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

Bing Yue1, Brady A. Vick2, Jerry F. Miller2, Xiwen Cai1, Jinguo Hu2

1Department of Plant Sciences, North Dakota State University, Fargo, ND 58105, USA
2U. S. Department of Agriculture, Agricultural Research Service, Northern Crop Science Laboratory, Fargo, ND 58105, USA, E-mail: jinguo.hu@ars.usda.gov

ABSTRACT

A linkage map containing 202 TRAP (target region amplification polymorphism) and 24 SSR markers was constructed in an F2 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 F2 and F3 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 F2 and F3 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 F2 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 F2 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 F2:3 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 F2 and F3 generations. At the flowering stage, leaves from each plant of the F2 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$_1$ 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.

<table>
<thead>
<tr>
<th>Code</th>
<th>EST accession no.</th>
<th>Sequences 5’—3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed primer</td>
<td>T99</td>
<td>QHB26P17.yg.ab1</td>
</tr>
<tr>
<td></td>
<td>T100</td>
<td>QHB33I23.yg.ab1</td>
</tr>
<tr>
<td></td>
<td>T101</td>
<td>QHB34F17.yg.ab1</td>
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<td>T102</td>
<td>QHG18P13.yg.ab1</td>
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<td>T103</td>
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<td></td>
<td>T104</td>
<td>QHK7L05.yg.ab1</td>
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<td>T105</td>
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<td></td>
<td>T109</td>
<td>EB700927</td>
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<tr>
<td></td>
<td>T110</td>
<td>CV987281</td>
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<tr>
<td></td>
<td>T111</td>
<td>EC683354</td>
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<td></td>
<td>T112</td>
<td>QHJ12G10.yg.ab1</td>
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<td></td>
<td>T114</td>
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<td>T02</td>
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<td>QHA11D14F1.yg.ab1</td>
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<td></td>
<td>T131</td>
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<td>miR170</td>
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<td></td>
<td>T152</td>
<td>miR394a</td>
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<tr>
<td>Arbitrary primer</td>
<td>R03</td>
<td>TRAP03(IR-700)</td>
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<td></td>
<td>R19</td>
<td>SA12(IR-700)</td>
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<td></td>
<td>R20</td>
<td>SA14(IR-700)</td>
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<td></td>
<td>R21</td>
<td>SA4(IR-700)</td>
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<tr>
<td></td>
<td>R13</td>
<td>TRAP013(IR-800)</td>
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<tr>
<td></td>
<td>R22</td>
<td>GA3(IR-800)</td>
</tr>
<tr>
<td></td>
<td>R23</td>
<td>GA5(IR-800)</td>
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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 F₂ 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 F₂ and F₃ 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<sub>a/b</sub>, indicating that these traits (with the exception of chl<sub>a/b</sub>) fit a normal distribution (Table 2). HA379 had deep green leaf color with significantly higher values of chl<sub>a</sub>, chl<sub>b</sub>, chlt, and greenness degree than Lgl in both generations. On the other hand, the values of chl<sub>a/b</sub>, 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 F₃ generation was much higher than that in the F₂ 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 F₂ generation in the greenhouse and the F₃ generation in the field.

Table 2. The measurements of the traits in the F₂, F₂:3 populations and their parents.

<table>
<thead>
<tr>
<th>Traits</th>
<th>HA379&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Lgl&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Mean</th>
<th>F₂/F₂:3 Range</th>
<th>Skewness</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf shape</td>
<td>0.0/0.3</td>
<td>3.0 **/3.0 **</td>
<td>2.0/2.2</td>
<td>(0.0-4.0)/(0.5-3.8)</td>
<td>0.3/-0.1</td>
<td>-1.0/-0.4</td>
</tr>
<tr>
<td>Head shape</td>
<td>0.0/0.0</td>
<td>3.0 **/3.0 **</td>
<td>2.5/2.0</td>
<td>(0.0-4.0)/(0.0-3.9)</td>
<td>-0.5/0.3</td>
<td>0.1/-0.8</td>
</tr>
<tr>
<td>Plant height</td>
<td>38.1/63.9</td>
<td>102.2 **/161.1 **</td>
<td>71.9/108.8</td>
<td>(13.3-162.6)/(58.3-183.7)</td>
<td>0.6/0.3</td>
<td>-0.1/-0.7</td>
</tr>
<tr>
<td>Chl&lt;sub&gt;a&lt;/sub&gt;</td>
<td>1.8 **/-</td>
<td>0.5/-</td>
<td>1.4/-</td>
<td>(0.4-2.3)/-</td>
<td>-0.4/-</td>
<td>-0.6/-</td>
</tr>
<tr>
<td>Chl&lt;sub&gt;b&lt;/sub&gt;</td>
<td>0.5 **/-</td>
<td>0.1/-</td>
<td>0.4/-</td>
<td>(0.1-0.6)/-</td>
<td>-0.7/-</td>
<td>-0.6/-</td>
</tr>
<tr>
<td>Chlt</td>
<td>2.3 **/-</td>
<td>0.6/-</td>
<td>1.8/-</td>
<td>(0.5-2.7)/-</td>
<td>-0.5/-</td>
<td>-0.7/-</td>
</tr>
<tr>
<td>Chl&lt;sub&gt;a/b&lt;/sub&gt;</td>
<td>3.6/-</td>
<td>4.8 **/-</td>
<td>3.9/-</td>
<td>(3.3-6.5)/-</td>
<td>1.6/</td>
<td>2.8/-</td>
</tr>
<tr>
<td>GD</td>
<td>-/42.9 **</td>
<td>-/20.1</td>
<td>-37.7</td>
<td>-(26.0-46.7)</td>
<td>-.0/-</td>
<td>-.4/-</td>
</tr>
</tbody>
</table>

<sup>1</sup>GD is greenness degree, the values on the left side of the sign “/” are the data collected in the F₂ generation, and those on the right side were collected in the F₃ generation.

<sup>2</sup>*, the difference is significant at the 0.01 level between the two parents.

Four traits related to leaf color (chl<sub>a</sub>, chl<sub>b</sub>, chlt and greenness degree) were highly intercorrelated (0.6<r<1.0), especially for chl<sub>a</sub> and chlt (r=1.0). Head shape in the F₃ generation was positively and significantly correlated to leaf shape, chl<sub>a</sub>, chlt, and chlt in the F₂ generation (0.21<r<0.25) and to greenness degree in the F₃ generation (0.26). However, chl<sub>a/b</sub> was negatively correlated to chl<sub>a</sub>, chlt, and plant shape in the F₂ generation (-0.64<r<-0.27), and to greenness degree in the F₃ 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 F₂ and F₃ 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 22 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, \textit{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 \textsubscript{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 \textit{chl} \textsubscript{a}, \textit{chl} \textsubscript{b}, \textit{chalb} and \textit{chalt} in the F \textsubscript{2} generation, respectively. Individual QTL explained 8.8%-49.5% of phenotypic variation. Three QTL for greenness degree were identified in the F \textsubscript{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 \textit{T115R03-446} – \textit{T105R20-380} on linkage group 5 contained the QTL for the four chlorophyll-content-related traits, \textit{chla1}, \textit{chalb1}, \textit{chalt1} and \textit{gd1}. The interval \textit{ORS595} – \textit{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 \textit{a} to chlorophyll \textit{b}. Two QTL, \textit{ph3} and \textit{gd3}, clustered in the region \textit{T116R23-300} – \textit{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, \textit{chla2} and \textit{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 \textit{ORS 811}, a QTL for plant height, \textit{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, \textit{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.

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