Genetic characterization and molecular mapping of a chlorophyll deficiency gene in sunflower (*Helianthus annuus*)

Bing Yue, Xiwen Cai, Brady Vick, Jinguo Hu

Department of Plant Sciences, North Dakota State University, Fargo, ND 58105, USA

US Department of Agriculture, Agricultural Research Service, Northern Crop Science Laboratory, Fargo, ND 58105, USA

National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China

US Department of Agriculture, Agricultural Research Service, Western Regional Plant Introduction Station, Pullman, WA 99164, USA

Received 9 June 2008; received in revised form 2 September 2008; accepted 2 September 2008

Summary

A major gene controlling chlorophyll deficiency (phenotyped by yellow leaf color, *yl*) in sunflower was identified and mapped in an F₂ population derived from a cross between two breeding lines. Greenness degree was scored by a hand-held chlorophyll meter in the F₂ population. Leaf tissue from the parents, F₁ hybrids, and some F₂ progenies were also sampled to determine the chlorophyll content. All F₁ plants had normal green leaf color and the segregation of the plants in the F₂ population fits the monogenic ratio (3:1) **w** = 0.03, **p** > 0.9, indicating that leaf color is a monogenic trait with normal green dominant over yellow leaf color in this population. The contents of chlorophyll **a**, chlorophyll **b**, and total chlorophyll in the yellow-leafed lines were reduced by 41.6%, 53.5%, and 44.3%, respectively, in comparison with those in the green-leafed lines. Genetic mapping with molecular markers positioned the gene, *yl*, to linkage group 10 of sunflower. An SSR marker, ORS 595, cosegregated with *yl*, and a TRAP marker, B26P17ga5-300, was linked to *yl* with a genetic distance of 4.2 cM. The molecular marker tightly linked to the chlorophyll deficiency gene will provide insight into the process of chlorophyll metabolism in sunflower.

Published by Elsevier GmbH.

Abbreviations: AFLP, amplified fragment length polymorphism; Chl, chlorophyll; ESTs, expressed sequence tags; NCCL, North China confection line; QTLs, quantitative trait loci; RFLP, restriction fragment length polymorphism; SSR, simple sequence repeat; TRAP, target region amplification polymorphism; *yl*, yellow leaf.

*Corresponding author. Tel.: +1 509 335 3683; fax: +1 509 335 6654.
E-mail address: jinguo.hu@ars.usda.gov (J. Hu).

0176-1617/$ - see front matter Published by Elsevier GmbH.
Introduction

Cultivated sunflower is one of the most important annual oilseed crops of the world, and sunflower oil accounts for approximately 10% of the total world consumption of plant-derived edible oils (Janz and Seiler, 2007). Sunflower is a diploid species with 17 pairs of chromosomes. About a dozen linkage maps of various molecular markers, including restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA, amplified fragment length polymorphism (AFLP), and simple sequence repeat (SSR), have been published, and most of the maps had 17 linkage groups, each corresponding to an individual chromosome in different cultivated sunflower populations (Berry et al., 1995; Gentzbittel et al., 1995; Gedil et al., 2001; Tang et al., 2002). Recently, Lai et al. (2005) added 243 expressed sequence tags (ESTs) and Hu (2006) added over 400 target region amplification polymorphism (TRAP) markers to the sunflower SSR map. Linkage maps were also constructed in the populations from the crosses between cultivated and wild sunflowers (Wills and Burke, 2007; Baack et al., 2008). Moreover, some major genes and quantitative trait loci (QTLs) have been identified to the public sunflower linkage maps. A self-incompatible (S) gene (Gandhi et al., 2005), three nuclear male-sterile genes (Pérez-Vich et al., 2005; Chen et al., 2006), a branching gene (Rojas-Barros et al., 2008), and a lemon ray flower color gene (Yue et al., 2008) were mapped in sunflower. QTL mapping for some agronomic and morphological traits has also been conducted (Hervé et al., 2001; Bert et al., 2004; Gandhi et al., 2005; Tang et al., 2006).

Leaf color variation is one of the common visible morphological traits in higher plants, and the change in leaf color is usually caused by nuclear genes involved in chlorophyll metabolism. Fick (1978) summarized several reports on different kinds of chlorophyll deficiencies in sunflower, including one mutant that expressed a yellow leaf phenotype throughout the growing period. Studies on inheritance, mapping, and cloning of genes controlling leaf color in yellow-leaved mutants have been conducted in several crops (De Jong et al., 1998; Nothnagel and Straka, 2003; Wu et al., 2007; Wang et al., 2008). However, no major gene governing chlorophyll deficiency has been mapped in sunflower.

Here we report the identification and mapping of a major gene (yl) conferring chlorophyll deficiency with yellow leaf phenotype in a sunflower F₂ population derived from a cross between two breeding lines with different leaf colors. Although the phenotypes are visually distinctive, quantification of the chlorophyll content of the parental lines and their progeny was conducted in the field with a hand-held chlorophyll meter. Two types of molecular markers, TRAP and SSR, were employed to map the yl locus onto the public sunflower linkage map.

Materials and methods

Parental lines and the segregating population

Two sunflower (Helianthus annuus L.) lines were used in the current study. The yellow-leafed parental line was introduced from Australia and has been used to mark specific rows in our experimental plots because of its distinctive yellow leaf color throughout the growing season. The green-leafed parental line was a confection-type breeding line introduced from China designated as NCCL (North China confection line). The yellow-leafed line was emasculated and pollinated with pollen collected from NCCL to produce F₁ hybrids. Five F₁ seeds from the cross were grown in the greenhouse in the winter of 2006 to generate selfed seeds for genetic analysis. The F₂ generation was grown in the field in Fargo, ND, USA, in 2007. Two parental lines and the F₁ hybrids were also planted in one row each in the field for observation. The F₂ population consisted of 172 plants, and each of the F₂ plants was easily recognized as expressing either the yellow- or green-leafed phenotype.

Chlorophyll content measurement

Greenness degree, a parameter related to leaf chlorophyll content, was measured for each plant with a hand-held chlorophyll meter (SPAD-502, Minolta Camera Co., Ltd., Osaka, Japan). The SPAD-502 meter utilizes two light-emitting diodes, one red light with a wavelength of 650 nm at which chlorophyll absorbs light, and the other an infrared light with a wavelength of 940 nm at which no absorption occurs. The meter uses a photodiode detector to measure the red and infrared light transmission through leaves. Based on these two transmission values the instrument calculates an output value, the greenness degree, which is quite well correlated with the chlorophyll content (Richardson et al., 2002). Following the manufacturer’s instructions, we performed the measurements on the fully expanded uppermost leaves, a minimum of three measurements were taken per leaf, about 2 cm away from the leaf edge.
At the flowering stage, five plants each from the two parents, five F1 plants, and five plants from the two groups of the F2 population (one group with green leaf and the other group with yellow leaf) were sampled for chlorophyll content measurement using a spectrophotometer with the following procedures. Approximately 20 mg of leaf tissue of a fully expanded leaf from each plant was sampled for chlorophyll extraction with 1 mL of 95% ethanol in a micro test tube at 60°C overnight. The extract was measured at wavelengths of both 645 and 663 nm with a DU7400 spectrophotometer (Beckman Coulter). Chlorophyll \( a \), chlorophyll \( b \), and total chlorophyll contents were calculated using MacKinney’s (1941) specific absorption coefficients as reported by Chory et al. (1989), in which chlorophyll \( a = 12.72(A_{663}) - 2.59(A_{645}) \), chlorophyll \( b = 22.88(A_{645}) - 4.68(A_{663}) \), and total chlorophyll \( = 20.29(A_{645}) + 8.02(A_{663}) \). The total specific chlorophyll content is expressed as milligram of chlorophyll per gram of fresh leaf tissue as converted with the formula of chlorophyll (mg/g) = chlorophyll (mg/L) \( \times 0.001 \) (L)/fresh weight (g). In addition, the ratios of chlorophyll \( a \) to chlorophyll \( b \) (chlorophyll \( a/b \)) were also calculated. Significant differences among the parents, F1 hybrids, and the yellow and green F2 plants were estimated by analysis of variance with the levels of 95% and 99%.

**DNA preparation**

Total genomic DNA was isolated from about 50 mg (fresh weight) leaf tissue sampled from individual plants of the parental lines and of the F2 population using the Qiagen DNeasy™ 96 Plant Kit (Qiagen, Valencia, CA), following the manufacturer’s instructions. DNA concentration was determined with a DU7400 spectrophotometer (Beckman Coulter) and adjusted to approximately 10 ng/μL for PCR amplification.

**Genotyping**

The TRAP marker technique developed by Hu and Vick (2003), which uses one fixed primer and two arbitrary primers in each PCR reaction, was used for genotyping. The TRAP assays followed the procedures described by Hu (2006). In total, 22 fixed and eight arbitrary primers were used for TRAP marker generation. Nine fixed primers were designed against sunflower ESTs, which have homology to genes involved in chlorophyll synthesis (retrieved from the Compositae Genome Project Database, [http://cgpdb.ucdavis.edu](http://cgpdb.ucdavis.edu)). One fixed primer was designed from an apple (Malus domestica L.) EST annotated as chlorophyll \( b \) synthase ([http://ftp.dna.affrc.go.jp/pub/dna_all/C/V9/87/28/CV987281/CV987281](http://ftp.dna.affrc.go.jp/pub/dna_all/C/V9/87/28/CV987281/CV987281)) and 12 fixed primers were designed against sunflower ESTs showing homology to other genes. The arbitrary primers labeled with either IR (infrared) 700 or IR 800 dye were from our laboratory and used for other research projects.

SSR markers from each of the 17 linkage groups (Yu et al., 2003) were also selected and used in this study. SSR marker assays were conducted following the procedures described by Tang et al. (2002).

**Mapping the \( yl \) locus with molecular markers**

The bulked segregant analysis strategy (Michelmore et al., 1991) was used for rapid establishment of the linkage relationship between the phenotype and the polymorphic TRAP and SSR markers. Initially, two bulked DNAs, green and yellow bulks (each containing equal amounts of DNA from eight green-leafed plants and yellow-leafed plants, respectively), were established to screen polymorphic SSR and TRAP markers. Polymorphic markers (showing band intensity differences) between the two bulks were then confirmed by surveying in two groups of F2 individuals, one with eight yellow-leafed plants and the other with eight green-leafed plants. Only the markers showing a significant association with leaf color were run on 117 randomly chosen F2 plants for map construction. For SSR markers, first one or two SSR markers from each of the 17 linkage groups were screened. Once one SSR marker was found to be linked with the \( yl \) locus, additional SSR markers mapped to the same chromosomal region were run to generate data for map construction. Genetic distances between linked markers (LOD > 3.0) were calculated with the computer program Mapmaker/EXP 3.0 (Lander et al., 1987) using Kosambi’s (1944) mapping function.

**Results**

**Inheritance of leaf color**

The two parental lines displayed an obvious difference in leaf color, which can be easily assessed (Figure 1). When the parent with yellow leaf color was used as female in hybridization with the parent with green leaf color, all F1 hybrid plants expressed the normal green phenotype of the male parent. This not only indicated that yellow leaf color is recessive to normal green but also excluded
the possibility of maternal inheritance for this trait. In the F2 population of 172 plants, 130 were normal green leaved and 42 were yellow leaved. This fit the monogenic segregation ratio of 3:1 \((\chi^2 = 0.03, p > 0.9)\).

The above visual observation was supported by the measurement of the greenness degree with a hand-held chlorophyll content meter, SPAD-502. The average greenness degrees for the green-leafed parent, the yellow-leafed parent, and the F1 plants were 36.8, 21.4, and 37.6, respectively. The distribution of the greenness degree of the 172 F2 individuals was discontinuous, ranging from 15.3 to 27.1 in the yellow-leafed group and from 30.2 to 41.6 in the green-leafed group (Figure 2). The wide range of chlorophyll content within the two leaf color groups might indicate an involvement of QTLs. However, there was no ambiguity in classifying the F2 plants into the two groups. These observations led to the conclusion that leaf color is controlled by a major gene and the green color is dominant over the yellow color in the population under investigation.

Leaf color and chlorophyll contents

Laboratory quantification of chlorophyll contents with a spectrophotometer revealed a significant difference in the contents of chlorophyll \(a\), chlorophyll \(b\), and total chlorophyll among the two parental lines and their progeny (Table 1). The contents of chlorophyll \(a\), chlorophyll \(b\), and total chlorophyll in the yellow-leafed F2 progenies were reduced by 41.6%, 53.5%, and 44.3%, respectively, in comparison with those in the green-leafed progenies. The green-leafed parent, NCCL, had much more chlorophyll \(a\), chlorophyll \(b\), and total chlorophyll than the yellow-leafed parent. However, the yellow-leafed parent had a higher
value of the chlorophyll \(a/b\) ratio than NCCL. The \(F_1\) hybrid had higher values for chlorophyll \(a\), chlorophyll \(b\), and total chlorophyll contents than the green-leafed parent, NCCL, and a lower value for the chlorophyll \(a/b\) ratio than the green-leafed parent, but the difference was not significant (Table 1). In the \(F_2\) population, the plants with green leaves or yellow leaves had similar chlorophyll contents and chlorophyll \(a/b\) ratios to that of the green-leafed parent and the yellow-leafed parent, respectively. These results also demonstrated that a major gene controls the leaf color phenotype under investigation. We propose the symbol \(yl\) (yellow leaf) to denote the gene.

### Genetic mapping of the \(yl\) gene

Altogether, 176 TRAP primer combinations were used to screen for polymorphisms between the two bulks. Thirty TRAP primer combinations that disclosed polymorphism between the two bulks were then used to survey 16 \(F_2\) individuals, eight yellow-leafed plants and eight green-leafed plants. Two TRAP primer combinations confirmed to be associated with leaf color were used to genotype 117 randomly chosen \(F_2\) plants for map construction. The first marker CV987281ga3-260, amplified with the fixed primer (5'-CATACAAGGTGTC-GAAATT-3') designed from the apple EST, mapped 16.6 cM proximal to the \(yl\) locus. The second marker, B26P17ga5-300, amplified with a fixed primer (5'-GTTTTCGTCATACTCGTTA-3') designed against a sunflower EST with homology to a gene for geranylgeranyl reductase of *Arabidopsis thaliana*, mapped 4.2 cM distal to the \(yl\) locus.

In an attempt to map the \(yl\) gene onto the public sunflower SSR map (Yu et al., 2003), 20 SSR markers (one or two per linkage group) were selected from this map to determine the chromosomal location of the \(yl\) gene using the same approach described above. SSR marker ORS595 from linkage group 10 amplified two fragments, one in each bulk. The 145-bp fragment, originating from the female yellow-leafed parent, appeared only in the yellow-leafed bulk, whereas the 135-bp fragment, contributed by the green-leafed male parent, was preferentially amplified and appeared only in the green-leafed bulk. Genotyping the 117 \(F_2\) plants at this locus revealed a tight linkage of ORS595 with \(yl\) since we found no recombination between them. Additional SSR markers that mapped near ORS595 were identified and four polymorphic SSRs between the two parents were identified. We genotyped the \(F_2\) plants with these SSR markers to confirm the location of \(yl\) on linkage group 10. A linkage map.
with five SSR and two TRAP markers was constructed for the chromosome segment harboring the yl gene (Figure 3). In comparison to the published RHA 280 × RHA 801 SSR map (Yu et al., 2003), the map order of the five SSR markers remained the same and the genetic length increased by only 1.7 cM (from 44.1 to 45.8 cM) after incorporating three additional markers. The SSR marker, ORS595, cosegregated with the yl locus.

**Discussion**

The yellow-leafed parent of our mapping population was introduced from Australia and has been used as a marker line in our sunflower research nursery for many years. Although the yellow-leafed phenotype expresses through the whole life cycle and transmits from generation to generation, no genetic analysis has been conducted for this interesting and somehow useful trait. We report for the first time in sunflower that a single dominant gene controls the leaf color phenotype, yellow leaf versus green leaf. Individual plants in the F2 population were categorized visually into green-leafed and yellow-leafed groups in a 3:1 ratio. The hand-held chlorophyll content meter, SPAD-502, also recognized two discontinuous phenotypic groups in the F2 population based on the greenness degree of the leaves. The extensive variation of greenness degree among plants within the green-leafed group and the yellow-leafed group could be caused by the influence of leaf thickness on the reading by the SPAD-502 meter (Peng et al., 1993; Reeves et al., 1994) and/or by small QTLs for chlorophyll content. However, no significant differences were detected among the green-leafed parent, F1 plants, and green-leafed progenies, or between the yellow-leafed parent and yellow-leafed progenies. In addition, the variation among the F2 progenies from the same leaf color group was relatively small (Table 1). This indicated that the leaf color was controlled by a major chlorophyll deficiency gene in this segregating population.

The estimated haploid genome size of sunflower ranged from 3.0 Gb (Arumuganathan and Earle, 1991) to 3.5 Gb (Baack et al., 2005) or even 3.6 Gb (Price et al., 2000). Molecular techniques have revealed the extensive duplication in the sunflower genome. It has been reported that approximately 30–35% of the RFLP probes could detect duplicated loci (Berry et al., 1995; Gentzbittel et al., 1995), and that about 40% of the SSR primer pairs could amplify multiple loci (Tang et al., 2002). However, many agronomic or morphological traits controlled by one or two major genes have also been reported in sunflower. For example, a self-incompatible gene (S) was found to be associated with a major gene located on linkage group 17 of the SSR map (Gandhi et al., 2005); a series of nuclear male sterility lines were each controlled by a single gene, and three of them, ms9, ms10, and ms11, were mapped by molecular markers (Pérez-Vich et al., 2005; Chen et al., 2006); apical branching was controlled by a recessive gene in the F2 population from the cross between HA 234 and RHA 271, b1, and it was mapped on linkage group 10 of the SSR map (Rojas-Barros et al., 2008); and lemon ray flower color was controlled by two recessive genes, and one of them was mapped on linkage group 11 of the SSR map (Yue et al., 2008). The mapped yl gene in the current study could be useful for map-based cloning of the gene, contributing to the understanding of chlorophyll biosynthetic pathways.

Laboratory assays with a spectrophotometer confirmed that the observed variation in leaf color resulted from the difference in chlorophyll contents, the yellow leaf color phenotype being caused by chlorophyll deficiency. The genetic basis of chlorophyll synthesis is very complex, since many metabolic steps and genes are involved (Narita et al., 1996; Porra, 1997; Suzuki et al., 1997). Although most previous studies mapped QTL underlying chlorophyll contents in various crops and documented that chlorophyll contents are under the control of multiple genes (Wang et al., 2003; Yang et al., 2003; Hu and Yan, 2004), chlorophyll content controlled by a single gene has also been reported. For example, a single dominant gene that increased chlorophyll b by 100% and total chlorophyll by 25% was reported in a rice population (Wang et al., 2008), and a single gene controlling yellow and green leaf colors was also reported in a diploid potato population and a yellow-leafed mutant of carrot (De Jong et al., 1998; Nothnagel and Straka, 2003). A single gene controlling chlorophyll deficiency, or yellow–green leaf (yl), was also identified and isolated recently in rice (Wu et al., 2007). In sunflower, Hervé et al. (2001) mapped four QTLs for chlorophyll contents onto linkage groups 5, 8, 10, and 18 of the AFLP map constructed from the recombinant inbred lines derived from the PAC2 × RHA 266 cross, and individual QTL explained 9.9–17.5% of phenotypic variation. It is not possible to determine whether there is a similar relationship between these mapped QTLs and the yl locus since there are no common markers between these two linkage maps.

TRAP was proposed in order to harness the annotated EST information to generate markers near the target sequence in order to map quantitative or
qualitative traits in plants with high efficiency (Hu and Vick, 2003). It has been successful in defining linkage group ends (Hu, 2006), in mapping a nuclear male-sterile gene (Chen et al., 2006) and a lemon ray flower color gene (Yue et al., 2008) in sunflower, and in mapping QTLs underlying disease resistance in common bean (Miklas et al., 2006) and wheat (Chen et al., 2007). Although two TRAP markers in the current study were detected to be linked to the yl locus, a TRAP marker cosegregating with yl was not identified. This could be due to the fact that there are many genes involved in controlling chlorophyll synthesis, and only one of them is different between the two parents. It is likely that none of the 22 annotated ESTs used for designing the fixed primer is the gene conditioning leaf color in the population used in this study.

Chlorophylls are light-harvesting pigments; leaves with higher chlorophyll contents will collect more energy and have a higher photosynthetic efficiency. It has been demonstrated that the chlorophyll content is positively correlated with the photosynthetic rate (Davis et al., 1979; Wang et al., 2003; Thomas et al., 2005). In sunflower, correlation analysis also revealed a significant and positive correlation between chlorophyll content and net photosynthesis (Hervé et al., 2001). The identification of a major gene controlling chlorophyll deficiency in this study offers the opportunity to understand the process of chlorophyll metabolism in sunflower. In addition, introgressing yl to sunflower restorer or male-sterile lines using the molecular markers identified in this study could produce a visible morphological indicator for seed purity testing and quality control in sunflower hybrid seed production. The work of selecting confectionery lines with yl is ongoing (Figure 1). Since this gene is recessive, the leaf color or chlorophyll content in the hybrids is normal. Therefore, seedlings from restorer/sterile lines with the yl gene mixed into the commercial hybrid seeds can be visibly identified and eliminated in the seedling stage in the sunflower field.

Acknowledgements

The authors greatly appreciate the technical assistance from Jerry Miller, Wenge Yuan, Ping Wang, and Angelia Hogness. The authors are also grateful to the two anonymous reviewers for their critical comments and constructive suggestions, which helped improving the quality of this report. This work was partially supported by USDA-Agricultural Research Service CRIS project 5442-21000-027-00D and a competitive grant from the National Sclerotinia Initiative.

References

Mapping of a chlorophyll deficiency gene in sunflower


Tang S, Leon A, Bridges WC, Knapp SJ. Quantitative trait loci for genetically correlated seed traits are tightly linked to branching and pericarp pigment loci in sunflower. Crop Sci. 2006;46:721–34.


