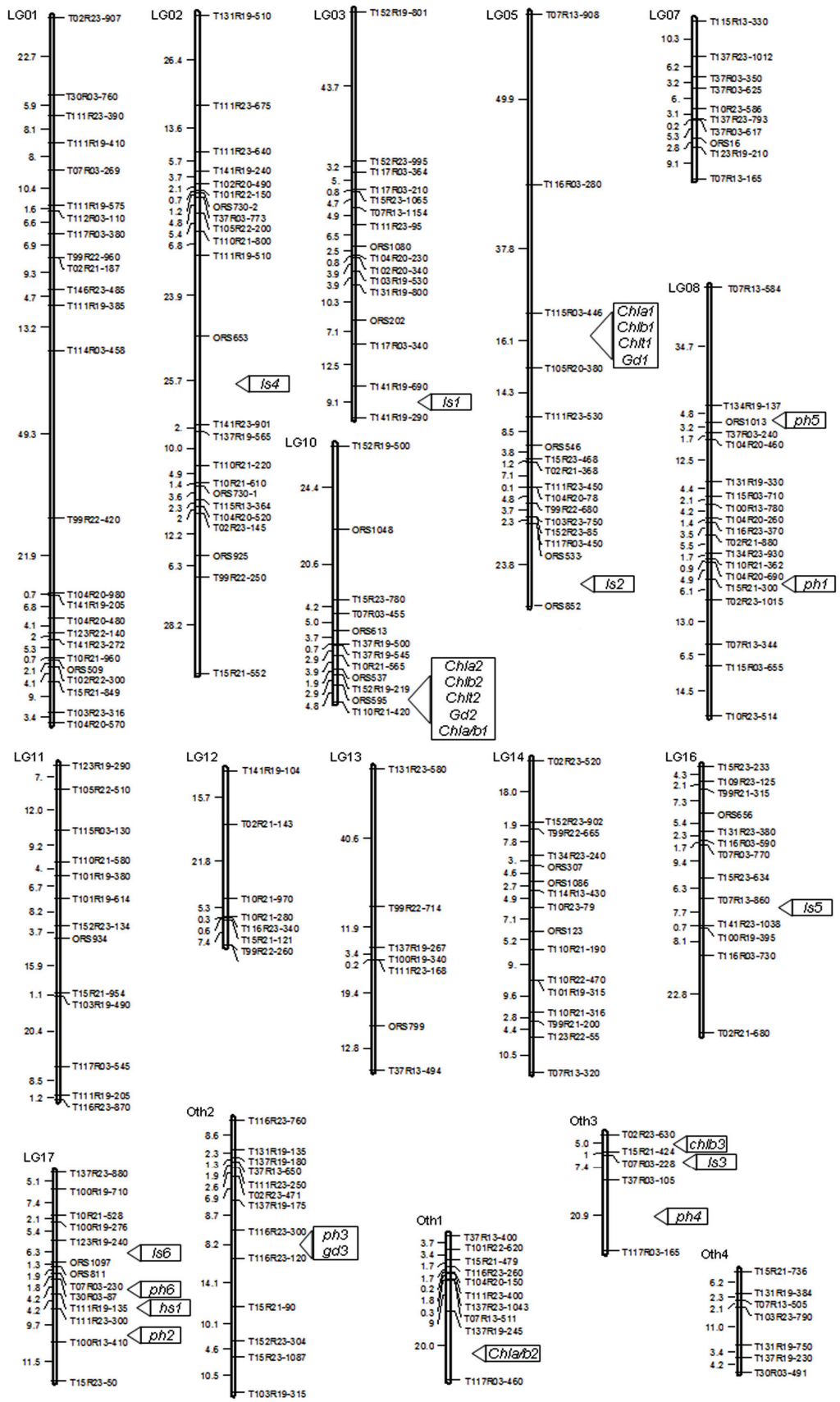

Fig. 1. The genetic linkage map and locations of the QTL detected in both generations. Designations of markers are on the right and genetic distances (cM) are on the left.

A total of six QTL for plant height were resolved in the two generations, including two detected in both generations. Individual QTL explained 6.7%-36.9% of phenotypic variation (Fig. 1). Among them, *ph3*, a major QTL for plant height, alone explained more than 30% of phenotypic variation in both generations. Six QTL were identified for leaf shape, which explained 7.8%-14.5% of phenotypic variation. None of them were detected in both generations. Only one QTL was detected for head shape in the F_3 generation, which explained 12.2% of phenotypic variation. Alleles from Lgl at nine of the QTL for these three traits had positive effects that coincided with the performance of this parent for these traits. For leaf-color-related traits, two, three, two, and two QTL were detected for chl*a*, chl*b*, chl*a*/*b* and chl*t* in the F_2 generation, respectively. Individual QTL explained 8.8%-49.5% of phenotypic variation. Three QTL for greenness degree were identified in the F_3 generation. Each explained 10.8%-27.2% of phenotypic variation.

Two chromosomal regions were identified that harbored more than three QTL for specific traits, and one region was identified that harbored two QTL (Fig. 1). The chromosomal interval *T115R03-446 – T105R20-380* on linkage group 5 contained the QTL for the four chlorophyll-content-related traits, *chla1*, *chalb1*, *chalt1* and *gd1*. The interval *ORS595 – T110R21-420* on linkage group 10 contained the QTL for all of the five chlorophyll-related-traits. The alleles from HA 379 at these loci increased chlorophyll content, but reduced the ratio of chlorophyll *a* to chlorophyll *b*. Two QTL, *ph3* and *gd3*, clustered in the region *T116R23-300 – T116R23-120* on linkage group Oth2, while the alleles from different parents had positive effects on the two traits, respectively. These results were consistent with the correlations observed among these traits.

DISCUSSION

TRAP markers, in combination with SSR markers, have been used to construct linkage maps in wheat, sunflower, and common bean (Liu et al., 2005; Miklas et al., 2006; Hu, 2006). The successful construction of a sunflower TRAP map and application of the map for QTL analysis in this study also indicates that TRAP is an efficient PCR-based marker technique for molecular mapping. For instance, each TRAP PCR reaction generated 12 polymorphic markers in the mapping populations of this study, whereas the SSR marker technique detected only 9.9% polymorphisms between the two parents. Moreover, TRAP takes advantage of the annotated EST information to generate markers at and near the target sequence (Hu and Vick 2003). The ESTs identified at the gene loci flanking the QTL of interest may provide useful information for the cloning of the QTL. In the present study, the TRAP markers flanking the two major QTL, *chla2* and *ph3*, were generated by the fixed primers designed against chlorophyll synthesis (CV987281) and gibberellin synthesis (QHB29B22.yg.ab1) related ESTs, respectively

The genetic basis of the four morphological traits is very complex, and it has been reported that some of the traits are under the control of multiple genes in sunflower (Hervé et al., 2001; Burke et al., 2002; Bert et al., 2003; Al-Chaarani et al., 2004). The results in this study also support this. It is difficult to compare the QTL in these studies with previous reports due to the unrelated markers used for map construction. However, near SSR marker *ORS 811*, a QTL for plant height, *ph6*, on LG17 detected in this study shared the same chromosomal region with a QTL for plant height reported by Burke et al. (2002).

In this study, one chromosomal region (ORS595 - T110R21-420) was identified to be involved in conditioning all the five leaf-color-related traits and explained more than 27.2% of the phenotypic variation (Fig. 1). A major QTL for chlorophyll content was also identified and positioned to the same chromosomal region in another sunflower mapping population (unpublished data). The identification of a major QTL controlling chlorophyll content in sunflower offers the opportunity to achieve a higher photosynthesis rate and to increase biomass and grain yield through genetic manipulation in the future. Moreover, the QTL for plant height, *ph3*, detected in two generations and which explained more than 30% of phenotypic variation in this study, will be also useful in sunflower plant breeding.

ACKNOWLEDGEMENTS

The authors greatly appreciate the technical assistance from Wenge Yuan, Ping Wang, and Angelia Hogness. This work was partially supported by USDA-ARS CRIS project 5442-21000-027-00D and a competitive grant from the National Sclerotinia Initiative to JH.

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