II.2 Genomics as Efficient Tools: Example Sunflower Breeding

A. Sarrafi and L. Gentzbittel[1]

1 Introduction

Sunflower (*Helianthus annuus* L.) is grown throughout the world mostly as a source of vegetable oil and protein. The main objectives of sunflower breeding programs are the development of productive F_1 hybrids with high oil and protein yield. As in all other crops, sunflower yield depends on many yield components which are controlled by several genes, the effects of which are modified by the environment. Conventional plant breeding methods are responsible for the improvement of plant yield which is provided by plant breeders in sunflower as well as in other crops.

Now, new technologies have introduced an additional means for improving sunflower yield and quality using molecular genetics. Molecular genetics and genomics are considered as tools for the genetic characterization of organisms. The aim of molecular genetics in sunflower breeding is to identify, isolate, amplify and modify genes or other sequences of DNA and to combine and express the novel or modified sequences in new genotypes. Despite some limitations, molecular genetics is now producing significant results by using the new technologies to influence basic and applied research in sunflower improvement.

Linkage map construction is usually the first step for identification of quantitative trait loci (QTLs) controlling different traits. Later, we will present some recent maps constructed for sunflower by different methods and also some QTLs identified for the traits related to yield improvement in this species. We will then present some recent results of genomics and the identification of genes of importance for sunflower breeding.

2 Linkage Mapping

The development of molecular marker techniques has provided an additional tool to determine linkage maps which are used for quantitative trait loci (QTLs) analysis. Molecular marker-based linkage maps are powerful tools for

[1] Department of Biotechnology and Plant Breeding, Pôle de Biotechnologie Végétale, ENSAT-BAP, 18 Chemin de Borde Rouge, BP 107, 31326 Castanet, France

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breeding programs. Genetic maps in sunflower constructed by different marker systems have been described by several authors. The most important markers are: restriction fragment length polymorphism, (RFLP), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSRs).

2.1 Restriction Fragment Length Polymorphism Markers

Restriction fragment length polymorphism genetic markers have unique characteristics for crop improvement and can be used to identify crop varieties or hybrids as well as to construct genetic maps. The association between specific DNA markers and agronomic traits will facilitate marker-assisted selection in breeding programs. Early genetic maps, constructed with RFLP markers (mainly cDNAs), were reported by Berry et al. (1995) and Gentzbittel et al. (1995). These maps were incomplete and were developed independently, thus showing very few common markers such as anchor points. Later, 232 unique cDNA probes were used for the RFLP linkage analysis of F_2 population of the cross 'RHA 271 × HA 234' and revealed 271 polymorphic loci (Jan et al. 1998). This map encompasses 20 linkage groups covering 1164 cM of the sunflower genome. Twenty linkage groups of the map are more than the 17 haploid chromosome number of sunflower (Table 1).

The rapid accumulation of markers and mapping populations are challenges for the management of information and the merging of separate sets of data in order to accumulate more valuable information for further research and the better use of genetics. Combined maps provide an easy and convenient way of comparing the component maps and other important information about the reliability of markers, order and distances between markers. With this aim, Gentzbittel et al. (1999) integrated seven individual maps and constructed a near-saturated linkage map, based on RFLPs and including major phenotypic traits (Table 1). This integrated map is arranged in 17 major linkage groups containing 238 loci and covers 1534 cM.

2.2 Amplified Fragment Length Polymorphism Markers

The amplified fragment length polymorphism (AFLP) assay is a powerful technique for genome mapping and genetic variability studies in sunflower. AFLP markers are typically dominant and most fragments correspond to a unique position on the genome and, hence, can be exploited as landmarks in genetic and physical mapping (Meksem et al. 1995). A total number of 19 AFLP primer pairs selected for sunflower were used to determine the linkage map of recombinant inbred lines (RILs) of the cross 'PAC-2 × RHA-266'. Out of 333 markers, 254 were placed in 18 linkage groups; the total length of the map is 2558 cM (Flores-Berrios 2002a; Table 1). The length of this map is significantly greater than that described by Jan et al. (1998) and Gentzbittel et al.

Table 1. Sunflower linkage maps: population, number of markers, linkage groups, kind of markers and map length (cM)

Cross	Population	Number of markers	Number of six linkage groups	Marker systems	Map length (cM)	Reference
RHA271 × HA 234	F_2	271	20	RFLP	1164	Jan et al. (1998)
SD × PA4	F_2	86	17	RFLP	774	Gentzbittel et al. (1999)
SD × CP73	F_2	106	18	RFLP	1056	Gentzbittel et al. (1999)
CP73 × PAC1	F_2	111	16	RFLP	1068	Gentzbittel et al. (1999)
GH × PAC2	F_2	95	18	RFLP	1008	Gentzbittel et al. (1999)
HA89 × RHA266	F_2	76	14	RFLP	176	Gentzbittel et al. (1999)
CX × RHA 266	F_2	99	18	RFLP	582	Gentzbittel et al. (1999)
PAC2 × RHA266	F_2	144	21	RFLP	763	Gentzbittel et al. (1999)
Composite map	F_2	238	17	RFLP	1534	Gentzbittel et al. (1999)
PAC2 × RHA266	RILs (F8)	254	18	AFLP	2558	Flores-Berrios et al. (2000a)
ZENB8 × HA89	F_3 and F_4	205	17	RFLP	1380	Leon et al. (2001)
HA370 × HA372	F_2	446	17	AFLP and RFLP	1326	Gedil et al. (2001b)
HA89 × CAS3	F_2	154	17	RFLP and AFLP	1807	Pérez-Vich et al. (2002)
HAOL9 × CAS3	F_2	137	17	RFLP and AFLP	1641	Pérez-Vich et al. (2002)
LI × L2	F_3	170	20	AFLP and SSR	2539	Mokrani et al. (2002)
CmsHA89 × Wild (H. *annuus Var annuus*)	F_3	107	17	SSR	972	Burke et al. (2002)

(1999), constructed by RFLPs, and corresponds to an expected twofold expansion of the distances, partly because of using RILs created through selfing. Sunflower possess 17 haploid chromosomes and 18 linkage groups, as well as unlinked markers presented in the map, which indicate that further mapping is still needed to obtain a saturated one.

2.3 Simple Sequence Repeat Markers

Simple sequence repeats (SSRs), called microsatellites, are also 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 fingerprinting and pedigree analysis (Plaschke et al. 1995; Rongen et al. 1995; Guilford et al. 1997). SSR markers are, thus, now recognized as one of the most efficient molecular markers. SSR identification in sunflower has been reported independently by at least two groups (Paniego et al. 2002; Yu et al. 2002). Burke et al. (2002) established a genetic map of an F_3 population derived from the cross 'cms HA 89 × wild (H. *annuus var. annuus*)' using SSR markers (Table 1). This map covers 17 linkage groups with 107 SSRs and the total map distance is 972 cM. The map length is thus considerably shorter than other maps presented in Table 1. This fact is most likely due, at least in part, to incomplete genome coverage.

The most complete SSR linkage map was published by Tang et al. (2002). They describe a map of 17 linkage groups, based on 408 SSRs, genotyped on 94 RILs, producing 462 SSR marker loci segregating in the mapping population. However, until now, this map has not been associated with any key traits for sunflower breeding and is thus of less interest. Important efforts toward the integration of previously described knowledge-associated data into the frame of a dense SSR map remain to be made. Many factors such as the nature of the populations studied, the number of individuals and the number of markers might change the recombination rate and, consequently, the distance between two loci. Some of the genetic maps summarized in Table 1 are constructed using AFLP and RFLP markers (Gedil et al. 2001b; Pérez-Vich et al. 2002), or AFLPs and SSRs (Mokrani et al. 2002). The advantage of these kinds of maps is that in cases where they have common SSR or RFLP markers with some other maps, they can be combined in order to construct new maps with more markers.

2.4 Cultivated Sunflower Genetic Polymorphisms and Heterosis Modelling

The discovery of genetic structures in cultivated sunflower was one of the first aims of genetic fingerprinting using molecular markers. Initial studies using RFLPs or RAPDs (Berry et al. 1994; Gentzbittel et al. 1994; Mosges and Friedt 1994; Teulat et al. 1994; Zhang et al. 1995) revealed a clustering of inbreds between restorer and maintainer lines of the 'classical' cytoplasmic male sterility to be the most important genetic structure found in cultivated sunflower. AFLPs (Hongtrakul et al. 1997) and minisatellite (Dehmer and Friedt 1998) analyses confirmed these results and also showed that the confectionery sunflower lines are clearly outgroups of oil-producing sunflowers. Using SSRs as high throughout tools for genetic fingerprinting allowed Yu et al. (2002) to show that the results obtained by this molecular tool are consis-

tent with the previously described structures. They also reconfirmed that polymorphism within cultivated sunflower is much more reduced than in other crops. Heterotic group modelling in sunflower using molecular tools was reported several times (Tersac et al. 1993, 1994; Cheres et al. 2000), but failed to reveal clear heterotic groups. At least AFLP-based genetic distances were poor predictors of hybrid seed yield.

3 Quantitative Trait Loci Identification

3.1 Agronomic Traits

The principal goal of sunflower breeding programs is the development of new cultivars with a high oil yield. Identification of the chromosome regions which affect grain yield, oil percentage in grain and other agronomic traits should increase our understanding of the genetic control of the characters and help us to develop marker-assisted selection programs. Days to flowering is one of these important traits because cultivars with certain ranges of cycle length provide optimum yield in specific environments. Leon et al. (2001) located six QTLs associated with growing degree days to flowering in an F_2 population presenting 76 % of phenotypic variation, whereas Mokrani et al. (2002) identified two QTLs for sowing to flowering date using 118 F_3 families coming from crosses between two others genotypes with a total effect of 89.30 % (Table 2).

In the same F_3 families, Mokrani et al. (2002) have also detected two QTLs for grain weight per plant, one QTL for 1000-grain weight and seven QTLs for oil percentage in grain with total phenotypic variation of: 50.70, 22.70 and 90.40 %, respectively (Table 2). QTLs for percentage of oil in grain and 1000-grain weight were also detected by Mestries et al. (1998). These results complement pioneer work describing molecular markers linked to oil characteristics and quantitative genetics analyses (Leon et al. 1995, 2003). As far as oil quality is concerned, four QTLs controlling stearic acid and three for oleic acid were identified by Pérez-Vich et al. (2002). Phenotypic variation for these traits are 84.4 and 58.4 %, respectively (Table 2).

3.2 Resistance to Disease

Plant improvement implies the ability to create genotype resistance to different diseases. Being originally breed in Russia under continental conditions, cultivated sunflower is susceptible to diseases under wetter conditions such as those encountered in western Europe. Downy mildew and black stem caused by *Plasmopara halstedii* and *Phoma macdonaldii* are considered important diseases in sunflower (Acimovic 1984; Mouzeyar et al. 1994; Tourvieille de Labrouhe et al. 1998).

Table 2. QTLs detected by different marker systems and their effects on some agronomic traits in sunflower

Reference	Traits	Abbreviation	Number of QTLs	Linkage groups	Total phenotypic variation (%)	Markers
Leon et al. (2001)	Growing degree days to flowering	*gdd*	6	A, B, F, I, J, L	76.0	RFLP
Mokrani et al. (2002)	Grain weight per plant	*gwp*	2	9	50.70	AFLP and SSR
	1000 grain weight	*tgw*	1	16	22.70	
	Oil percentage in grain	*pog*	7	9, 11, 12, 13	90.40	
	Sowing to flowering	*stf*	2	9, 10	89.30	
Pérez-Vich et al. (2002)	Stearic acid	C18:0	4	1, 3, 8, 14	84.4	AFLP and RFLP
	Oleic acid	C18:1	3	1, 8, 14	58.4	
Rachid Al-Chaarani et al. (2002)	Downy mildew	*dmr*	4	1, 9, 17	54.90	AFLP
	Black stem	*bsr*	7	3, 4, 8, 9, 11, 15, 17	93.10	
Hervé et al. (2001)	Chlorophyll concentration	*chl*	4	5, 8, 10, 18	53.5	AFLP
	Net photosynthesis	*pho*	3	9, 14	62.5	
	Stomatal conductance	*sco*	4	3, 8, 16, 17	61.9	
	Predawn leaf water potential	*pot*	3	8, 10, 14	34.1	
Flores-Berrios et al. (2000a)	Shoots per explant	*ose*	6	2, 4, 7, 9, 17	52.0	AFLP
	Shoots per regeneration explant	*osr*	7	2, 4, 6, 7, 8, 15, 17	67.0	
Flores-Berrios et al. (2000b)	Total embryogenic explants	*toe*	4	1, 3, 13, 15	48.0	AFLP
Flores-Berrios et al. (2000c)	Total protoplast division	*ptd*	12	1, 7, 8, 10, 13, 14, 15, 17	72.0	AFLP

Downy mildew is the most studied system, probably because it is considered to be a simple gene-for-gene resistance model. Several resistance clusters were described (Mouzeyar et al. 1995; Roeckel Drevet et al. 1996; Vear et al. 1997; Bert et al. 2001), and at least one of them was independently confirmed by Gedil et al. (2001a). These results are accompanied by the descriptions of molecular markers tightly linked to the downy mildew resistances, often based on candidate gene approaches, and putatively used as tools for breeding (Gentzbittel et al. 1998; Brahm et al. 2000; Gedil et al. 2001a; Bouzidi et al. 2002). Using a quantitative model, Rachid Al-Chaarani et al. (2002) identified four QTLs for resistance to downy mildew and seven QTLs for resistance to black stem using recombinant inbred lines of the cross 'PAC 2 × RHA 266' (Table 2). The four detected loci explained 54.9% of the total phenotypic variance for resistance to downy mildew, whereas the seven detected QTLs for black stem explained 92% of the variance.

Candidate genes for disease tolerance to *Sclerotinia* were described (Mouzeyar et al. 1997; Gentzbittel et al. 1998) and were evaluated in subsequent QTL analyses (Bert et al. 2002). Under semi-dry conditions (Spain, Israel, Northern Africa), *Orobanche cumana*, a parasitic weed, appears to be a potential important disease. Genetic mapping of several sources of resistance to *O. cumana* were described (Lu et al. 1999, 2000) leading the way to putative breeding for appropriate sunflower inbreds. Linkage of molecular markers with resistance genes to rust were also described (Lawson et al. 1998).

Thus, several tools are already available to breeders for the improvement of breeding processes for disease resistance.

3.3 Photosynthesis and Water Status Traits

Yield components and oil production are positively correlated with photosynthesis and water status traits in sunflower. Hervé et al. (2001), conducted an experiment to identify QTLs for photosynthesis and water status traits using RILs from the cross 'PAC 2 × RHA 266' and the results are summarized in Table 2. Four QTLs detected for chlorophyll concentration accounted for 53.5% of phenotypic variation for this trait and three QTLs for net photosynthesis accounted for 62.5% of total phenotypic variation. As far as stomatal conductance is concerned, four QTLs with 61.9% of phenotypic effect were detected, whereas predawn water potential was associated with three QTLs and only 34.1% of phenotypic variance.

3.4 In Vitro Regeneration

The ability to regenerate large numbers of plants is important for the development of biotechnology as regards genetic transformation in sunflower. In vitro regeneration was investigated in 75 RILs and their parents (PAC 2 and

RHA 266). The results summarized in Table 2 show that six putative QTLs for the number of shoots per explant and seven for shoots per regenerating explants were detected in cotyledon organogenesis culture (Flores-Berrios et al. 2000a). The same RILs were also used for somatic embryogenesis by epidermic layers; four QTLs were identified for the total number of embryogenic explants which explained 48% of the phenotypic variation for this trait (Flores-Berrios et al. 2002b). The above-mentioned RILs were also used in another experiment in which 12 QTLs were identified for protoplast division with a total phenotypic variation of 72% (Flores-Berrios et al. 2000c). Some segments of the linkage groups 1, 15 and 17 are likely to contain genes important for organogenesis, somatic embryogenesis and protoplast division. The QTLs identified in these three linkage groups should be involved in cell division in early events associated with cell differentiation.

4 Sunflower Genomics: Towards Genes and Functions

Sunflower genomics is still in its infancy, as compared to *A. thaliana*, rice or maize genomics initiatives. However, significant programs have been recently developed, with the aim of bringing to sunflower breeders the tools and knowledge to answer the major challenges of sunflower breeding. In this section, we will present results of the characterization of the oil synthesis pathways and the emergence of genomics programs for sunflower.

4.1 Oil Synthesis

Despite being biochemically well characterized, the lipid metabolism in sunflower remains undescribed at the molecular level and, as stated in previous sections, the genetics of oil (fatty acids) synthesis still remains to be discovered and understood. The 'high oleic acid' trait has mainly been studied because of its economic importance. It is unclear if other fatty acids are considered as breeding and research targets.

Sunflower possesses the unique ability to produce oleic acid at both a high percentage and high yield. This feature was obtained after mutagenesis treatment on Vnimk 8931 population (known as the Pervenets mutation). Several commercial varieties derived from Pervenets and breeding materials with a high oleic acid content have been marketed. However, the genetics of this trait are still not fully understood by breeders. To characterize the Pervenets mutation, several groups have tried to clone and discover mutations in the genes associated with oleic acid synthesis (Hongtrakul et al. 1998a, b; Lacombe and Berville 2001). Tightly linked markers were discovered, some of them displaying the characteristics of candidate genes (Lacombe and Berville 2001). However, the exact mechanism by which the Pervenets mutations provide the 'high oleic' phenotype remains unknown.

4.2 Functional Genomics

For functional genomics, the objectives are to optimize the targets for breeding by deciphering the genetics of simple or quantitative traits, for example by identifying expressed sequence tags (ESTs) putatively underlying QTLs. This method is based on the massive and parallel analysis of the expression levels of thousands of genes in key situations and rely heavily, in a first step, on massive EST sequencing and the use of so-called DNA chips. For sunflower, two major programs are emerging.

A US program for the massive sequencing of ESTs is underway. As a result of this program, the increase in GenBank entries for sunflower ESTs has been spectacular: from 191 entries in December 2000 to 49,264 entries in January 2003. These ESTs were obtained from two inbred lines (RHA801 and RHA280) and cover 11 different organs or physiological situations: callus, roots, disk and ray flowers, pre-fertilized flowers, developing kernel, chemical induction, root environmental stress, shoot environmental stress, germinating seeds, flower environmental stress and hulls. A French program of massive sunflower EST sequencing is also on the way (Caboche and Boucly 2000). More than 20 different cDNA libraries covering key traits for sunflower breeding were constructed and subjected to sequencing. The targets are plant and seed development, drought tolerance and disease resistance. The results will be deposited in public databases and are already available through a high value-added database after bioinformatics work (Samson 2003).

As a consequence of the large number of available sequences (47,000 in March 2003), high-throughput design of primers for EST-based SSRs or single nucleotide polymorphism (SNP) discovery is currently possible to significantly increase the number of expressed sequence-based molecular markers in sunflower.

The expected result of these functional genomics programs is the identification of key genes by temporal, spatial or conditional study of the gene expression level. The steps of gene validation could, however, be difficult as genetic transformation for sunflower does not reach the efficiency needed to be used as a routine tool. To overcome this problem, programs leading to the creation of large mutant collections should be underway. In any case, the functional genomics programs will led to the discovery of new targets for breeding, or to the definition of molecular covariates for the resolution of QTLs for major agronomic traits in sunflower.

4.3 Sunflower Genome Structure

As presented in previous sections, molecular linkage maps for sunflower are already available, though based on different tools and with contrasting efficiencies and knowledge-associated data. In order to facilitate positional gene cloning and to provide a public tool for the improvement of sunflower

genome analyses, a BAC library of about four genome equivalents of the inbred HA821 was constructed (Gentzbittel et al. 2002).

As the sequence of the dicot model plant, *Arabidopsis thaliana*, becomes available, several groups are working towards the analysis and the fine characterization of the synteny conservation between the sunflower and *A. thaliana* genomes. Massive EST sequencing also provides a large amount of information that could be used in many different ways. For example, it could provide physical anchors to an EST-derived marker-based (SNPs, SSRs, CAPS, etc.) genetic map, which could be extended to regional or global contig building of the sunflower genome. These contigs will, thus, be of valuable use in the fast cloning of important genes. As another example, ESTs could be used to globally align the sunflower genome with that of *A. thaliana* by in silico hybridization and genetic mapping, thus using the *A. thaliana* sequence as a predictor of the sunflower genome sequence in the regions where synteny exists. For example, a set of conserved orthologous sequences (COS) between different dicots is being defined. In this respect, two websites are proposing tools in a first attempt to graphically present the results of the synteny analyses: the Composite Genome Project Database(http://cgpdb.ucdavis.edu/) and the ICCARE/HeliantSynteny information server (http://genopole.toulouse.inra.fr/~cmuller/accueil.html)

5 Conclusions

Taking into account the large size of the sunflower genome (estimated 3.10^9 bp), a global physical map and genome sequencing project are not realistic in the near future. Research programs will probably focus on a few regions of the sunflower genome governing key agronomic traits, such as oil and protein synthesis, seed development and disease resistance. Numerous molecular tools are already available to develop such programs.

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