AN ABSTRACT OF THE THESIS OF

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The level of polymorphisms of many biochemical and DNA markers are low in cultivated sunflower (*Helianthus annuus* L.). The number of mapped public DNA markers is limited. Molecular markers have not been developed for the most important diseases of sunflower, such as downy mildew. The objectives of this study were (i) to help alleviate the problem of low DNA marker polymorphisms by developing simple sequence repeat (SSR) markers, (ii) to build an integrated AFLP-RFLP linkage map by using previously described probes and newly developed AFLPs, and (iii) to clone and characterize candidate disease resistance genes. Forty-four polymorphic SSR markers were developed from a genomic DNA library. Diversity analysis of these SSRs for variability among 10 public inbred lines produced an average of 1.86 alleles per locus and mean heterozygosity of 0.21. The number of alleles ranged from 1 to 5. Trinucleotide SSRs were less polymorphic than dinucleotide and mononucleotide SSRs. Cluster analysis and multidimensional scaling separated elite inbred lines from wild species. There was more polymorphism in wild species than in elite lines.

Three hundred and six AFLP markers were developed using 18 primer combinations. Two sets of previously mapped RFLP markers were tested for segregation in an F_2 mapping population. A total of 401 markers were assigned to 17 linkage groups covering 1326 cM with a mean spacing of 3.3 cM between adjacent markers. The RFLP markers were well spaced and well distributed

throughout the genome. Some linkage groups are sparsely populated with common markers. There were two gaps of 30 or more cM in two linkage groups.

We cloned candidate disease resistance genes for downy mildew resistance based on sequence homology among resistance genes in other species. Eleven unique nucleotide binding sequence (NBS) containing clones were isolated and showed similarity to the corresponding domains of cloned disease resistance genes in other plant species. Seven clones mapped to four linkage groups and identified nine loci. A cleaved amplified polymorphic sequence (CAPS) marker that was 3.7 cM from the *Pl1* resistance gene was developed by analysis of NILs. This CAPS marker should facilitate marker-assisted selection in sunflower.

Marker Development, Genome Mapping, and Cloning of Candidate Disease Resistance Genes in Sunflower, *Helianthus annuus* L.

by

Melaku Ayele Gedil

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Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Melaku Ayele Gedil, Author

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CONTRIBUTION OF AUTHORS

Professor Steven J. Knapp initiated, advised, and supervised all aspects of the projects. Dr. Gordon M. Huestis was involved in library screening and primer designing for the SSR project. Dr. Mary B. Slabaugh contributed and helped in CDR cloning and sequence analysis. Dr. Simon Berry and Dr. Richard Johnson have taken part in SSR screening, mapping of CDR clones, and developing RFLP markers for the integrated map study. Sergio Feingold and Dr. Alberto Leon assisted in growing plants for the mapping population and preparation of sample leaf tissues for DNA extraction. Dr. Jerry F. Miller and Dr. Tom J. Gulya performed screening for downy mildew resistance. Johan Peleman, Rindert Peerbolte, and Crispin Wye generated AFLP data and assisted in linkage analysis. Alex Kahler conducted genotyping of USDA/BGS RFLP probes. Dr. Richard Michelmore provided lettuce NBS clones and sequences.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF TABLES

Table **Page**

Marker Development, Genome Mapping, and Cloning of Candidate Disease Resistance Genes in Sunflower, *Helianthus annuus* L.

CHAPTER 1

INTRODUCTION

The level of polymorphisms of many biochemical and DNA markers are low in cultivated sunflower (*Helianthus annuus* L.) even though this species is highly allogamous and found in diverse habitats throughout the western half of the US (Cronn et al. 1990; Rieseberg and Seiler 1997). An analysis of elite US breeding lines confirmed the narrowness of the genetic base of sunflower (Cheres and Knapp 1998). They showed that public inbred lines trace to 58 germplasm sources with 70% of the genetic diversity among both confectionery and oilseed sunflower being derived from ancestral germplasm sources, most of which were developed in the former Soviet Union (Cheres and Knapp 1998).

Development of a variety of molecular markers and subsequent utilization of these markers for constructing high-density genetic linkage maps is the first step towards molecular plant breeding. Molecular markers are powerful tools for marker-assisted breeding, map-based cloning, DNA fingerprinting and germplasm identification, and quantitative trait locus (QTL) analysis (Lee 1995; Stuber 1992; Staub et al. 1996). A variety of molecular markers have been invented in the past few decades and the utility of these markers depends on their unique properties such as reproducibility, information content, multiplex ratio, cost and convenience (Powell et al. 1996a; Russel et al. 1997; Jones et al. 1997). Saturated molecular marker maps have been developed in rice, *Oryza sativa* L. (Harushima et al. 1998), soybean, *Glycine max* (Keim et al. 1997), and tomato, *Lycopersicum esculentum* L. (Tanksley et al. 1992).

In sunflower, molecular markers have been utilized (i) to study genetic diversity using random amplified polymorphic DNAs (RAPDs) (Lawson et al. 1994; Arias and Rieseberg 1995), restriction fragment length polymorphisms

(RFLPs) (Berry et al. 1994; Gentzbittel et al. 1992, 1994; Zhang et al. 1995), amplified fragment length polymorphisms (AFLPs) (Hongtrakul et al. 1997), and simple sequence repeats (SSRs) (unpublished data), (ii) for construction of genetic linkage maps (Berry et al. 1995, 1997; Gentzbittel et al. 1995; Jan et al. 1998), (iii) for mapping and characterizing genes controlling qualitative traits such as disease resistance and fatty acid content (Vear et al. 1997; Lawson et al. 1998; Besnard et al. 1997; Hongtrakul et al. 1998a, 1998b) and (iv) for dissecting quantitative traits (Leon et al. 1995, 1996; Mestries et al. 1998).

Even though a relatively large number of RFLP markers have been developed for sunflower, very few SSR markers have been developed (Brunel 1994; Whitton et al. 1997). SSR makers are powerful tools for molecular breeding and genome analysis. They are often multiallelic, can be multiplexed and automated for high throughput genotyping, and easy and convenient to exchange between laboratories (Powell et al. 1996b; Chen et al. 1997). Rebaut et al. (1997) demonstrated the usefulness and reliability of SSRs in a marker-assisted selection program as a tool for pre-selection in very large populations.

Our goal was to alleviate the problem of low DNA marker polymorphism by developing a collection of DNA markers including simple sequence repeats (SSRs), restriction fragment length polymorphisms (RFLPs), and amplified fragment length polymorphisms (AFLPs) and to provide genetic mapping resources for sunflower breeders in the public and private sectors. Chapter 2 presents the development of SSR markers in sunflower via screening a genomic DNA library with di- and tri-nucleotide repeat oligonucleotide probes. Polymorphic markers were screened for variability across 22 sunflower genotypes representing diverse inbred lines and wild *Helianthus* species. Heterozygosities and other statistics were estimated for each marker. Genetic distances between lines were estimated and used to construct a phenogram depicting the genetic relationships among genotypes.

Although the number and density of molecular markers on the genetic map of sunflower has rapidly increased since the publication of the first RFLP maps (Berry et al. 1995; Gentzbittel et al. 1995), the number of mapped public DNA

markers is still limited, many gaps persist in the map, and the mean density of mapped public co-dominant markers needs to be increased. Furthermore, currently available genetic maps of sunflower were constructed independently based on proprietary RFLP markers. Probes for many of the RFLP markers have not been widely distributed and maps have not been developed to link map positions and mapping data from different studies (Knapp et al. 1999). One of the aims of this study was to develop an integrated genetic map of sunflower using AFLP markers and two sets of previously mapped RFLP markers. The map was built using 180 HA370 x HA372 F_2 progeny (Chapter 3).

Sunflower (*Helianthus annuus* L.) is afflicted by several biotic diseases, including downy mildew caused by *Plasmopara halstedii* (Farl.) Berl. & de Toni. The sunflower seed industry devotes significant resources to disease resistance breeding. Most of the disease resistance genes of sunflower have not been mapped, and the genetics of resistance to important sunflower diseases have yet to be investigated. Wild species of sunflower are known to be an important source of disease resistance genes and have been utilized as a source of genes for introgression into cultivated sunflower (Skoric 1993). Molecular markers can accelerate the introgression of genes from wild to cultivated sunflower and reduce the problem of linkage drag. The third study (chapter 4) involves the cloning and characterization of candidate disease resistance (CDR) genes in sunflower based on the recently reported PCR-based approach (Kanazin et al. 1996; Yu et al. 1996; Leister et al. 1996, 1998; Speulman et al. 1998; Feuillet et al. 1997; Ohmori et al. 1998; Shen et al. 1998) and the development of a cleaved amplified polymorphic sequences (CAPS) marker for a downy mildew resistance gene. This strategy capitalizes on conserved domains among cloned disease resistance genes in plants (Michelmore 1995b; Staskawicz et al. 1995). Unique clones that showed similarity to cloned R genes were examined for linkage to disease resistance genes by hybridization-based and PCR-based methods. A CAPS marker was developed for downy mildew resistance. The CAPS marker should facilitate marker-assisted selection for downy mildew resistance genes in sunflower.

CHAPTER 2

Simple Sequence Repeat Markers Developed from a Genomic DNA Library of Sunflower, *Helianthus annuus* L.

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ABSTRACT

The level of polymorphisms of many biochemical and DNA markers are low in cultivated sunflower (*Helianthus annuus* L.), even though this species is highly allogamous and found in diverse habitats throughout the western half of the US. Our goal was to alleviate the problem of low DNA marker polymorphisms by developing a collection of simple sequence repeat (SSR) markers. A random genomic DNA library was developed with inserts ranging from 200 to 600 base pairs. This library was screened for five tri-nucleotide repeats (TNRs) (AAT, AAC, AAG, TCG, ACC) and two di-nucleotide repeats (DNRs) (AT and AC) using radio-labeled oligonucleotide probes. Clones were put through two rounds of colony screening, positive clones were sequenced, and sequences harboring SSRs were selected for marker development and testing. A total of 313 clones were selected for sequencing of which 109 had SSRs. After eliminating redundant sequences and those unsuitable for primer design, oligonucleotide primers were developed for 96 clones. Primers were designed to facilitate multiplexing by varying the lengths of target sequences, and by selecting primers that functionioned under a narrow range of PCR conditions. Testing of 120 primer sets (96 new and 24 previously developed SSR markers) produced 44 polymorphic markers (2 mono-, 6 di-, and 34 tri-nucleotide repeats and 2 others). Testing of these SSRs for variability among 10 public inbred lines produced an average of 1.86 alleles per locus and mean heterozygosity of 0.21. The number of alleles ranged from 1 to 5. Heterozygosity ranged from 0 to 0.7 with a mean of 0.17 for trinucleotide SSRs and from 0.18 to 0.76 with a mean of 0.35 for dinucleotide SSRs. The number of alleles ranged from 1 to 4 (mean=1.7) and 2 to 5 (mean=2.7) for trinucleotide and dinucleotide SSRs, respectively. Polymorphisms of all classes of SSRs (mono, di, and tri) were higher in wild species than in elite lines. Dinucleotide SSRs showed more alleles per locus and higher heterozygosity than trinucleotide SSRs. A total of 23 SSR markers showed polymorphism in at least two inbred lines. In wild species 41 out of 44 markers were polymorphic. Cluster analysis and multi-dimensional scaling

clearly separated elite lines from wild species. Elite lines were not separated into maintainer and restorer lines as reported before. The SSR markers developed can serve as an easily assayable sequence-based molecular marker that complement commonly used markers such as RFLP, AFLP, and RAPD in sunflower.

INTRODUCTION

Simple sequence repeats (SSRs) are present in the genomes of many species and are a source of highly polymorphic sequence-based genetic markers (Morgante and Olivieri, 1993; Powell et al. 1996; Wang et al. 1994; Brown et al. 1996). Polymorphisms are produced by repeat number variants (alleles) by PCR amplification of short (1-4) tandem repetitive DNA sequences using oligonucleotide primer sets designed to flank the SSR locus. In a recent study, Loridon et al. (1998) found that length polymorphism occurs not only due to variation in repeat number but also due to variation in flanking sequences. The length variants produced by difference in repeat number can be assayed using a variety of methods. The conventional method involves separation of radiolabeled PCR products on standard denaturing polyacrylamide gels and detection by autoradiography. Modification of the conventional methods of detection of SSR polymorphism include separation of unlabeled PCR products on nondenaturing polyacrylamide gel and staining with ethidium bromide (Mellersh and Sampson, 1993), or simultaneous detection of several loci by multiplexing and silver staining (Chen et al. 1997). Recently, automated high throughput genotyping (Ziegle et al. 1992; Schwengel et al. 1994; Perlin et al. 1995) of microsatellites have been demonstrated in maize (Smith et al. 1997), wheat (Roder et al. 1998a), tomato (Bredemeijer et al. 1998), and *Brassica* sps. (Mitchell et al. 1997).

Outstanding features responsible for ubiquity of SSRs as valuable genetic markers include high polymorphism, codominance inheritance, ease of detection

based on PCR using a small amount of DNA, suitability for automated genotyping, and extensive genome coverage (Powell et al. 1996; Morgante and Olivieri 1993). Furthermore, unlike restriction fragment length polymorphism (RFLP), SSR markers can be maintained and disseminated more easily, through publication or internet, since only primer sequence data are needed to describe a marker. The setback in utilization of SSRs is the high cost and time incurred in screening and sequencing clones. For plant species with numerous DNA sequence data, SSR markers can be developed in short period of time by searching for repeat-containing sequence in various databases (Akkaya et al. 1992; Saghai Maroof et al. 1994; Akagi et al. 1996; Becker and Heun 1995). An alternative and more common strategy of isolating SSRs is screening of different types of DNA libraries including genomic, cDNA, and large insert (McCouch et al. 1997; Loridon et al. 1998; Chen et al. 1997; Struss and Plieske 1998; Roder et al. 1998a). The efficiency of screening can be enhanced by using a library enriched for one or more repeat motifs (Karagrozov et al. 1993; Ostrander et al. 1992; Edwards et al. 1996). Various microsatellite enrichment methods have been described in Powell et al. (1996). Methylation sensitive enzymes (Roder et al. 1998a) and physically sheared DNA (Chen et al. 1997), have been successfully utilized to ensure fairly uniform representation of the genome. Roder et al. (1998a) reported that the proportion of useful SSR markers was almost doubled in microsatellite clones isolated from hypomethylated regions of the wheat genome.

Survey of various repeat motifs in the database and genomic library showed that AT repeats are most abundant in plant genomes (Akkaya et al. 1992; Lagercrantz et al. 1993; Morgante and Olivieri 1993; Wang et al. 1994). A study on limited number of sunflower DNA sequences suggested that AT repeats are the most abundant (unpublished data). Despite its abundance in plant genomes, AT repeats could not be utilized due to self-annealing of probes (Bell and Ecker 1994; Dehmer and Friedt 1998). Abundance of SSRs did not correlate with nuclear DNA content (Wang et al. 1994). Based on the assessment of genomic libraries, Condit and Hubbell (1991) estimated 5×10^3 to 3×10^5 (AC)n and (AG)n

sites per genome in six plant species including maize. In general, (AC)n and (AG)n repeats were estimated to be present every few hundreds to thousand kb in rice (Wu and Tanksley 1993), wheat (Roder et al. 1995), maize (Condit and Hubbell 1991), and *Arabidopsis* (Bell and Ecker 1994). Hybridization of oligonucleotide containing repeats showed that the sunflower genome contains abundant poly-A, poly-AC and poly-AG repeats (Dehmer and Friedt 1998).

SSR markers tend to be more polymorphic than other classes of markers in some species. SSRs have been successfully utilized to determine genetic variability of closely related genotypes in rice (Yang et al. 1994; Wu and Tanksley 1993), soybean (Morgante et al. 1994; Wang et al. 1998), barley (Saghai Maroof et al. 1994; Struss and Plieske 1998), wheat (Roder et al. 1995; Plaschke et al. 1995; Bryan et al. 1997), maize (Smith et al. 1997), *Arabidopsis* (Innan et al. 1997; Loridon et al. 1998), and sunflower (unpublished data). Olufowote et al. (1997) demonstrated the use of SSRs to detect within cultivar variation in rice. Due to the apparent advantage of SSRs, the number of SSR markers being developed and integrated into an existing framework RFLP maps is increasing rapidly in several crop species including rice (Akagi et al. 1996; McCouch et al. 1997; Chen et al. 1997; Cho et al. 1998), barley (Becker and Heun 1995), wheat (Roder et al. 1995; Roder et al. 1998a), maize (Senior et al. 1996), potato (Milbourne et al. 1998), soybean (Akkaya et al. 1995), and sorghum (Taramino et al. 1997).

Hypervariability of SSRs made these markers a powerful tool for germplam identification (Akagi et al. 1997; Russell et al. 1997; Smith et al. 1997), evolutionary studies, and pedigree analysis (Akagi et al. 1997; Saghai Maroof et al. 1994; Yang et al. 1994). Once assigned to genetic linkage maps, SSRs are markers of choice for marker-assisted selection by virtue of their ease and efficiency of assayability. Data on linkage of SSRs with economically important genes is emerging in several plant species (Ribaut et al. 1997; Senior and Heun 1993; Yu et al. 1994). In sunflower, an SSR marker was developed from poly-T repeat found in the intron of stearoyl-acyl carrier protein desaturase (SAD) gene and was polymorphic among elite inbred lines (Hongtrakul et al. 1998).

SSRs have been found in several sunflower (*Helianthus annuus* L.) gene sequences and have been used to develop 14 SSR markers (unpublished data; Hongtrakul et al, 1998; Whitton et al., 1997). Additionally, seven SSRs have been developed by screening genomic libraries for specific repeats (Brunel, 1994; Whitton et al., 1997). Even though genetic diversity among elite and wild sunflower have been determined by using RAPD (Arias and Rieseberg 1995; Lawson et al. 1994), RFLP (Berry et al. 1994; Gentzbittel et al. 1992, 1994; Zhang et al. 1995), and AFLP (Hongtrakul et al. 1997), it has not been investigated by using SSR markers. Dehmer and Friedt (1998) followed a hybridization based approach to determine the abundance of various mono-, di-, tri-, and tetra-nucleotide repeat motifs and to examine the genetic relationship among 10 inbred lines of sunflower. Two tri-nucleotide repeats (ACA and CAT) and 3 tetra-nucleotide repeats (CATA, GACA, and GATA) were found to be polymorphic. These markers showed that the relationship among North American inbred lines corresponds to expected relationship based on pedigree.

Because known sequences are a limited source of SSRs in sunflower, random or enriched genomic libraries must be developed and screened to develop large collections of SSR markers. Our objectives were to (i) develop and describe the properties of a collection of SSR markers from a random genomic DNA library of sunflower and (ii) assess the polymorphisms of SSR markers across a diverse set of inbred lines.

MATERIALS AND METHODS

Plant Material

A screening panel comprising 10 elite inbred lines of sunflower (*Helianthus annuus*), representing different heterotic groups, market and germplasm classes (Cheres et al. 1998) and 12 wild species in the genus *Helianthus* (10 from section Helianthus and 2 from section Ciliares) were examined for polymorphism. The inbred lines are parents of different mapping populations and single cross hybrids used in our project (Table 2.1).

Table 2.1. Germplasm accessions of cultivated and wild sunflower (*Helianthus*) species screened for simple sequence repeat (SSR) polymorphisms.

Accession	Species	Description
HA89	*H. annuus*	Oilseed Maintainer (B) Line
HA292	*H. annuus*	Confectionery B Line
HA370	*H. annuus*	Oilseed B Line
HA372	*H. annuus*	Oilseed B Line
HA383	*H. annuus*	Oilseed B Line
HA821	*H. annuus*	Oilseed B Line
RHA274	*H. annuus*	Oilseed Restorer (R) Line
RHA280	*H. annuus*	Confectionery R Line
RHA377	*H. annuus*	Oilseed R Line
RHA801	*H. annuus*	Oilseed R Line
10280	*H. debilis* ssp. *cucumorifolis*	Wild Species
Kermit TX 2	*H. neglectus*	Wild Species
	H. praecox ssp. *runyoni*	Wild Species
303	*H. bolanderi*	Wild Species
1474	*H. deserticola*	Wild Species
1471	*H. paradoxus*	Wild Species
1098	*H. niveus* ssp. *niveus*	Wild Species
IU-CAN	*H. niveus* ssp. *canesceas*	Wild Species
1015	*H. argophyllus*	Wild Species
Keith CO	*H. petiolaris*	Wild Species
IU-CUS	*H. cusikii*	Wild Species
IU-PUM	*H. pumilis*	Wild Species

Genomic library construction and screening

Genomic DNA was isolated from *H. annuus* L. cv. HA89 using a modified CTAB technique (Webb and Knapp 1990). DNA was digested with the restriction enzyme *Mbo*I and electrophoresed on a low-melting point agarose gel (FMC). Fragments from 200-600 base pairs were excised and isolated from the gel using a QIAquick Gel Extraction Kit (Qiagen). Selected fragments were cloned into the *Bam*HI site of Lambda ZAP Express vector following the manufacturer's specification (Stratagene). Plaques were plated at 2500 to 25000 pfu/plate and lifted onto 137mm Magna Lift filters following the manufacturer's specifications (MSI). Filters were pre-hybridized in 6X SSPE, 1% SDS, 5X Denhardt's solution at 48°C, then probed with end-labeled $(AT)_{12}$, $(AC)_{12}$, $(AAT)_7$, $(AAC)_7$, $(AAG)_7$, $(TCG)_6$, $(ACC)_6G$ oligonucleotides with polynucleotide kinase (New England Biolabs) and [gamma-^{33}P]ATP (New England Nuclear) in hybridization solution of 6X SSPE, 1% SDS at 48°C. Filters were washed in 0.1% SDS, 2X SSC at 48°C for 30 min. to 1 hour while background was monitored with a Geiger counter. Positive clones were re-plated at 100-500 pfu/plate and re-screened as above. Insert DNA was isolated by picking putative positive plaques with a toothpick and amplifying directly using T7 and T3 primers in PCR buffer with the addition of 1% tween-20. Amplified DNA was purified in a QIAquick PCR Purification Kit (Qiagen) and sent to the Center for Gene Research and Biotechnology (CGRB) at Oregon State University for sequencing.

Primer design and PCR

Primers flanking microsatellite sequences were designed using the PRIMER computer program (Lincoln et al. 1991), and chosen having target melting temperatures of 60°C, and obtained at CGRB at Oregon State University. To simplify programming and facilitate multiplex gel loading, all primers were designed with melting temperature (TM) in the range of 57° - 63°C and an

optimum of 60°C. PCR amplifications were performed in 10 or 20μL using 1X buffer as supplied by the manufacturer of *Taq* polymerase (Gibco BRL), 20 ng genomic DNA, 0.25 μM each primer, 2.5 mM $MgCl_2$, 0.125 mM each dGTP, dCTP, dTTP, 0.7 μCi [α-^{33}P], 0.0625 mM dATP, and 1 U *Taq* polymerase using a touch-down PCR protocol (Don et al. 1991): 1 cycle: 95° for 3 min., then 1 cycle: 94°C for 30 sec., 64°C for 30 sec., 72°C for 30 sec. In each subsequent cycle, annealing temperature was reduced 1°C until 55°C was reached, then amplification was continued for 30 cycles at 94°C for 30 sec., 55°C for 30 sec., 72° for 30 sec followed by 1 cycle of 72° for 10 min. The PCR products amplified from HA-370, and HA-372 genomic DNAs were assayed on agarose gels to test the efficacy of the primers. Primer pairs which successfully amplified the target sequence were then run on standard sequencing gels and scored for allele length differences as follows: 10 μl of stop solution (95% formamide, 0.05% each bromophenol blue and xylene cyanol) was added to the amplification products and 6.5 μl of each reaction was denatured for 3 min at 94°C and analyzed on a 6% denaturing polyacrylamide gel containing 7.5M urea at 60 watts constant power for 2 hr 15 min. Sequencing reactions of M13mp18 ssDNA (USB) were included on the gel as molecular weight standards. Gels were dried and exposed to x-ray film for 1-2 days, then developed in a Kodak Xomat film processor.

SSR assay for polymorphism

Genomic DNA from 22 sunflower genotypes were amplified and subsequently run on polyacrylamide sequencing gels as described above. Primer sets for final evaluation on the screening panel were selected based on the result from amplification in HA370 and HA372 described above.

Heterozygosities were estimated for each SSR using $1-\sum_{i=1}^{k} p_i^2$, where p_i is the frequency of the *i*th allele and *k* is the number of alleles (Ott, 1991). This

parameter, termed heterozygosity by Ott (1991), estimates the polymorphic information content (PIC) of a marker and ranges from 0.0 (monomophic) to 1.0 (polymorphic). The genetic distance of Rogers as modified by Wright (1978) was estimated by using the computer software NTSYS-pc (Rohlf 1993). The genetic distance matrix was subsequently used to perform non-metric multidimensional scaling (MDS, Kruskal 1964) using the MDSCALE program of NTSYS-pc. MDS coordinates were estimated for three dimensions and stress statistics computed. Cluster analysis based on an average linkage (UPGMA) was performed using the genetic distance matrix (Rohlf 1993).

RESULTS

Genomic Library Screening

We screened 897,000 plaques for the presence of AT, AC, AAT, AAC, AAG, TCG, and ACC repeats. Primary screening produced 916 positive clones (0.1%). Secondary screening of the 916 primary clones resulted in 313 (34%) positive clones. We sequenced almost all of the positive clones and found 109 (35% of sequenced) clones to contain SSR repeats. Eight clones were isolated by PCR analysis using direct sequencing of positive primary clones or by three-reaction assay of the primary clones, a modified method of Grist et al. (1993). Direct sequencing of secondary clones was not successful due to large number of false positive clones. Most of the clones containing SSRs were isolated by screening a genomic library by hybridization with oligonucleotide repeats.

Of the 2 DNRs and the 5 TNRs searched in the genomic library, (AAG) showed the highest frequency (35%) in the final 96 SSR markers followed by (AAC) (23%) and (ACC) (14%). Altogether, the TNRs accounted for 86% of the clones for which primers were designed. While two useful AC-containing clones were isolated from the genomic library, no AT-containing clones were found. The

MNRs and the DNRs accounted for 9% and 5% of the SSR markers, respectively. Three non-targeted DNR (AG/CT), and 8 MNR (A/T)n were found in the sequenced clones and utilized for designing primers. This finding suggests the abundance of poly-A and (AG/CT) repeats in the sunflower genome. Twelve redundant sequences and four sequences containing repeats close to one end of the sequences were encountered in the course of genomic library screening, hence reducing the proportion of useful markers.

Testing of SSR primer sets

All 120 SSR primer sets were tested on 1% agarose gel for PCR amplification of the expected product size on the reference allele (HA89). Eight SSRs failed to amplify any product whereas 5 produced faint products. Included were 15 previously published SSR markers (Brunel, 1994; Whitton et al. 1997) of which 5 failed to amplify under our optimal PCR conditions.

We tested primers for 105 of the new SSR markers (ORS-1 to ORS-105) on denaturing polyacrylamide gel using two inbred line parents of a mapping population – HA370 and HA372. Seventy-seven SSRs (73%) showed scorable band pattern and, hence, were selected for diversity analysis of 22 sunflower genotypes. Twenty-eight primer pairs (27%) produced either no product or an unresolvable banding pattern. Ten of the 15 previously described SSRs were polymorphic. Therefore, 28 ORS SSRs and 5 of the fifteen SSRs obtained from other labs, a total of 33, were excluded from further assays.

Screening of primers for variability

The 77 SSR markers selected based on prescreening testing, along with 10 primer sets obtained from other labs, were included in the final screening. Ten sunflower inbred lines and 12 wild species of *Helianthus* were profiled using

these 87 SSR markers. Forty-four (37%) markers showed polymorphic and easily scorable band whereas 76 (63%) markers failed. Twenty-seven of the failed primers showed stutter bands whereas eleven markers (9%) were monomorphic. The list of 44 SSR markers and their primer sequences is presented in Table 2.2. We used the 44 unambiguously scored SSR markers to determine number of alleles, heterozygosity and diversity pattern. The twenty-seven stuttering SSRs showed polymorphism, particularly in the wild species, but the pattern of the bands was complex and difficult to score unequivocally. Scoring was complicated due to stuttering, gene duplication, and heterozygosity of the wild species. Some of the monomorphic markers produced easily scorable single bands. These monomorphic markers may show polymorphism in other *Helianthus* species not included in the present study.

Genetic Diversity and number of alleles

Testing of the 44 SSRs for variability in the 10 elite inbred lines produced an average of 1.86 alleles per locus and mean heterozygosity of 0.21. The number of alleles ranged from 1 to 5. ORS-8 (a dinucleotide) was the most polymorphic with 5 alleles and a heterozygosity of 0.76. This marker was the second most polymorphic with 7 alleles and 0.71 heterozygosity in the previous study performed on 28 elite lines (unpublished data). Twenty-one markers (48%) were monomorphic among elite lines (a single allele) whereas 12 (27%) SSRs had 2 alleles, 8 (18%) SSRs had 3 alleles, 2 (5%) SSRs had 4 alleles each. ORS-78 and ORS-31 (both trinucleotides) had the second (0.70) and the third (0.67) highest heterozygosity. Eight SSRs (18%) showed heterozygosity ranging from 0.41 to 0.60, whereas 5 (11%) SSRs showed heterozygosity of 0.21 to 0.4.

Heterozygosity ranged from 0.18 to 0.76 with a mean of 0.35 for dinucleotide SSRs and 0 to 0.7 with a mean of 0.17 for trinucleotide SSRs. The number of alleles ranged from 2 to 5 (mean=2.7) and 1 to 4 (mean=1.7) for dinucleotide and trinucleotide SSRs, respectively. The two mononucleotide SSRs, ORS-53 and

ORS-57, showed an average of 3 alleles per locus and 0.61 mean heterozygosity. IUB5, a hexanucleotide SSR that showed a single allele in several species (Whitton et al. 1997), was monomorphic in this study. Polymorphisms of all classes of SSRs (mono, di, and tri) were higher in wild species than in elite lines. Dinucleotide SSRs showed more alleles per locus and higher heterozygosity than trinucleotide SSRs. In wild species, 41 out of 44 markers were polymorphic. Seventeen (39%) had 2-4 alleles, 22 (50%) had 5-10 alleles, and 2 (4%) had 11-12 alleles per locus. Nineteen SSRs (43%) showed high heterozygosity, ranging from 0.76 to 0.92. Seventy-four percent of these highly polymorphic SSRs contained perfect or imperfect trinucleotide repeats. Also, three of the six dinucleotide SSRs were among these highly polymorphic markers. Eleven showed heterozygosity ranging from 0.51 to 0.75, nine showed 0.26 to 0.50, five showed 0-0.25.

The screening panel in this study comprises 10 elite lines that are commonly used as parents of mapping populations in our breeding program. Seven of the 10 inbred lines are the parents of four mapping populations (Table 2.1). A total of 23 SSR markers showed polymorphism in at least two inbred lines. Nine markers showed co-dominant polymorphism between HA370 and HA372, four between HA383 and HA372, six between HA377 and RHA274, eight between RHA280 and RHA801. In addition, there were 1-4 dominant polymorphic markers in these pairs of parents. Four markers (ORS-8, ORS-13, ORS-31 and ORS-90) were polymorphic in three pairs and 7 markers (ORS-10, ORS-16, ORS-31, ORS-34, ORS-53, ORS-70, and ORS-78) were polymorphic in two pairs of mapping population parents.

Table 2.2 SSR marker names, repeat classes, length of the reference allele (HA89), and oligonucleotide primer sequences for amplifying SSRs and sequences flanking SSRs for 44 SSR markers.

Name	Repeat	Length	Left Primer	Right Primer
ORS-1	$(ta)_{11}$	178	cacttctcacactttgggca	ccaaataattaccatcatgcca
ORS-2	$(ta)_{11}$	169	ttcacgcagttcactcttgg	tatttgatcaaaagcatgggc
ORS-3	$(agt)_6$	225	agaactggcagcttggaaaa	gtccaaatggtggaaaactacc
ORS-4	$(agg)_6$	148	acaagtcggctggtgagc	acatgaaacacgagctaaacc
ORS-5	$(aac)_4$	311	atgtggagcagcaaattcag	ctgctgcccaccatactg
ORS-6	$(agg)_4$	237	gtggagagaggtgtagagagca	cacccctcaccctgacac
ORS-7	$(tca)_4$	262	ccctcactaccgtgtggtg	ttgaaagagacgaagcgaaa
ORS-8	$(ct)_8$	195	ttggatcgattgatgattgttg	gaatccgtcatgtataaaacga
ORS-10	$(aat)_9$	237	ctccaccacacaatctccg	tgtggagtttgccagaactg
ORS-11	$(gag)_4$	247	gggttatattgccgggatg	tcgtcaagctggttctcatg
ORS-12	$(aat)_7$	138	gctttgagaaaccgcttcac	ttgatatggtaactaacgaacacaa
ORS-13	$(att)_9$	212	gaataaccttgtggagtttgcc	cctcattctcattctctccacc
ORS-16	$(aat)_9$	138	gaggaaataaatctccgattca	gcaaggactgcaatttaggg
ORS-21	$(ttg)_4$	237	ttcaccttgaccaatcacga	tgcaaaggattgcagaagg
ORS-26	$(ttg)_4$	188	gacccagcacgaaagcac	gggctgaaggtcatttcaaa
ORS-27	$(ctt)_{10}$	110	atcatggcacaatcacagaca	aaaccactgtctgacccctg
ORS-30	$(ctt)_9$	184	tttctcagcttcagcttcagc	ggccaaagagatgtccaaaa
ORS-31	$(aag)_{10}$	286	aattcatgccccaagagatg	cacaattcatgcatttctctgg
ORS-32	$(aat)_3n(aat)_3$	150	agttggtttgtttcttgcagc	aaccccacaacctgtttcat
ORS-34	$(ttg)_5$	105	ttgttgatgagtttggagatgg	aagaagctaccagaacaatggc
ORS-35	$(ctt)_6$	200	ttttctcagcttcagtttcagc	tttggatcaagaactcatggc
ORS-36	$(gtt)_3$	236	ggcaaaaagaatggtgaaatg	caagcctttgtgatgaacca
ORS-45	$(ctt)_7$	203	agaacgtatctatcacgtgcct	gatattgagcctgacactcacc

Table 2.2 Continued.

Name	Repeat	Length	Left Primer	Right Primer
ORS-46	$(cga)_5$	182	gccatatgatcatggaattgg	tgaattcgacatttgaatttgg
ORS-53	$(t)_{30}$	450	gctggcaatttctgatacacgat	catctagacaacgacagaagatg
ORS-54	$(ttc)_6$	139	tgttgctggagctgtttttg	cgataagcgagtcaagtctgg
ORS-57	$(t)_{31}$	250	ttccatttccaccattttgg	cattcatggcctaaaaggttc
ORS-59	$(tcg)_4n(ag)_6$	167	gcttcgtagctttcttcttcgc	aagtagcttcgcaagtattcgg
ORS-60	$(ttg)_4n(ttg)_4$	379	tagcacaaaagcacgaccc	ccaggggcagagtggttata
ORS-65	$(aat)_{12}$	204	tctcttattttgtgcatcgtgg	gccttgtgcggaattaaaaa
ORS-66	$(aag)_6$	154	tcaaaggaaaaagaaggcga	gtcatcttcaccaccttctgg
ORS-70	$(ctt)_9$	126	gaccctggtcaccgaagtta	atctgaaatcggacaagattca
ORS-75	$(aac)_8$	250	tcgatgtaatttgcaccatagc	ttctgattttggttcagctgg
ORS-76	$(ctt)_7$	261	cttacggggttagcatcttcc	cgtgactagtcttcttctccgg
ORS-78	$(aag)_{10}$	161	gttcgtcgagtacatgttctgc	tttccctctggaaagttgtca
ORS-79	$(gtt)_7n_6(gtt)_4$	329	tcactgcatctcctcgacc	tggccagtgtaataaggttcg
ORS-85	$(aac)_5$	396	gtcagcaatcctctggtggt	tagcacaaaagcaggaccac
ORS-90	$(ac)_8$	125	ggatttcgcgtgattgttg	agcagttacgagtgtgtgtgtg
ORS-102	$(ctt)_7$	149	aacggcggttttgaataatg	gaaatatag$(a)_5$gaagat$(aag)_3$aa
ORS-103	$(ag)_7$	138	tgttggtgtgatggtggc	ccatgatgacactaggttggg
OBS-3	N/A	N/A	caccaccaagaaggctaacgact	tggccacacctgaacaagaaat
OBS-4	$(tat)_n$	N/A	tgaagcccatgaaagcagt	atggtataacaagtaaaaagtaag
IUB-5	$(tgaaaa)_n$	116	cctgaaagccagtgaatc	tcaagatgagacttgattgaa
IUB-6	$(gt)_n$	360	tcggtatcgtttgctaatgg	ggtaactctaaagctctgtc

Comparison of polymorphisms of MNR, DNR, and TNR

Only two MNRs (20%) detected polymorphism among the screened lines. ORS-53, composed of (t) 30, was developed from the SAD17 gene of sunflower (Hongtrakul et al. 1998). In the diversity analysis of 8 inbred lines, ORS-53 showed three alleles and 0.59 heterozygosity (Hongtrakul et al.1998). ORS-57 was isolated from genomic library while searching for other di- and tri-nucleotide repeats. Both MNRs revealed 3 alleles each in elite inbred lines in the present study. ORS-53 exhibited 5 alleles in exotic lines whereas ORS-57 could not show any product in exotic lines. Out of the 16 DNR-containing markers, 6 (38%) showed scorable polymorphism, 4 showed stutter bands that were difficult to score, and six were unclear or failed but none of the DNRs were monomorphic. The main focus of this experiment was to isolate TNRs. Therefore, the majority of the polymorphic SSRs (77%) belonged to this group. IUB5 (a hexanucleotide SSR) showed polymorphism in exotic germplasm whereas OBS-3 showed polymorphism in both elite and exotic lines.

The mean number of alleles for MNRs, DNRs, and TNRs were 4, 8.7 and 5.7, respectively. The mean heterozygosities were 0.67, 0.74, and 0.56, respectively. DNRs showed the highest mean number of alleles per locus and mean heterozygosity. Heterozygosity ranged from 0 to 0.92 whereas the number of alleles per locus ranged from 1-13 (Table 2.3). In the previous study ORS-2 had 14 alleles (unpublished data) compared to only 2 alleles in the present study. ORS-5, on the other hand, exhibited moderate polymorphism (0.29) and three alleles among elite lines in a previous study (unpublished data). Almost all TNRs showed higher rate of polymorphism in wild germplasm than in elite lines (Table 2.3). Eleven TNR markers were not polymorphic in elite lines but were polymorphic in wild species.

The number of repeats for tri-nucleotide markers in the reference allele ranged from 3 to 12 with a mean of 7.5. Among TNRs the maximum total heterozygosity was displayed by ORS-31 (0.85; 10 repeats) followed by ORS-78 (0.83; 10 repeats), ORS-12 (0.82; 7 repeats), and ORS-7 (0.82; 4 repeats). No

correlation was found between the repeat length of microsatellite markers and the number of alleles (r=0.08) or heterozygosity (r=0.25). In general, the TNRs that exhibited total heterozygosity of 0.7 or greater and 7 or more alleles per locus comprise a repeat length ranging from 4 to 12. Furthermore, different TNRs with the same repeat length showed a range of alleles per locus and total heterozygosity (Table 2.3). For instance, the number of alleles and heterozygosity for TNRs with 4 tandem repeats ranged from 3 to 13 and 0.36 to 0.82, respectively. ORS-36 had the shortest length and showed low number of alleles and heterozygosity. Five DNR markers had repeat lengths ranging from 7 to 11. The maximum number of alleles was detected by ORS-2 (11 repeats) and ORS-8 (8 dinucleotide repeats). ORS-8 also showed the maximum total heterozygosity (0.82) among DNRs.

Cluster analysis and Patterns of Diversity

Genetic distance estimated based on 44 markers tested on all 22 genotypes ranged from 0.34 to 0.84. Among elite lines, the distance estimated based on 23 polymorphic markers varied from 0.51 to 0.88 whereas in the wild species 41 polymorphic markers detected a genetic distance of 0.56 to 0.83.

Cluster analysis (Figure 2.1), based on average linkage, revealed sub-clustering of elite inbred lines and wild species but no clear separation of maintainer (B lines) and restorer (R) lines as reported previously with coancestry (Cheres et al. 1998) and AFLP (Hongtrakul et al. 1997). One cluster comprises 3 lines each from oilseed B-line (HA383), confectionery B-line (HA292), and confectionery R-line (RHA280). RHA801 was subclustered with HA370. Three of the oilseed B-lines (HA89, HA821, and HA372) were grouped together.

Table 2.3. Number of repeats, size of reference allele, number of alleles and heterozygosity of 44 SSR markers in 10 inbred lines of *Helianthus annuus* and 12 wild *Helianthus* species.

Locus	Repeat	Ref Allele length (bp)	Number of Alleles			Heterozygosity		
			Elite	Wild	Total	Elite	Wild	Total
ORS-1	$(ta)_{11}$	178	3	9	11	0.34	0.86	0.79
ORS-2	$(ta)_{11}$	169	2	12	13	0.18	0.92	0.77
ORS-3	$(agt)_6$	225	1	3	3	0.00	0.50	0.46
ORS-4	$(agg)_6$	148	3	6	8	0.34	0.81	0.69
ORS-5	$(aac)_4$	311	2	12	13	0.18	0.92	0.77
ORS-6	$(agg)_4$	237	1	4	4	0.00	0.62	0.36
ORS-7	$(tca)_4$	262	2	6	8	0.44	0.80	0.82
ORS-8	$(ct)_8$	195	5	9	13	0.76	0.83	0.89
ORS-10	$(aat)_9$	237	2	6	7	0.44	0.79	0.75
ORS-11	$(gag)_4$	247	1	3	3	0.00	0.63	0.46
ORS-12	$(aat)_7$	138	3	6	9	0.37	0.81	0.82
ORS-13	$(att)_9$	212	2	5	5	0.49	0.72	0.63
ORS-16	$(aat)_9$	138	3	2	4	0.46	0.50	0.51
ORS-21	$(ttg)_4$	237	1	9	10	0.00	0.88	0.74
ORS-26	$(ttg)_4$	188	1	6	6	0.00	0.90	0.66
ORS-27	$(ctt)_{10}$	110	1	5	5	0.00	0.56	0.34
ORS-30	$(ctt)_9$	184	1	2	3	0.00	0.22	0.54
ORS-31	$(aag)_{10}$	286	4	6	8	0.67	0.78	0.85
ORS-32	$(aat)_3n(aat)_3$	150	2	5	6	0.18	0.80	0.60
ORS-34	$(ttg)_n$	105	2	2	3	0.42	0.28	0.38
ORS-35	$(ctt)_6$	200	1	4	4	0.00	0.61	0.39
ORS-36	$(gtt)_3$	236	1	2	3	0.00	0.29	0.17
ORS-45	$(ctt)_7$	203	1	5	5	0.00	0.74	0.51
ORS-46	$(cga)_5$	182	1	3	3	0.00	0.50	0.23
ORS-53	$(t)_{30}$	450	3	5	5	0.62	0.79	0.74
ORS-54	$(ttc)_6$	139	1	4	4	0.00	0.63	0.52
ORS-57	$(t)_n$	250	3	-	3	0.59	-	0.59
ORS-59	$(tcg)_4n(ag)_6$	167	1	10	11	0.00	0.89	0.74
ORS-60	$(ttg)_4n(ttg)_4$	379	2	7	8	0.18	0.71	0.72
ORS-65	$(aat)_{12}$	204	1	6	7	0.00	0.78	0.70
ORS-66	$(aag)_6$	154	3	1	4	0.57	0.00	0.73
ORS-70	$(ctt)_9$	126	3	8	10	0.46	0.84	0.80
ORS-75	$(aac)_8$	250	1	2	2	0.00	0.44	0.30
ORS-76	$(ctt)_7$	261	1	2	2	0.00	0.44	0.46
ORS-78	$(aag)_{10}$	161	4	7	8	0.70	0.84	0.83

Table 2.3 Continued

Locus	Repeat	Ref Allele length (bp)	Number of Alleles			Heterozygosity		
			Elite	Wild	Total	Elite	Wild	Total
ORS-79	$(gtt)_7n_6(gtt)_4$	329	1	2	2	0.00	0.49	0.39
ORS-85	$(aac)_5$	396	1	2	2	0.00	0.50	0.24
ORS-90	$(ac)_8$	125	2	4	5	0.32	0.52	0.69
ORS-102	$(ctt)_7$	149	1	8	9	0.00	0.86	0.73
ORS-103	$(ag)_7$	138	2	1	3	0.18	0.44	0.61
OBS-3	N/A	N/A	2	7	8	0.18	0.81	0.70
OBS-4	$(tat)_n$	N/A	1	4	4	0.00	0.61	0.31
IUB-5	$(tgaaaa)_n$	116	1	2	2	0.00	0.17	0.09
IUB-6	$(gt)_n$	360	2	6	7	0.32	0.66	0.74
Mean			1.86	5.12	6.0	0.21	0.64	0.59

Among wild species *H. bolanderi* and *H. deserticola* were separated from the rest with *H. bolanderi* being the farthest. *H. cusikii* and *H. pumilis* (section Ciliares) were clustered together. *H. debilis* was closer to the two members of section Ciliares than to Helianthus section. *H. niveus* ssp *niveus* was closer to *H. praecox* than to *H. niveus* ssp *canesceas*.

Figure 2.2 shows a two-dimensional presentation of the genetic distance. The MDS algorithm (Rohlf 1993) converged with a final stress of 0.088 when three dimensions were estimated using genetic distance estimated based on 44 SSRs among 22 genotypes. This level of stress suggests the fit in the 3 dimension was excellent (Kruscal 1964). The first and the second as well as the first and the third coordinates separated the genotypes into elite and wild (Figure 2.2). Within elite lines RHA280 and HA292, both confectionery types were separated from other elite lines. Plots of coordinate 1 and 2 performed only among elite lines (data not shown) placed HA383 (oilseed B-line) close to HA292 (confectionery B-line). HA89 was separated from other elite lines.

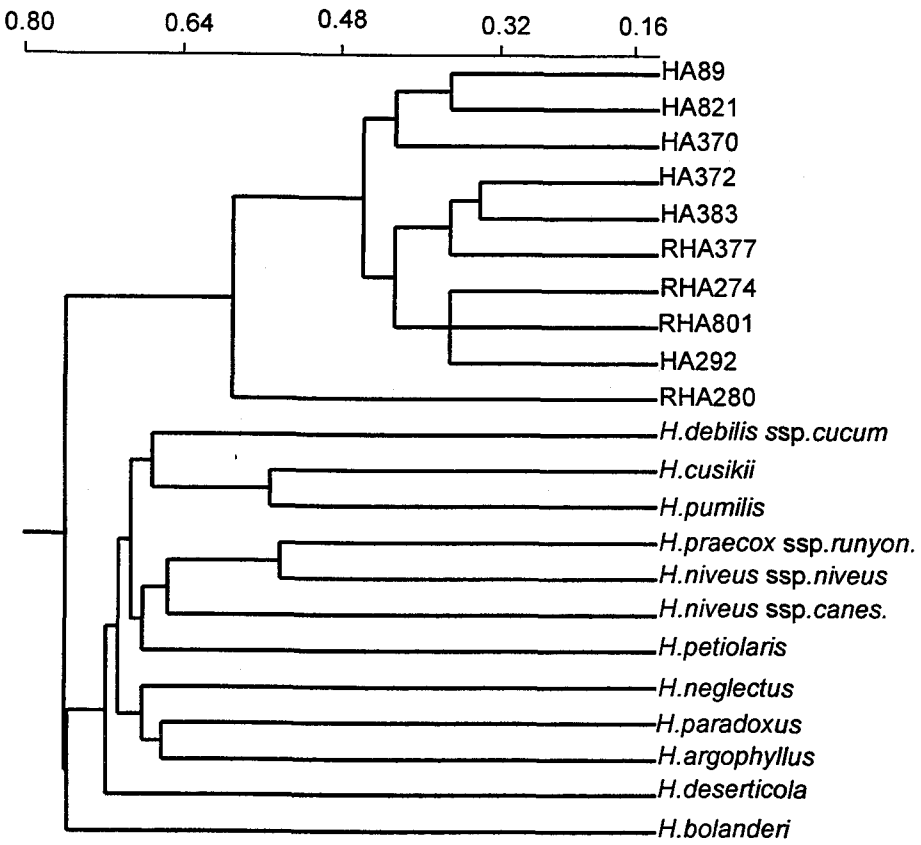

Figure 2.1. A dendrogram produced by UPGMA clustering of Rogers-W genetic distance among ten elite inbred lines of sunflower and twelve wild species.

Three of the four R-lines (except RHA280) were clustered with the B-line. Consistent with the cluster analysis, MDS showed that *H. bolanderi* and *H. debilis* were separated from the other wild species. *H. cusikii and H. pumilis* were clustered. One sub-cluster comprises *H. neglectus*, *H. argophyllus*, and *H. paradoxus* whereas the other sub-cluster comprises *H. niveus* ssp *niveus* and *H. deserticola* and this sub-cluster was close to another sub-cluster comprising *H. petiolaris* and *H. niveus* ssp *canesceas*.

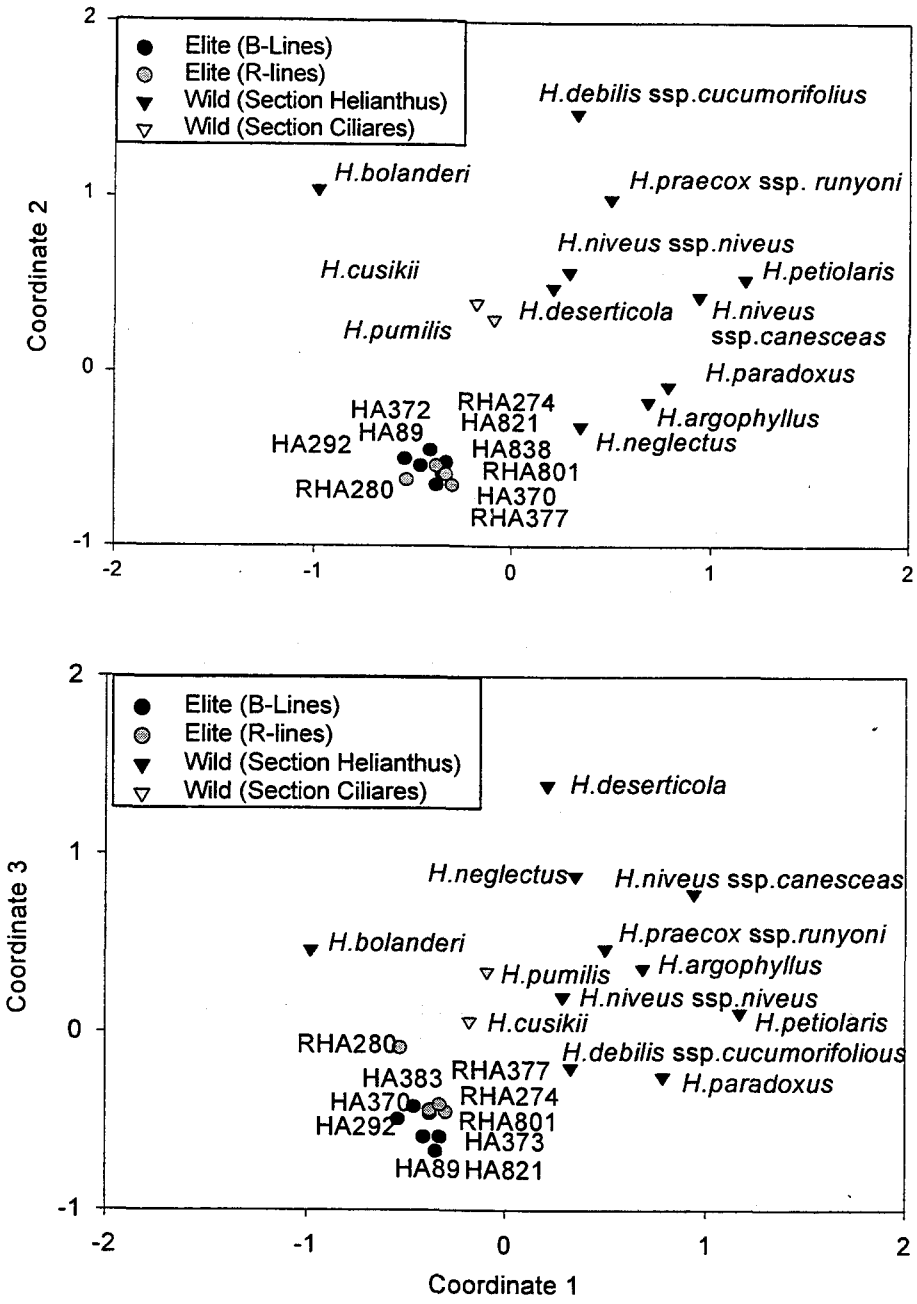

Figure 2.2. First and second (panel A), first and third (panel B) multidimensional scaling coordinates for 22 sunflower genotypes (ten elite oilseed and confectionery female (B) and male (R) inbred lines, and 12 wild *Helianthus* species.

DISCUSSION

Screening of approximately 897000 genomic clones yielded only 0.1% putatively positive primary clones which, in turn, produced 34% positive clones in the rescreening step. Screening of a large number of genomic or cDNA libraries gave very low numbers of useful markers in previous studies in sunflower (Whitton et al. 1997) and other species (Chen et al. 1997; Struss and Plieske 1998). Among the five TNRs targeted for screening, (AAG)n showed the highest frequency followed by (AAC)n and (ACC)n. The TNR repeat (AAT)n was not abundant as reported by Wang et al. (1994). However, (AAC)n was the second most frequent TNR both in our study and a previous study (Wang et al. 1994). The most frequent TNRs in the survey made by Morgante and Olivieri (1993) were TAT and TCT. The abundance of repeat motifs could be species specific as suggested by Dehmer and Friedt (1998), or it may depend on the type of the library screened. Different types of libraries (Chen et al. 1997; McCouch et al. 1997; Roder et al. 1998a) represent different regions of a genome. Presumably, representation of the genome in the library may have a bearing on the difference in abundance of repeat motifs in different plant species (Wang et al. 1994).

Among DNRs, AG and AC repeats are known to be very abundant in plant genomes, the most frequent being AT (Wang et al. 1994; Morgante and Olivieri 1993; Wu and Tanksley 1993; Condit and Hubbell 1991; Bryan et al. 1997). In agreement with previous studies, we found 3 non-targeted (AG) repeats suggesting the abundance of this class of repeats in sunflower. In addition, poly-A (or poly-T) repeats showed up randomly in our sequenced clones confirming the abundance of poly-A repeats in plants (Morgante and Olivieri 1993; Wang et al. 1994). Markers based on poly-T repeats found in the sunflower SAD gene proved very polymorphic, distinguishing between closely related elite inbred lines (Hongtrakul et al. 1998). The repeats poly-A, poly-GA and poly-CA were abundant whereas poly-GCG was rare in the study of Dehmer and Friedt (1998) in sunflower.

Testing of 120 primer sets for variability in 22 sunflower genotypes resulted in 44 useful SSR markers and 27 markers with complex banding patterns. Scoring for the 27 markers was complicated by stutter or multiple bands and products with larger than expected size. Some of these unresolvable markers showed polymorphism between elite lines and wild species as well as among wild species. Therefore, resolution of these markers can be improved by optimizing the PCR condition for each primer individually (Whitton et al. 1997) or by redesigning the primers (Smith et al. 1997). Bryan et al. (1997) suggested the use of different primers for each microsatellite sequence in order to produce unequivocally scorable bands of expected size. In the present study, scoring of the bands was complicated due to duplication of loci, the occurrence of stutter bands, and the heterozygosity of the wild species. Stutter bands result from non-template nucleotide addition to the ends of PCR products resulting in doubled bands (Clarke 1988). It was demonstrated that the tendency of DNA polymerase to add a non-template nucleotide depends on the terminal nucleotide present in the reverse primer (Xu 1993). Reverse primers designed to contain adenine residue in the terminal position helps to eliminate addition of non-template dATP, and hence double bands (Magnuson et al. 1996). Stutter bands could reduce the utility of DNR microsatellite for molecular marker based applications in plant improvement (Smith et al. 1997; Bryan et al. 1997). Larger-than-expected alleles could occur due to the presence of random sequence similar to the SSR primer sequences somewhere in the genome as hypothesized by Senior and Heun (1993). Multiple bands were observed in this study suggesting duplication of SSR loci. Mapping of simple sequence length polymorphisms (SSLPs) in other crop species revealed duplicated loci. In rice several SSRs detected two loci that mapped to different chromosomes (Chen et al. 1997; McCouch et al. 1997). Bryan et al. (1997) found that 14 out of 49 (29%) of the SSRs mapped detected more than one loci in wheat.

We found 12 redundant sequences accounting for 11% of the repeat-containing clones. Redundant sequences are responsible for reduced efficiency in the development of SSR markers. Chen et al. (1997) observed high level of

redundancy (2-4 copies) from enzyme digested library as compared to physically sheared library. Redundancy can partly be explained by the fact that a size-selected genomic library may represent only a subset of DNA fragments in the genome (Chen et al. 1997). Nearly one-third of the SSR primer sets failed to amplify any product or resulted in smear and spurious banding pattern. Screening for SSR repeats by hybridization produces a high level of false positives or repeat-containing clones that are not suitable for designing a primer. The use of pre-sequencing screening, based on PCR, helps to confirm the presence of repeats and to increase the proportion of useful (polymorphic) markers (Senior and Heun 1993; Chen et al. 1997).

Mapping of a large number of SSR markers demonstrated an even distribution and genome wide coverage (Akkaya et al. 1992; Cho et al. 1998; Roder et al. 1998a). Roder et al. (1998b) performed physical mapping of 31 microsatellites and observed that the markers were distributed evenly along the chromosomes. Wang et al. (1994) suggested the use of a mixture of different repeat classes in order to ensure whole genome coverage as some types of repeats were found to be more frequent in the non-coding region than others.

Genetic diversity and Number of alleles

SSRs were shown to be more polymorphic than RFLPs in soybean (Akkaya et al. 1992; Rongwen et al. 1995), bread wheat (Plaschke et al. 1995; Roder et al. 1995), rice (Wu and Tanksley 1993; Olufowote et al. 1997), barley (Saghai Maroof et al. 1994), and rapeseed (Charters et al. 1996; Kresovich et al. 1995). However, in maize, the amount of polymorphism exhibited by SSRs was similar to that exhibited by RFLP (Smith et al. 1997). In the present study, the level of genetic diversity in both elite lines and wild species was less than the genetic diversity detected by RFLP in sunflower (Berry et al. 1994; Gentzbittel et al. 1994; Zhang et al. 1995). Among the different repeat classes, DNRs had the highest mean heterozygosity (0.35). This level of heterozygosity was less than

the mean heterozygosity for DNRs in the previous study (unpublished data). MNR markers showed relatively smaller mean number of alleles per locus but their variability was greater than both DNR and TNR markers. Dinucleotide SSRs exhibit high level of polymorphism but their practical utility is reduced due to stuttering and difficulty of scoring (Smith et al. 1997). DNRs showed higher level of polymorphism and number of alleles than TNRs in barley (Saghai Maroof et al. 1994; Struss and Plieske 1998), rice (Akagi et al. 1997; Olufowote et al. 1997), wheat (Plaschke et al. 1995), maize (Smith et al. 1997), and soybean (Rongwen et al. 1995).

In the present study, TNRs showed less polymorphism and number of alleles than DNRs and MNRs. The level of genetic diversity detected by TNRs in the present study (0.17) was significantly less than trinucleotide SSRs in maize (0.53) (Smith et al. 1997) and in wheat (0.51) (Bryan et al. 1997). Rongwen et al. (1995) found relatively high level of genetic diversity (0.5 to 0.8) for trinucleotide microsatellites evaluated in 96 soybean genotypes. Considering the occurrence of stutter and difficulty of detecting polymorphism (scoring) with DNRs, TNRs show promise as genetic markers for molecular breeding in sunflower.

Cluster analysis and patterns of diversity

Convergence of the MDS algorithm (Rohlf 1993) with a final stress of 0.088 when three dimensions were estimated suggested that the fit in the 3 dimension was excellent (Kruscal 1964). This level of final stress was better than the final stress achieved previously in sunflower using SSRs (unpublished data). Unlike the previous studies where maintainer (B) and restorer (R) lines were separated, there was no clear separation of maintainer and restorer lines in the present study. Even though genetic diversity studies based on enzymatic analysis showed low diversity among cultivated sunflower (Cronn et al. 1997; Rieseberg and Seiler 1990), studies based on SSRs (unpublished data), RFLPs (Berry et al. 1994; Gentzbittel et al. 1994; Zhang et al. 1995), and AFLPs (Hongtrakul et al.

1997) were able to separate cultivated sunflower into maintainer and restorer lines.

Both cluster analysis and principal coordinates clearly separated elite lines from wild species. Among wild species, separation of *H. bolanderi* from the rest of the wild species and clustering together of *H. argophyllus* with *H. paradoxus* and *H. cusikii* with *H. pumilis* (members of section Ciliares) is in agreement with the previous study based on RFLP (Gentzbittel et al. 1992). However, neither *H. petiolaris* and *H. neglectus* nor *H. debilis* and *H. praecox* were sub-clustered as reported (Gentzbittel et al. 1992). Isozymes and chloroplast DNA (Rieseberg and Seiler 1990) and RAPD (Arias and Rieseberg 1995) analysis showed high degree of genetic similarity between wild and domesticated sunflower. However, allozyme study on variability among domesticated and cultivated *H. annuus* showed distinct separation between cultivated and wild *H. annuus* and between *H. annuus* and two wild species, *H. argohyllus* and *H. petioralis* (Cronn et al. 1997).

Application of SSR to sunflower breeding

Twenty-three markers were polymorphic among elite inbred lines whereas 41 markers were polymorphic among wild species. The 10 elite inbred lines represent parents of four different mapping populations. There were 4 to 8 polymorphic markers in each population. The number of mappable SSRs can be maximized by using different populations as has been done in maize (Senior et al. 1996). SSRs in this study showed low polymorphism among elite lines. The polymorphism exhibited between elite lines and wild species could be exploited for analysis of interspecific crosses and marker-assisted introgression of desirable genes within the genus *Helianthus* (Skoric 1993). Even though SSR primer sequences were not conserved across the Asteraceae, primers were found that amplify homologous fragments from within the tribe Heliantheae (Whitton et al. 1997). Conservation of SSR primer sequences across species

(Kresovich et al. 1995; Rongwen et al. 1995; Thomas and Scott 1993; Wu and Tanksley 1993) and even across genera (Roder et al. 1995) have been documented in other crops. Wild species of sunflower are sources of many important genes governing important agronomic and quality traits. Interspecific hybridization provides an opportunity to broaden the narrow genetic base of cultivated sunflower and introgression of useful genes such as disease resistance genes (Skoric 1993). Introgression of important genes could be facilitated by using molecular markers. SSRs were demonstrated to be very suitable for marker-assisted introgression due to their convenience and efficiency for assaying large populations (Ribaut et al. 1997). The SSR markers developed in this study provide the ease and convenience of applying microsatellites to sunflower genetics and breeding. Detailed information on sunflower SSR markers including primer sequences and other details will be made available on the Internet.

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CHAPTER 3

An Integrated RFLP-AFLP Linkage Map for Cultivated Sunflower

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ABSTRACT

Genetic maps of sunflower (*Helianthus annuus* L.) have been developed using a variety of molecular markers but with few or no common markers across maps. This has impeded integrating knowledge gained from different mapping projects. Our aim was to develop an integrated genetic map of sunflower using amplified fragment length polymorphism (AFLP) markers and two sets of previously mapped RFLP markers. The map was built using 180 HA370 x HA372 F_2 progeny. HA370 and HA372 genomic DNA samples were screened for AFLPs using 42 AFLP primer combinations and for RFLPs using 131 probes and three restriction enzyme (*EcoRI*, *EcoRV*, and *HindIII*) digests. Eight-two percent of the RFLP markers were polymorphic between HA370 and HA372. The AFLP assays produced 1,547 bands, of which 306 (20%) were polymorphic between HA370 and HA372. Four hundred and one loci (105 RFLP and 296 AFLP loci) were mapped into 17 linkage groups covering 1326 cM with a mean spacing of 3.3 cM between markers. RFLP markers were well distributed in 14 of the 17 linkage groups, integrated 14 linkage groups from two previously published maps, and had a mean spacing of 9.2 cM.

INTRODUCTION

Several molecular genetic maps have been developed for cultivated sunflower (*H. annuus* L.) (Knapp et al. 1999). The first was a RAPD map developed by Rieseberg et al. (1993) from an interspecific (*H. annuus* x *H. anomalus*) cross. The number and density of molecular markers on the genetic map has steadily increased since this map was published. Three groups produced RFLP maps using a variety of proprietary RFLP probes (Berry et al., 1995, 1996; 1997; Gentzbittel et al. 1995; Jan et al. 1998). The map developed by Berry et al. (1997) has the highest density of RFLP markers. The Berry et al. (1997) map has a mean density of 2.3 cM and integrated 635 RFLP loci from

nine proprietary F_2 maps. Although more than 1100 RFLP markers have been mapped in cultivated sunflower, only 81 cDNAs from the map of Berry et al. (1996, 1997) have been shared with the public sector and an integrated public map has not been developed.

The development of genetic maps has been greatly aided by the development of amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995; Zabeau and Vos 1993). AFLPs have high multiplex ratios (Russell et al. 1997; Powell et al. 1996) and require no prior sequence data. They are powerful tools for rapidly increasing the density of a map and can be assayed using universal primer sets. AFLPs have been added to the RFLP maps of several crop plants, e.g., barley (*Hordeum vulgare* L.) (Becker et al. 1995), soybean (Keim et al. 1997), rice (Maheswaran et al. 1997), and sunflower (Peerbolte and Peleman 1996), and have been used in a multitude of mapping and map-based cloning projects. Several proprietary AFLP maps have been developed and integrated for sunflower (Peleman and Peerbolte, personal communication).

The development of a dense public map is needed for sunflower. Such maps have been developed and have become powerful tools for genome analysis in most economically important crop plants, e.g., rice (*Oryza sativa* L.) (Harushima et al. 1998), barley (*Hordeum vulgare* L.) (Qi et al. 1998), soybean (*Glycine max* L.) (Keim et al. 1997), and tomato (*Lycopersicon esculentum* L.) (Tanksley et al. 1992). Without a dense public map comprised of locus specific markers, e.g., RFLPs and simple sequence repeats (SSRs), the results of genome and molecular breeding analyses performed in different laboratories cannot be integrated. Our aim was to develop an integrated genetic map of sunflower using AFLPs and two sets of RFLP markers.

MATERIALS AND METHODS

Mapping Population and DNA Extraction

F_2 progeny were produced from a cross between two sterility maintainer lines, HA370 and HA372 (Miller and Gulya 1990). The cross was produced in the greenhouse using pollen from one HA372 plant and one manually emasculated HA370 plant. One hundred and eighty F_2 progeny were grown in a field nursery at Balcarce, Argentina in 1995-96. Leaves were harvested, lyophilized, and ground to a fine powder. F_3 seed was produced from each F_2 by bagging each inflorescence with an insect-proof sack and separately harvesting each head. A variety of protocols were used to extract DNA from the lyophilized leaf samples. The DNA for mapping the ZVG RFLP probes was extracted as described by Berry et al. (1995). The protocols of Webb and Knapp (1990) and Jan et al. (1998) were used to extract DNA for mapping the USDA-BGS (UB) RFLP probes.

RFLP and AFLP Marker Analyses

Eighty-four cDNA probes from the genetic maps of Berry et al. (1995; 1996; 1997) were screened for RFLPs between HA370 and HA372 using three restriction enzyme (*EcoRI*, *Hind*III, and *EcoR*V) digests. The 84 cDNAs comprised 81 publicly released cDNAs (Berry et al. 1996) and three proprietary cDNAs (Berry et al. 1997) from each linkage group (x = 17). The polymorphic probes (56 total) were assayed for RFLPs on 180 F_2 progeny. The screening and mapping assays were done using ^{32}P as per Berry et al. (1995) and the autoradiographs were manually scored.

One hundred and thirty proprietary cDNA probes from the genetic map of Jan et al. (1998) and a cDNA for a Δ12 oleate desaturase (OLD-7) (Hongtrakul et al. 1998) were screened for RFLPs between HA370 and HA372 using three restriction

enzyme (*EcoR*I, *Hind*III, and *EcoR*V) digests. The polymorphic probes (46 total) were assayed for RFLPs on 92 F_2 progeny. The screening and mapping assays were done using ^{32}P as per Jan et al. (1998) and the autoradiographs were manually scored.

HA370 and HA372 were screened for polymorphisms using 42 *EcoRI/MseI* AFLP primer combinations with three selective nucleotides (Table 3.1). Eighteen AFLP primer combinations (E32/M57, E32/M60, E32/M61, E35/M48, E35/M49, E35/M52, E38/M47, E38/M48, E38/M50, E38/M51, E38/M54, E39/M53, E39/M61, E39/M62, E40/M49, E40/M61, E41/M59 and E41/M62) were selected and assayed on 180 F_2 progeny using protocols described by Vos et al. (1995). The autoradiographs were electronically scanned and co-dominantly scored using proprietary software developed by KeyGene (Wageningen, the Netherlands). If a marker could not be unambiguously co-dominantly scored, then it was scored dominantly.

Materials and methods for mapping eight candidate disease resistance (CDR) clones, one CAPS marker, and the downy mildew resistant gene, *Pl1*, were as described in Chapter 4.

Genetic Analyses

Genetic maps were first constructed using RFLP markers alone to compare locus orders between the integrated map and previously published individual maps (Berry et al. 1997; Jan et al. 1998). We used MAPMAKER (Lander et al. 1987) and G-MENDEL (Holloway and Knapp 1993; www.css.orst.edu/g-mendel) for these analyses. Loci were assigned to groups using minimum LOD scores of 3.0 and maximum recombination frequency estimates of 0.35. Locus orders were produced and compared using the MAP function of MAPMAKER and the ORDER function of G-MENDEL. Multipoint likelihood was the objective function for comparing orders

Table 3.1. Names and sequences of oligonucleotide adapters and primers for amplified fragment length polymorphism (AFLP) assay of sunflower mapping population.

Name	Sequence
EcoRI adapter	5'-CTCGTAGACTGCGTACC
	CATCTGACGCATGGTTAA-5'
EcoRI primers	
E01	5'-AGACTGCGTACCAATTCA
E32	5'-AGACTGCGTACCAATTCAAC
E35	5'-AGACTGCGTACCAATTCACA
E38	5'-AGACTGCGTACCAATTCACT
E39	5'-AGACTGCGTACCAATTCAGA
E40	5'-AGACTGCGTACCAATTCAGC
E41	5'-AGACTGCGTACCAATTCAGG
MseI adapter	5'-GACGATGAGTCCTGAG
	TACTCAGGACTCAT-5'
MseI primers	
M02	5'-GATGAGTCCTGAGTAAC
M47	5'-GATGAGTCCTGAGTAACAA
M48	5'-GATGAGTCCTGAGTAACAC
M49	5'-GATGAGTCCTGAGTAACAG
M50	5'-GATGAGTCCTGAGTAACAT
M51	5'-GATGAGTCCTGAGTAACCA
M52	5'-GATGAGTCCTGAGTAACCC
M53	5'-GATGAGTCCTGAGTAACCG
M54	5'-GATGAGTCCTGAGTAACCT
M57	5'-GATGAGTCCTGAGTAACGG
M59	5'-GATGAGTCCTGAGTAACTA
M60	5'-GATGAGTCCTGAGTAACTC
M61	5'-GATGAGTCCTGAGTAACTG
M62	5'-GATGAGTCCTGAGTAACTT

produced by MAPMAKER, while sum of adjacent recombination frequencies (SAR) or map length was the objective function for comparing orders produced by G-MENDEL. We used multipoint likelihood to select the final locus order estimate for each linkage group. If the likelihood for a locus order produced by MAPMAKER was greater than the likelihood of the locus order produced by G-MENDEL, then we

selected the MAPMAKER estimate. Similarly, if the likelihood for a locus order produced by G-MENDEL was greater than the likelihood of the locus order produced by MAPMAKER, then we selected the G-MENDEL estimate.

Monte Carlo analyses of each linkage group were performed using the MONTE function of G-MENDEL (Holloway and Knapp 1993). This function produces *n* locus order estimates from *n* repeat runs of the simulated annealing algorithm and estimates mean locus orders (Monte Carlo maps) by ranking the rank mean locus orders (Holloway and Knapp 1993). Monte Carlo maps were estimated using n = 100 and were compared to maps produced by the MAP function of MAPMAKER and the ORDER function of G-MENDEL. Discrepancies between the maps could often be traced to individual loci with extraordinary sampling errors and were typically only found for linkage groups with concordance estimates less than 0.96. Problem loci were discarded and locus orders were re-estimated using the same process until the concordances were $\geq$ 0.98 for each linkage group.

The combined RFLP-AFLP map was constructed using JoinMap (Stam, 1993; Stam and Van Ooijen 1996). Kosambi's mapping function (Kosambi, 1944) was used for the calculation of map distances, with a recombination threshold value of 0.49 and a LOD threshold value of 0.1. The data set was analyzed for markers that give rise to unlikely double recombination events in the map position and for the occurrence of distorted segregation using the JMSLA module of JoinMap 2.0. Only markers that were predominantly scored as co-dominant were included in this analysis. The data set was split up at a LOD of 4.0, except for groups 5 and 14 which were split up at a LOD of 5. All linkage groups of markers were stable up to approximately a LOD of 8.0.

RESULTS

Fifty-six of the 84 ZVG (67%) probes detected a total of 64 polymorphic RFLP loci in the HA370 x HA370 population. Example of segregation of RFLP probes in the F_2 mapping population was shown in Fig. 3.1. Forty-eight ZVG cDNAs detected one locus each and eight ZVG cDNAs (ZVG05, ZVG61, ZVG11,

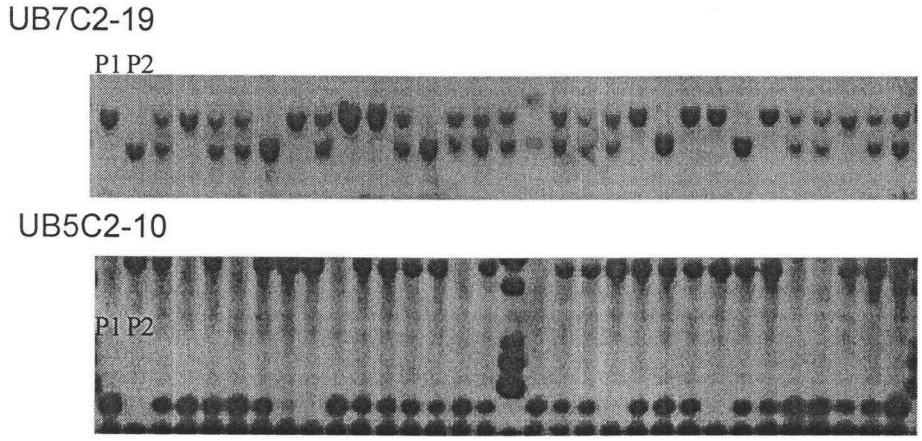


Figure 3.1. Segregation of UB7C2-19 and UB5C2-10 markers in F_2 mapping populations of sunflower derived from the cross HA370 x HA372. Genomic DNA was digested with *EcoRI*

ZVG22, ZVG58, ZVG32, ZVG61, and ZVG42) detected two loci each. The RFLPs for five UB markers were either monomorphic or could not be unequivocally genotyped. Forty UB cDNAs detected one locus each and two UB cDNAs (6B2 and 11C4) detected two loci each. The OLD-7 probe was not polymorphic in the HA370 x HA372 population. The *Pl1* gene mapped on LG8, 3.7 cM away from HR-4W2. Eight ZVG RFLP markers (ZVG11B-7, ZVG17-4, ZVG19B-8, ZVG3-1, ZVG44B-5, ZVG50B-17, ZVG58B-7, and ZVG61B-3) and two USDA-BGS RFLP markers (UB6B2B-3 and UB7D1-6), and four candidate

disease resistance (CDR) clones (HR-1W22A, HR-1W53, HR-1W48A, and HR-1W23) were dominant.

The locus groups and orders in this map were identical to those reported by Berry et al. (1997). There were, however, some gaps in the ZVG RFLP map that forced linkage groups apart. Specifically, there were gaps of 30 cM in group 1, 43 cM in group 4, 32 cM in group 5, and 35.1 cM in group 5. The Monte Carlo analysis did not detect any prominent locus ordering errors.

The second analysis was done using USDA-BGS marker data alone. There was complete agreement between the MAPMAKER and G-MENDEL analyses of these data. There were too few loci to order and evaluate the validity of the order in several groups. Only 4 groups had more than 3 loci. There were, however, several differences in the locus groups and orders between the HA370 x HA372 and Jan et al. (1998) maps. Details are described below.

The third analysis was done by combining the data for ZVG and USDA-BGS RFLP markers. A total of 121 RFLP markers were subjected to segregation analysis using G-MENDEL and/or MAPMAKER. These include 64 RFLP probes selected from the map of Berry et al. (1996; hereafter referred to as ZVG markers), 48 RFLP probes selected from the map of Jan et al. (1998; hereafter referred to as UB markers), and 9 CDRs. There was a general agreement between the MAPMAKER and G-MENDEL analyses of these data except for some minor local ordering differences between closely linked markers. This analysis produced a map with 105 loci distributed over 17 linkage groups, covering 961 cM with an average marker to marker distance of 9.2 cM. (Fig. 3.2). Evaluation of orders using Monte Carlo produced a concordance estimate of 0.9 in LG5 and LG9 to 1.0 in most groups.

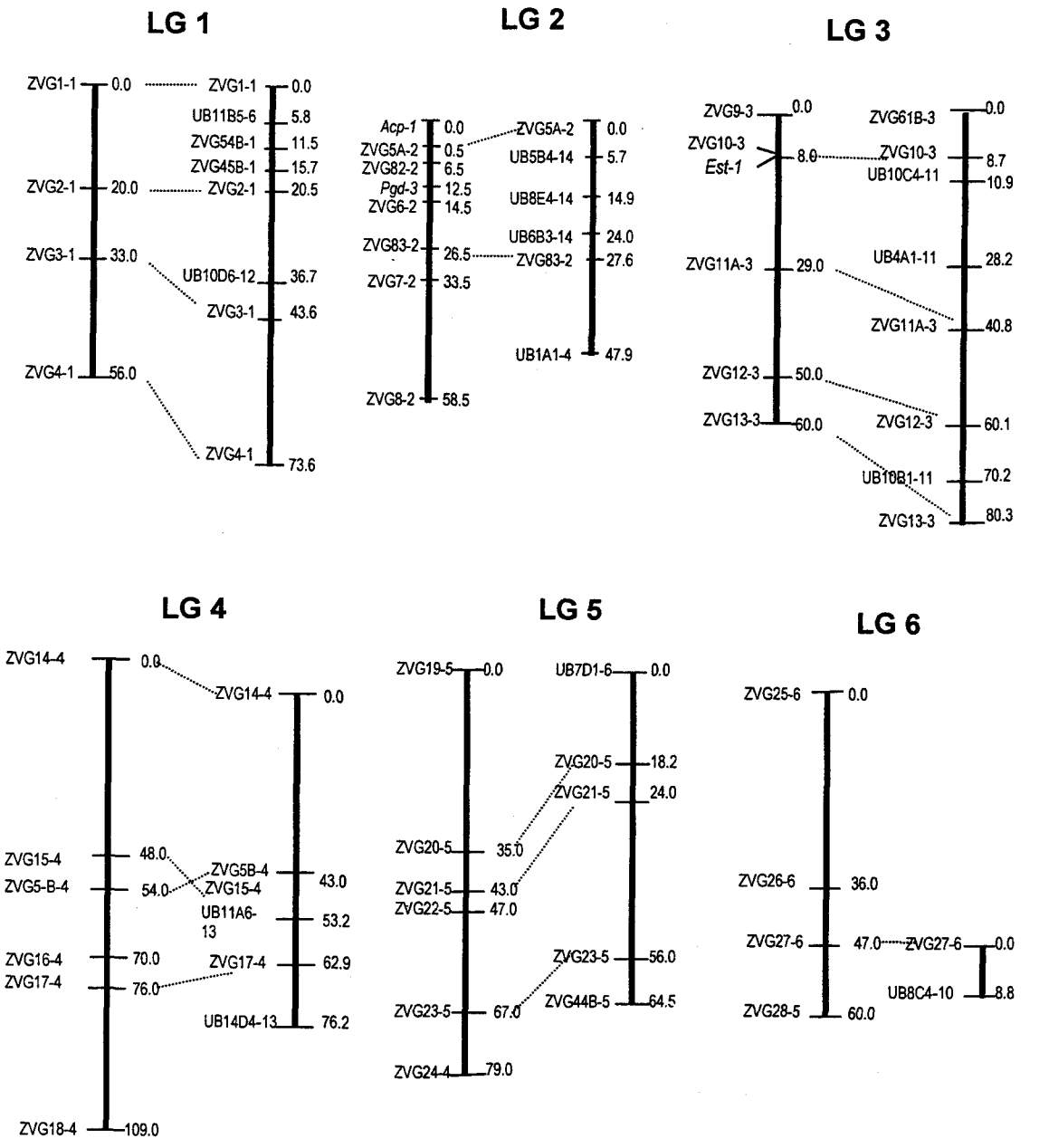
The average number of loci per linkage group (LG) was 6.1. Assignment of loci to linkage groups was based on Berry et al. (1996). LG13 and LG17 consist of 14 and 11 loci, respectively. Five of the loci in LG13 were a cluster of 5 CDRs. Therefore, the largest number of RFLP probes was mapped to linkage group 17. Eight linkage groups had 6 to 9 loci whereas 7 groups contain 1 to 4 loci. LG12 had only 1 ZVG locus. ZVG markers showed fairly even distribution among the

17 groups with a range of 1 to 7 loci and an average of 3.2 loci per LG. The UB probes were distributed among 14 LGs with a range of 1 to 7 and a mean of 2.9 loci per linkage group. No UB probe mapped to LG12, LG14, and LG15.

LG13 was the largest (105.4 cM) and consists of the highest number of loci (14) whereas LG6 was the smallest (8.8 cM) with only 2 loci. The CDR probes accounted for a 20 cM extension of map distance on LG13. The average distance between markers was least in LG7 (2.2 cM) and highest in LG5 (16.1 cM). Four linkage groups contain gaps of greater than 30 cM. The largest gap was 43 cM in LG4 followed by a 35.1 cM gap in LG14. LG5 and LG1 contain a gap of 32 cM and 30 cM, respectively. Ten gaps of 20-30 cM exist in 7 linkage groups. LG8, LG9, and LG15 each contain two gaps of 20 to 30 cM. Seventeen loci, 9 ZVG, 7 UB, and 1 CDR, were excluded from the final linkage map because these markers were either unlinked, loosely linked, or could not be ordered properly. Five of the 9 ZVG and 4 of the 7 UB markers and the one CDR markers were duplicate loci. Evaluation of locus order was performed using the Monte Carlo function of G-MENDEL and the order with the highest concordance chosen (Holloway and Knapp 1993). Furthermore, the final order was compared to the reference map (Berry et al. 1996).

Correspondence to published RFLP maps

Compared to the map described by Berry et al. (1996) the only minor change was on LG8 where the order of ZVG19B-8 and ZVG36-8 was reversed (Fig. 3.2). This discrepancy may be the result of close linkage between the two markers, 1 cM apart in the previous map and 1.7 cM apart in the present map, which impaired precise ordering. Even though the distances between markers were essentially the same in the two maps, some discrepancies were observed in several linkage groups. For example, on LG1 one of the three intervals showed the same distance whereas the remaining two intervals showed a 7 to 8 cM difference. In the second interval in this group, the distance was expanded by 8



Figrue 3.2. Linkage map of sunflower based on RFLP markers only. The map on the left (Berry et al. 1996) was included for comparison. Locus names are on the left and map distances (cM) on the right. Assignment of linkage group number is according to Berry et al. (1996). Locus names starting with ZVG- refer to probes from Berry et al. (1996), and those starting with UB- refer to probes from USDA/Biogenetic Services (Jan et al. 1998). The number at the end of the locus name refers to the location of the probes in the previous maps.

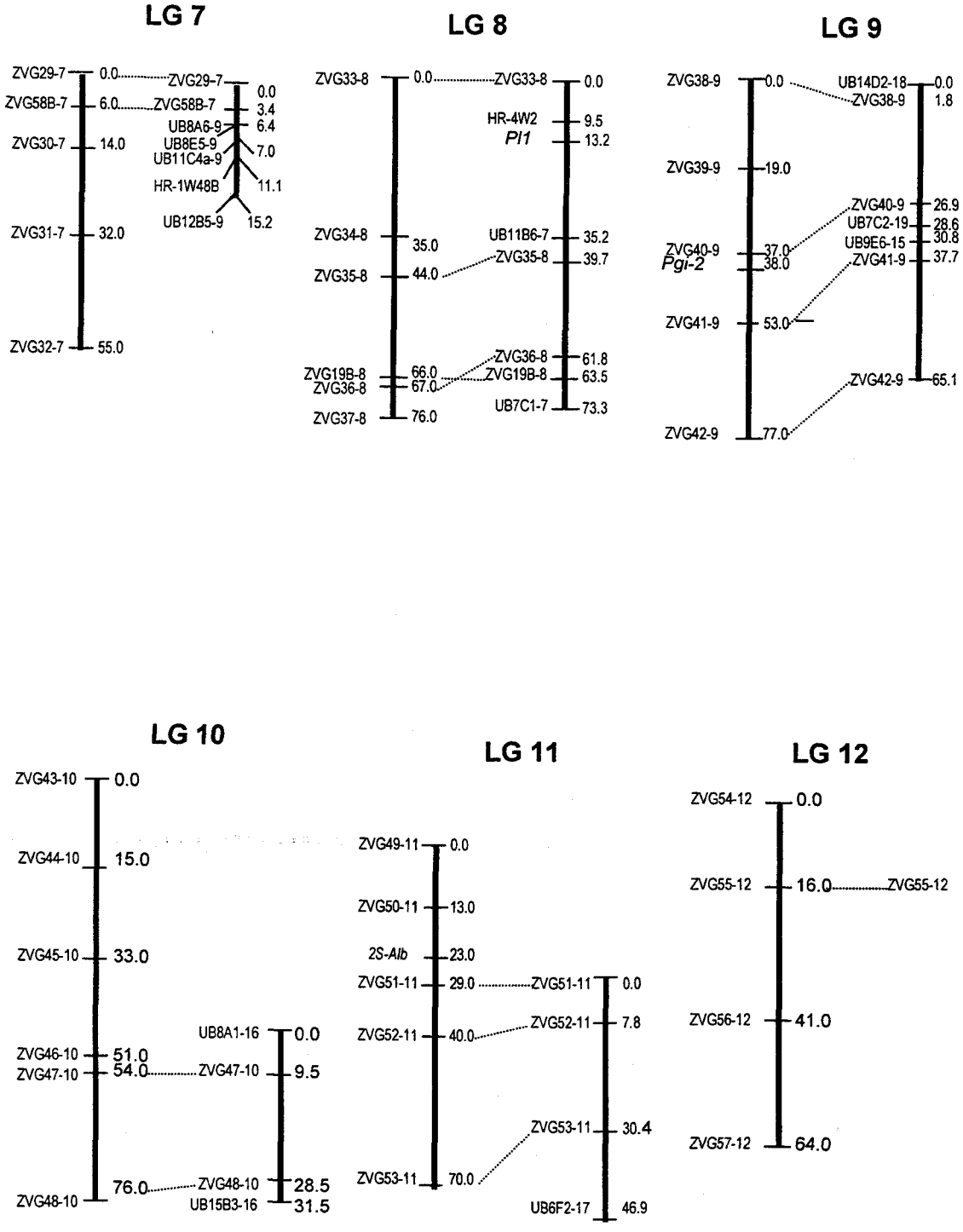


Figure 3.2. Continued

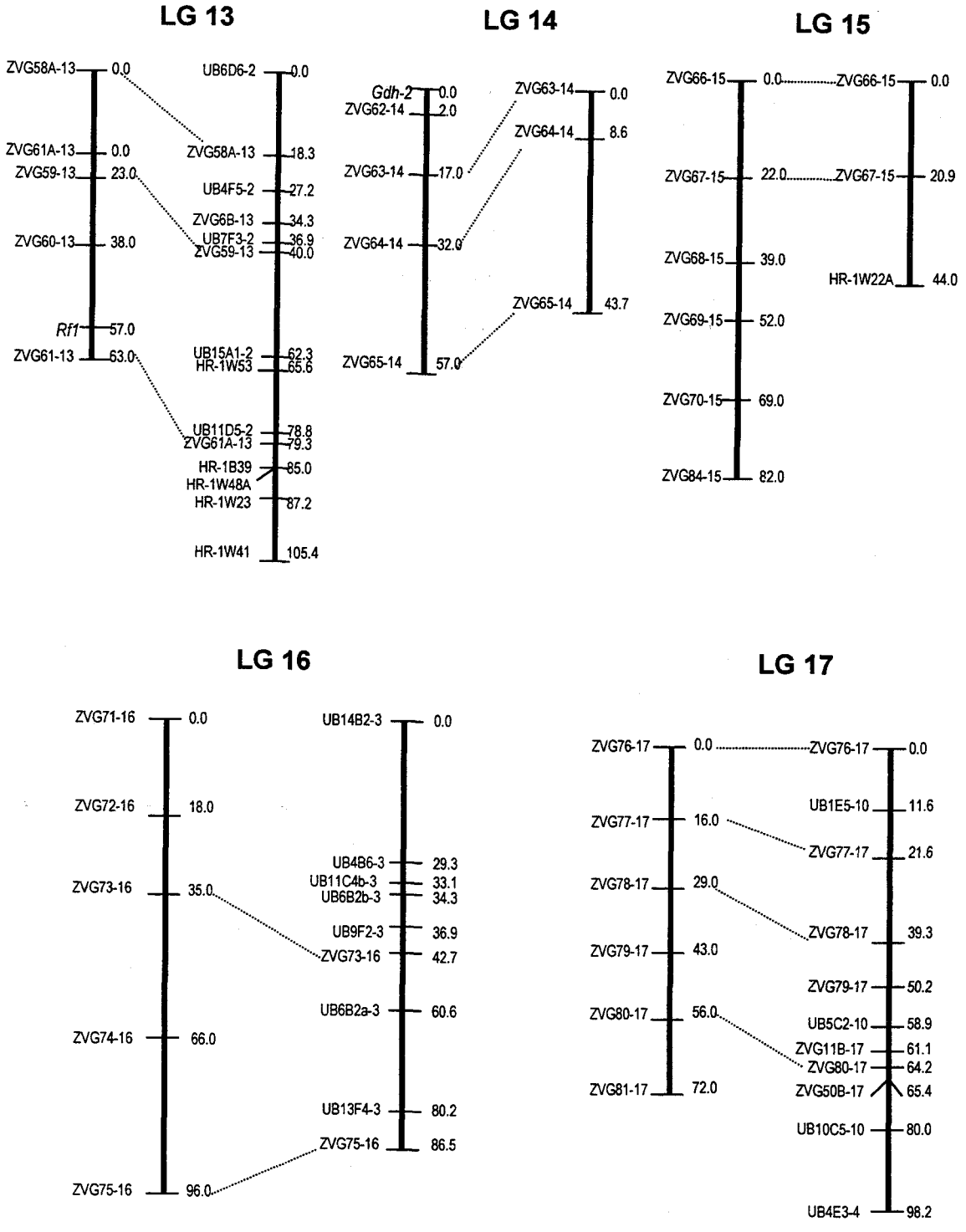


Figure 3.2. Continued

cM due to the presence of UB10D6 probe in the present map. In most cases, differences were observed where there are additional markers mapped between the two markers in one or the other map. The present map contains UB markers that were not mapped in the previous map whereas the latter contains additional ZVG markers that could not be mapped in the present mapping population. For example, the distance between ZVG10-3 and ZVG11A-3 was 21 cM in the previous map but it was 32 cM in the present map. However, two additional UB probes mapped between this interval perhaps causing map expansion. There were distance discrepancies even without additional loci between two markers common to both maps. The most noticeable difference is the distance between ZVG15-4 and ZVG5B-4 on LG4, which was 6 cM in the previous map but 0 in the present map. There was a 12 cM map expansion in the present map between ZVG22-5 - ZVG23-5 interval as compared to the interval in the previous map. Similarly, ZVG52-11 and ZVG53-11 had an interval of 30 cM in the previous map but 22.6 cM in the present map. ZVG64-14 and ZVG65-14 showed a 25 cM distance in the previous map but 35.1 cM in the present map. In several cases, the distance between two markers remained consistent whether additional loci exist between them (e.g. ZVG1-1 and ZVG2-1, ZVG5A-2 and ZVG83-2, ZVG59-13 and ZVG61-13). The UB markers mapped to some of the gaps in Zeneca's map, thereby increasing marker density in these regions. These include the intervals between ZVG1-1 and ZVG2-1, ZVG10-3 and ZVG11A-3, ZVG73-16 and ZVG75-16. However, some gaps of 20 to 30 cM persisted in linkage groups 1, 4, 8, 9, 11, 14, and 15.

Forty-one UB markers were assigned to 14 linkage groups. Linkage groups 12, 14, and 15 contain no UB markers. Locus grouping in 10 linkage groups corresponded to the map described by Jan et al. (1998). However, lack of correspondence was observed in four linkage groups (LG1, LG2, LG9, and LG17). Linkage groups 7, 13, and 16 showed the same locus grouping but slightly different order whereas LG3 showed the same grouping and locus order but slightly different map distance. The other linkage groups contain only 1 to 2 UB markers. In LG1 two markers, UB11B5-6 and UB10D6-12, from two different

groups in the previous map were grouped together (numbers at the end of the markers indicate the linkage group of the marker in the previous map). LG2 contains four UB markers, 3 from former LG14 and one from former group 4. The order of the 3 UB markers from group 14 was conserved in the present map but there was slight variation in the distance between markers. In LG9, three markers from previous group 15, 18, and 19 were grouped together. UB7C2-19 and UB9E6-15 were only 2.2 cM apart on the present map. These two markers were located at the end of their respective groups in the previous map suggesting that these two previous groups merged end to end in the present map. It can also be deduced that the former LG18 was connected to the distal end of LG19. Further study is required to verify this assumption by mapping more markers from the previous map. In LG17, three markers from former group 10 were joined with one marker from former group 4. The order of the three UB markers from group 10 was not conserved in the present mapping population. UB4E3-4 was located at the end of group 4 in the previous map and it is likely that these two groups were joined in the present map.

RFLP-AFLP integrated map

A total of 1547 bands were produced by 18 of the 42 AFLP primer combinations tested. The number of polymorphic bands in the HA370 x HA372 population was 306 (20%). The integrated map was produced using data from 105 RFLP and 296 AFLP loci (Fig. 3.3). For the AFLP markers no significant distortion away from the expected segregation ratio of 1:2:1 (for the three possible genotype classes: homozygous 'parent 1', heterozygous, and homozygous 'parent 2') was identified. Among RFLP markers only UB6D6 was identified with segregation ratios significantly distorted from the expected 1:2:1 ratio.
The parent HA370 contributed 140 of the AFLP alleles whereas HA372 contributed 166 of the alleles. All but 10 AFLP markers were placed on 17 linkage groups corresponding to the haploid chromosome number of sunflower, *Helianthus*

annuus. The 10 AFLP markers were excluded from the map because they were either unlinked to any other marker or showed unlikely double recombination. The mapped 401 loci covered 1326 cM of the sunflower genome with an average distance between adjacent loci of 3.3 cM. Size of the 17 linkage groups ranged from 16.3 cM in LG12 to 113.9 cM in LG13 (Table 3.2). The shortest and the longest linkage groups were the groups with the least and the largest number of RFLP loci, respectively. A total of 28 cosegregating markers were observed on seven linkage groups. Most of the cosegregating markers involve 2 or more AFLP markers. Clustering of, mostly AFLP, markers with a density of 4 to 8 markers per cM, was observed on linkage groups 3, 4, 5, 7, 10, 16, and 17. Co-segregating or clustered RFLP loci were also observed on LG4 (ZVG5B-4 and ZVG15-4), LG7 (UB8A6, UB8E5, and UB11C4a), and LG13 (ZVG61A and UB11D5).

Table 3.2. Number of AFLP and RFLP markers, total and average distance (cM) for RFLP alone and AFLP-RFLP (A/RFLP) integrated genetic map of sunflower.

	Number of			Distance (cM)			
LG	AFLP	RFLP	Total	RFLP	AVG	A/RFLP	AVG
1	14	8	22	73.6	9.2	80.0	3.6
2	10	5	15	47.9	8.0	35.4	2.2
3	21	8	29	80.3	10.0	90.8	3.1
4	14	6	20	76.2	12.7	79.9	4.0
5	39	4	44	64.5	16.1	119.5	2.8
6	7	2	9	8.8	4.4	48.0	5.3
7	21	7	28	15.2	2.2	36.5	1.3
8	17	7	24	73.3	10.5	107.9	4.5
9	19	7	26	65.1	9.3	93.8	3.6
10	27	4	32	31.5	7.9	101.4	3.3
11	14	4	18	46.9	11.7	69.6	3.9
12	4	1	5	0.0	-	16.3	3.3
13	14	14	28	105.4	7.5	113.9	4.1
14	4	3	7	43.7	14.6	61.4	8.8
15	4	4	8	44.0	14.7	94.5	13.5
16	26	9	34	86.5	9.6	65.5	2.1
17	41	11	52	98.2	8.9	111.9	2.2
Total	296	105	401	961.1	9.2	1326.3	3.3

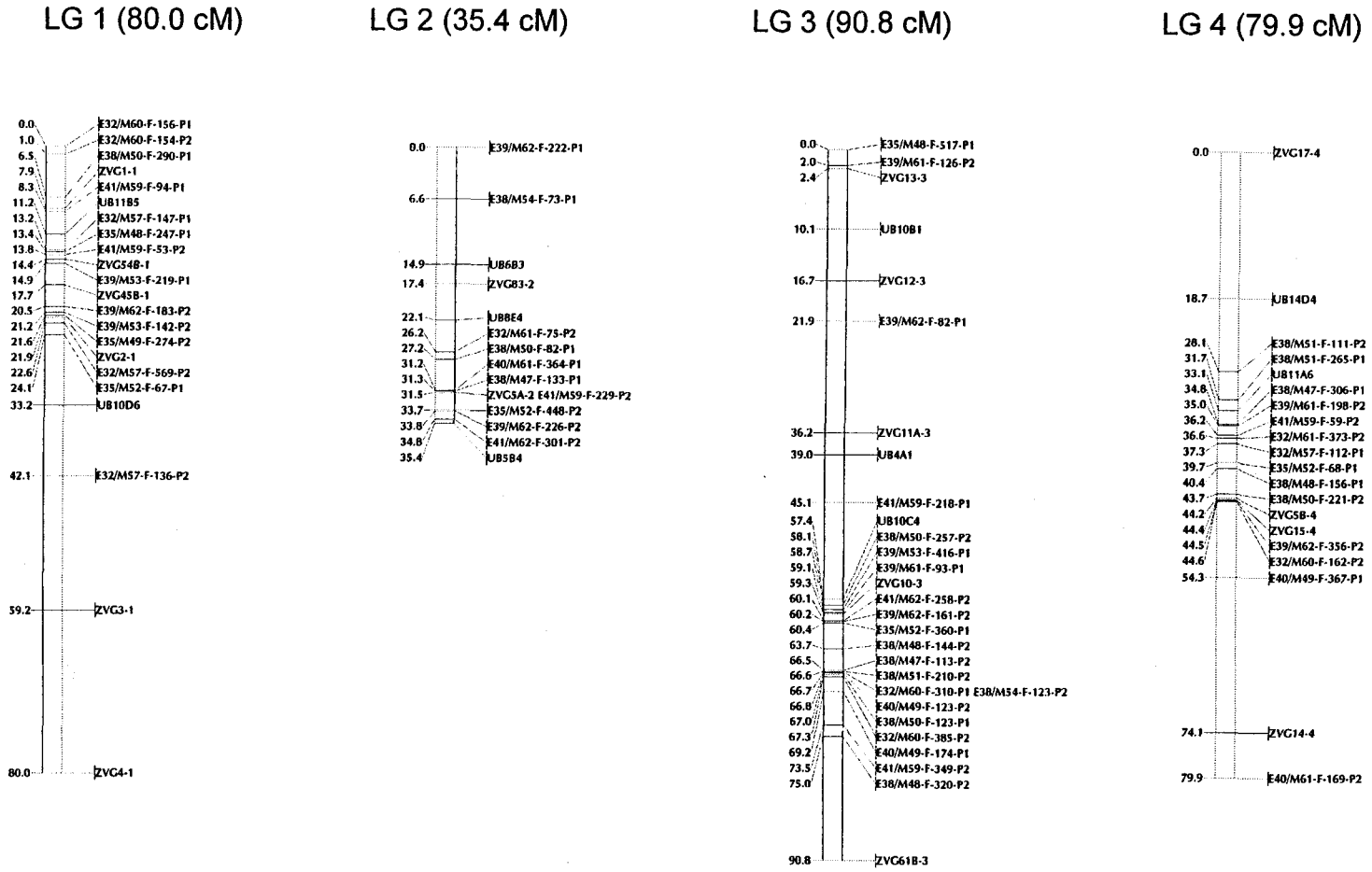


Figure 3.3. AFLP-RFLP integrated map of sunflower based on segregation analysis of 180 F_2 plants derived from the cross HA370 x HA372. Locus names are on the rightt and map distances (cM) on the left. Assignment of linkage group number is according to Berry et al. (1996). Locus names starting with ZVG- refer to probes from Zeneca, those starting with UB- refer to probes from USDA/Biogenetic Services (Jan et al. 1998), markers with HR- refer to sunflower RGA clones. The number at the end of the locus name refers to the location of the probes in the previous maps.

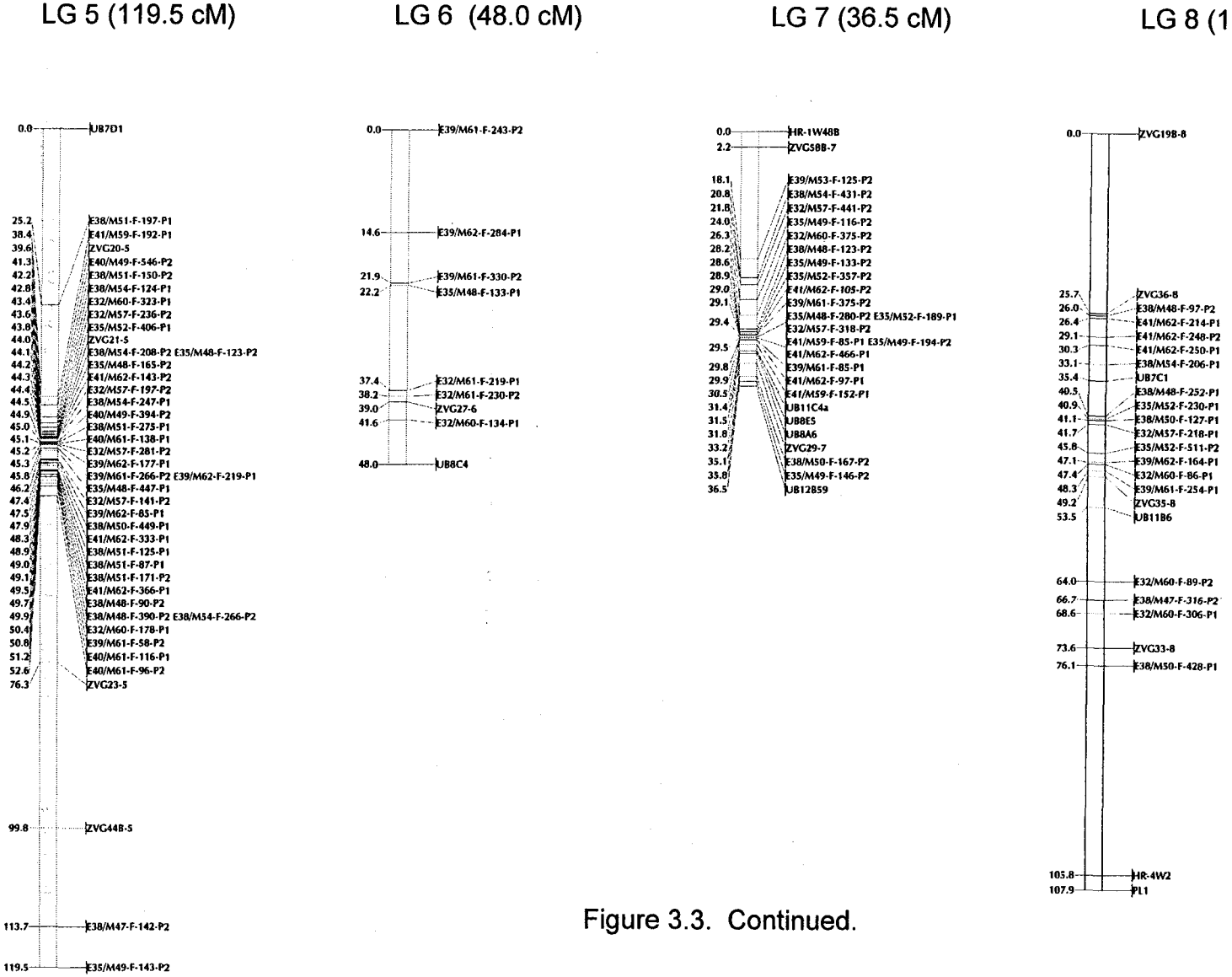


Figure 3.3. Continued.

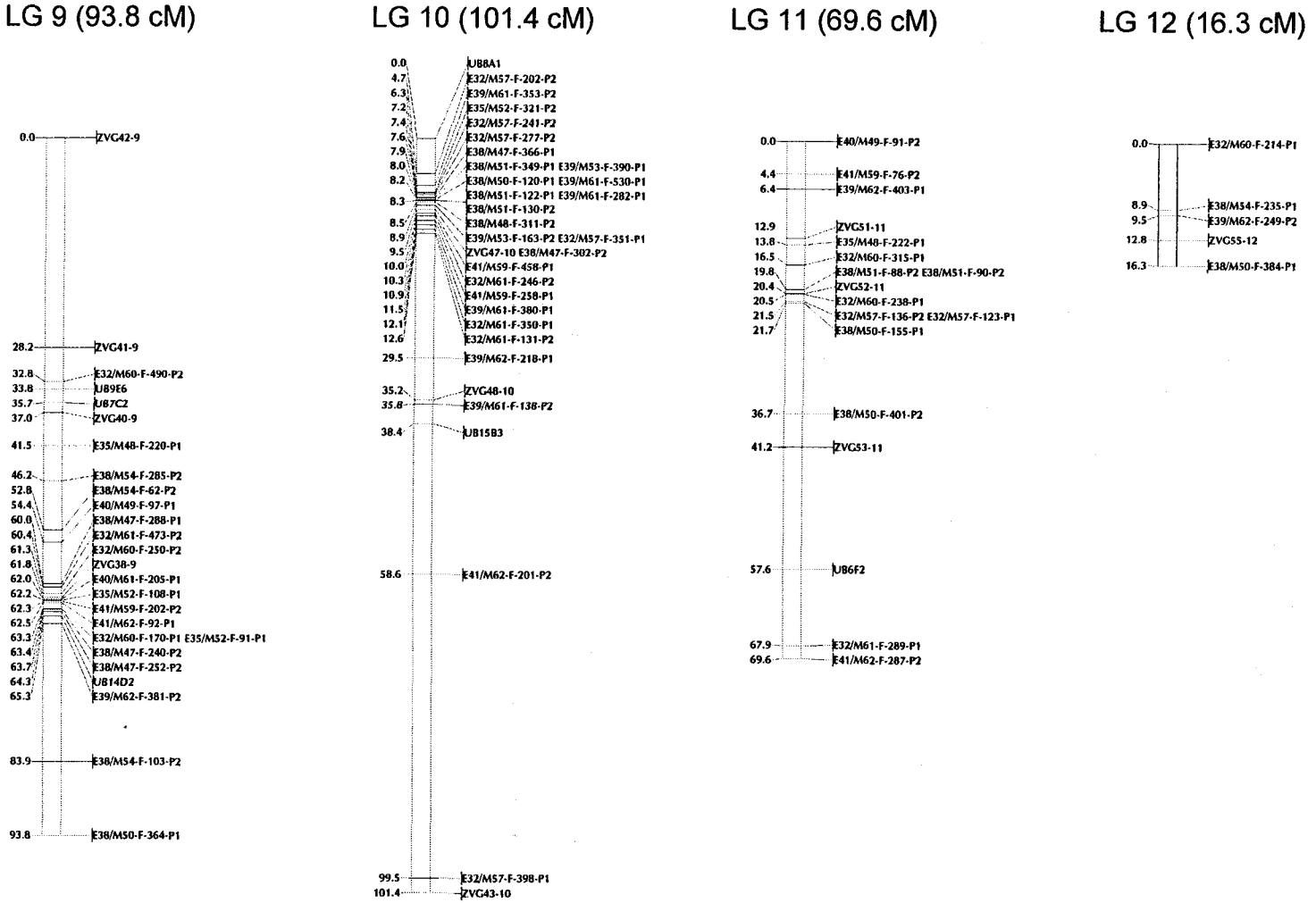


Figure 3.3. Continued

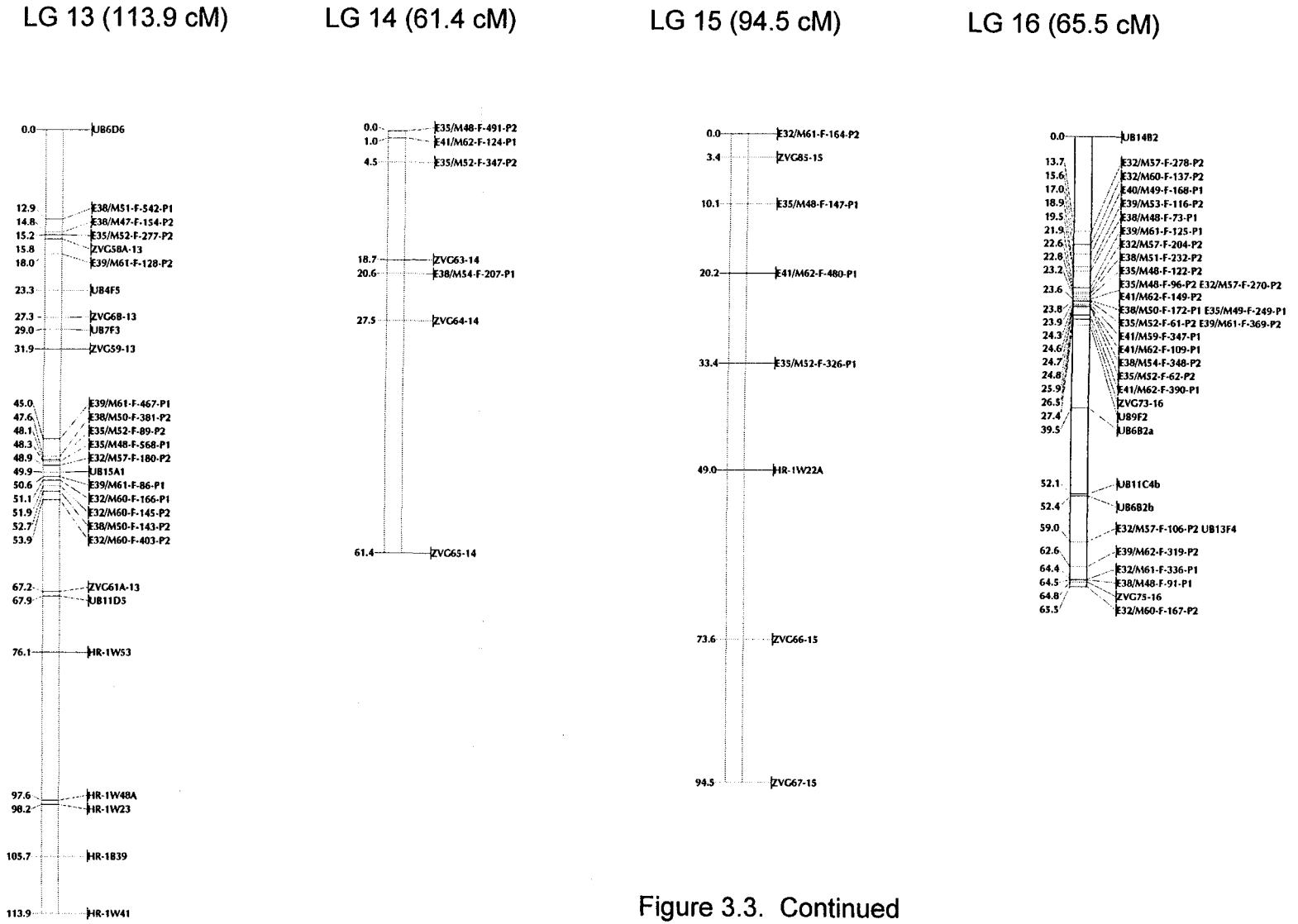


Figure 3.3. Continued

LG 17 (111.9 cM)

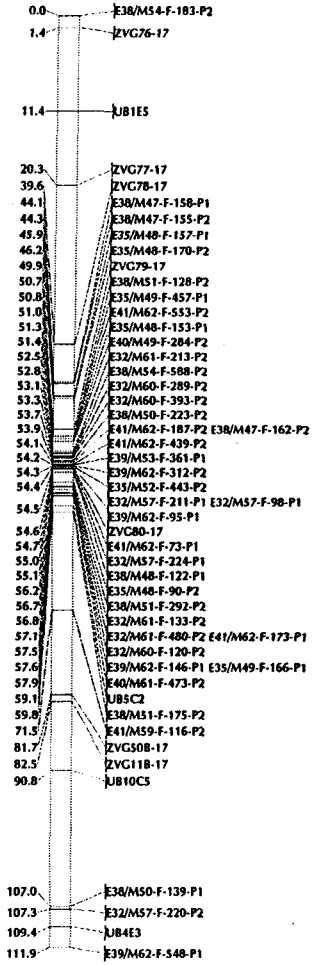


Figure 3.3. Continued.

The AFLP markers showed fairly even distribution over all linkage groups with a range of 4 to 41 markers per linkage group. Thirteen out of 17 linkage groups contain at least 10 AFLPs (Table 3.2). Both ZVG and UB RFLPs showed fairly even distribution in all linkage groups in the combined map (Fig. 3.3). Linkage groups 10 and 14 had gaps of 40.9 cM and 33.9 cM, respectively. Only one of the four gaps (> 30 cM) in the RFLP only map (Fig. 3.2) persisted in the combined map whereas the gap on LG10 was introduced due to mapping of ZVG43-10 that was found to be unlinked in the RFLP only linkage analysis. AFLPs filled the gap between ZVG5B-4 and ZVG14-4 and reduced the map distance from 43 cM to 20 cM. The gap between ZVG21-5 and ZVG23-5 was 32 cM in the RFLP map but 23 cM in the combined map. Additional 11 gaps of 20 to 30 cM existed on groups 1, 5, 8, 9, 10, 13, and 15.

The integrated map was significantly stretched in linkage groups 5 to 11, and 15 (Table 3.2). On LG5, about half of the increment in map distance was due to AFLPs mapped to the end of the group thereby extending the map, whereas the remaining was due to AFLPs stretching the distance between RFLP markers. The change in map distance on linkage groups 6, 9, and 11 was due to addition of AFLPs at either or both ends of the group. Map expansion on LG7 and LG8 was due to AFLPs added distance between RFLPs. Inclusion of previously unlinked or loosely linked RFLP markers, ZVG43-10 and ZVG85-15, in the integrated map caused map extension on LG10 and LG15, respectively.

DISCUSSION

The marker content in the genetic map of sunflower is rapidly increasing. The average interval size between markers in sunflower genetic map has been successively reduced as different maps in different mapping populations were produced. The average map distance per marker in previous maps was 7 cM (Gentzbittel et al. 1995), 5.9 cM (Berry et al. 1995), 2.3 cM (Berry et al. 1996) and 4.6 cM (Jan et al. 1998). In the present study we reported an integrated genetic

linkage map based on RFLP and AFLP covering 1326 cM with an average distance of 3.3 cM per marker. Genome coverage of this map falls in the same range of 60-80% reported earlier (Berry et al. 1995; Gentzbittel et al. 1995).

In the map constructed with RFLP markers only, the ZVG probes showed conserved grouping and map order. This conservation suggests that these markers can be used in various populations and for comparative mapping in the *Helianthus* species. However, even though many of the BGS-USDA probes showed conserved grouping in the previous and the present map, some of the probes tended to map to different linkage groups in the present map despite their tight linkage in the previous map. In the present map, three linkage groups contain loci from 2 previous groups and one linkage group contains loci from three short previous groups in the map described by Jan et al. (1998). The differences are not trivial and can only be settled by mapping the markers in question in new mapping populations. However, the genotypes for the disputed markers were very clear and the results were reproduced with separate analyses done with MAPMAKER and G-MENDEL.

The present map contains 2 large gaps (>30 cM) on two linkage groups and 11 medium gaps (20–30 cM) on 7 linkage groups. Keim et al. (1997) observed large gaps in a map containing 840 markers and attributed the presence of large gaps to the use of intraspecific recombinant inbred populations and a small size of mapping population. These gaps could be filled as more polymorphic markers are identified and added to the map. The development of simple sequence repeat (SSR) markers is underway in a few laboratories. This will provide an opportunity to saturate the existing sunflower map with multiallelic, highly polymorphic SSR markers. Aggregation of AFLP markers in some regions of the map has been observed in this and previous studies (Keim et al. 1997; Tanksley et al. 1992; Qi et al. 1998). Jan et al. (1998) observed 39 clusters with 2-9 tightly linked RFLP markers with no recombination. In this study we observed clustering of markers on several linkage groups. It was hypothesized that reduced recombination in certain chromosomal regions, particularly, in regions near centromere and possibly near telomeric regions is responsible for clustering of markers (Tanksley et al. 1992).

The regions with high density of markers in the present map may be the centromeric regions. In this study map stretching resulted from AFLP markers filling gaps between RFLP markers and in some cases added to the tip of linkage group. Similar finding has been reported in barley (Becker et al. 1995). Stretching could occur due to AFLP markers scanning different genomic regions that has not been detected by RFLPs (Maheswaran et al. 1997).

Accumulation of a variety of markers allows for saturation of the existing map and provides opportunities and flexibility for utilization of markers for different breeding purposes including marker-assisted selection (MAS) and map-based cloning. Particularly, PCR-based markers such as AFLP, SSR and single strand conformational polymorphism (SSCP) are preferable because of ease of handling. Despite the time-consuming and labor intensive procedure, RFLPs are routinely used in plants for constructing linkage maps, DNA fingerprinting, and tagging of agriculturally important genes. RFLPs are especially suitable for comparative mapping due to their locus specificity (Tanksley 1989).

It has been well documented that AFLP markers allow for efficient generation of reproducible genetic data for various applications in crop improvement. Examination of reproducibility of several PCR-based marker systems showed that AFLPs were highly reproducible and had the highest effective multiplex ratio, i.e., number of loci revealed per assay (Becker et al., 1995; Rusell et al. 1997; Powell et al. 1996). AFLPs have proven suitable for detecting polymorphism among 24 sunflower inbred lines (Hongtrakul et al., 1997). Polymorphic fragments generated by AFLP can be successfully excised from a sequencing gel for re-amplification and conversion into other convenient, PCR-based markers such as sequence characterized amplified region (SCAR) or for screening cDNA or genomic libraries (Cho et al. 1996; Cnops et al. 1996; Thomas et al. 1995). The present map combines the advantage of both RFLP and AFLP markers for utilization in molecular breeding of sunflower.

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CHAPTER 4

Cloning and Analysis of Candidate Disease Resistance Genes in Sunflower: Linkage Between a Cluster of LRR Genes and a Gene for Downy Mildew Resistance in Sunflower, *Helianthus annuus* L.

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ABSTRACT

Candidate disease resistance (CDR) genes have been cloned from a variety of species using highly conserved CDR gene sequences. We used degenerate oligonucleotide primers based on conserved sequences in disease resistance genes from other species to PCR amplify CDR gene fragments from sunflower (*Helianthus annuus* L.). Twenty-one PCR clones were selected and sequenced. These clones were shown to belong to 11 unique groups. Derived amino acid sequence analysis revealed that seven classes were more similar to the tobacco *N* gene and the flax L^6 gene (44-58%) than to the *Arabidopsis RPS2* gene whereas clones from the other four classes were more similar to *RPS2* (36-43%) than N and L^6. BLAST search of the GENBANK database revealed that the sunflower CDR clones are homologous to previously cloned plant disease resistance genes such as *RPS2*, *N*, L^6, *M*, *RPP1*, and *RPP5*. Seven clones mapped to four linkage groups and identified nine loci. Two clones each produced two polymorphic loci suggesting chromosomal rearrangements such as duplication of resistance genes in sunflower. We identified a CDR gene (HR-4W2) that was closely linked to downy mildew resistance gene and utilized this clone to develop cleaved amplified polymorphic sequence (CAPS) marker, linked to downy mildew resistance at 3.7 cM. Single strand conformational polymorphism (SSCP) and Southern analysis of NILs also confirmed the association of HR-4W2 with downy mildew resistance. This CAPS marker could be useful for marker-assisted selection for downy mildew resistance in sunflower.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is afflicted by several biotic diseases including downy mildew (caused by *Plasmopara halstedii* (Farl.) Berl. & de Toni). Downy mildew has long been considered one of the production constraints in commercial sunflower production. Resistance to downy mildew is controlled by major genes designated *Pl* and function in accordance with the gene-for-gene model (Sackston 1992; Miller and Gulya 1991; Zimmer and Kinman 1972). Even though the pathogen is able to infect both susceptible and resistant lines, hypersensitive like reactions limit further growth and spread of the initial inoculum in resistant lines (Mouzeyar et al. 1994; Viranyi 1992). The sunflower seed industry devotes significant resources to disease resistance breeding. Most of the disease resistance genes of sunflower have not been mapped and the genetics of important sunflower diseases are yet to be investigated. A large number of investigations have been conducted to identify sources of resistance and to determine the nature of inheritance of downy mildew resistance genes. Since the first report of downy mildew resistance in mid 60's, several pathogen races and corresponding genes conditioning resistance to these races have been identified (Sackston et al. 1990; Miller and Gulya 1991). It is not well understood, however, if resistance to different races is conditioned by different genes or by a family of clustered genes segregating together. Downy mildew resistance genes previously thought to be different were later found to be the same (Sackston 1992). The works of Roeckel-Drevet et al. (1996) and Vear et al. (1997) suggest that *Pl1*, *Pl2*, and *Pl6*, the genes conferring resistance to the most important races of downy mildew, map to the same location. Further investigations are required to see whether the *Pl* genes are organized in cluster of resistance (R) genes as in lettuce downy mildew. In lettuce, the three major disease resistance gene clusters contain 2-8 *Dm* genes, the genes conferring resistance to lettuce downy mildew caused by *Plasmopara lactucae-radicis* (Witsenboer et al. 1995).

Lack of segregation between different *Pl* genes and the fact that some sunflower lines are resistant to multiple races (Miller and Gulya 1988; Miller and

Gulya 1991; Viranyi and Gulya 1995) led to an investigation of the relationship between different *Pl* genes. The first sunflower R gene mapped was *Pl1* (Mouzeyar et al. 1995), the gene conditioning resistance to Race 1 of downy mildew. In this study, two RFLP markers positioned at 5.6 and 7.1 cM on each side of *Pl1* locus were identified. In a subsequent study, *Pl6*, a gene conferring resistance to several races of downy mildew, was found to be linked to the same RFLP marker loci as those determined for *Pl1* suggesting that *Pl1* and *Pl6* might be either allelic or closely linked (Roeckel-Drevet et al. 1996). Similarly, Vear et al. (1997) showed that *Pl2*, a gene known to give resistance to Race 1 and 2, was located at the same position as previously mapped *Pl1* and *Pl6* genes. It has been concluded that *Pl6*, previously thought to give resistance to all downy mildew races, is a member of cluster of genes controlling resistance to downy mildew races.

Recently, genes conferring resistance to several diseases caused by a wide range of pathogens including viruses, fungi, bacteria, and nematodes have been isolated using map-based cloning or insertional mutation. To date, successful cloning of R genes have been reported for both dicotyledenous and monocotyledenous species (reviewed in Michelmore 1995a, 1995b; Staskawicz et al. 1995; Parker and Coleman 1997; Baker et al. 1997; Hammond-Kosack and Jones 1997). These breakthroughs have opened up several avenues of research on plant diseases. Accumulation of cloned R genes greatly facilitate the development of cultivars with enhanced resistance to major diseases via marker-assisted selection or genetic engineering of crop plants (Staskawicz et al. 1995; Michelmore 1995b). Many cloned R genes have common structural motif known as leucine rich repeats (LRR) which is thought to be involved in protein-protein interactions and ligand binding in plants (Parker and Coleman 1997; Baker et al. 1997). Recent studies provide evidence that the LRR domain is the specificity determinant. Simons et al. (1998) found that deletion or duplication of LRR repeats in the tomato *I2* gene (a gene conferring resistance to *Fusarium oxysporium*) revealed that these regions are involved in determination of specificity. *Cf-4* and *Cf-9* are R genes known to map to the same location but

recognize different avirulent determinants in tomato. It was demonstrated that the recognitional specificity in these genes was caused by deletion of two LRR motifs in *Cf-4* (Thomas et al. 1997). Wang et al. (1998) provided functional and evolutionary evidence that race-specific pathogen recognition in the rice *Xa21*D gene is determined by LRR domain.

The tomato *Pto* gene (Martin et al. 1993), which encodes a cytoplasmic serine/threonine protein kinase, is an exception to the LRR-containing R genes. Parker and Coleman (1997) and Baker et al. (1997) classified plant R proteins into several groups by comparing predicted structural motifs. In general, LRR-containing R genes can be grouped into (i) transmembrane R proteins (LRR-TM) that lack nucleotide binding site (NBS) such as *Cf-2* (Dixon et al. 1996), *Cf-4* (Takken et al. 1998), *Cf-9* (Jones et al. 1994), *Xa-21* (Song et al. 1995) and $HS1^{pro1}$ (Cai et al. 1997) and (ii) cytoplasmically located R proteins, which include L^6 (Lawrence et al. 1995), *N* (Whitham et al. 1996), *RPP5* (Parker et al. 1996), *RPM1* (Grant et al. 1995), *RPS2* (Bent et al. 1994), *I2C-1* (Ori et al. 1997), and *Prf* (Salmeron et al. 1996).

The presence of conserved sequences among R genes provides an opportunity for isolation of new R genes based on PCR methods. Recently, a number of candidate disease resistance (CDR) genes have been isolated from various plant species based on sequence homology among R genes and shown to be tightly linked to known disease resistance loci and clustered in the genome (Aarts et al. 1998; Collins et al. 1998; Kanazin et al. 1996; Yu et al. 1996; Leister et al. 1996, 1998; Speulman et al. 1998; Feuillet et al. 1997; Ohmori et al. 1998). In sunflower, Gentzbittel et al. (1998) isolated clones homologous to resistance genes by using the NBS and protein kinase conserved motifs. Linkage analysis showed that at least three NBS-like clones were mapped to linkage group 1 where downy mildew resistance genes are located. The protein kinase-like clone exhibited an association with QTL for *Sclerotinia* white rot suggesting the feasibility of using both NBS-like and PK-like clones as molecular markers for resistance breeding. Two of the four lettuce resistance gene candidate (RGC) classes mapped to four known downy mildew R gene clusters and showed

multiple copies. RGC1 cosegregated with *Dm13* whereas RGC2a cosegregated with *Dm3* (Shen et al. 1998).

The recent cloning and analysis of CDR genes from several species has opened new avenues of research on plant diseases and produced genetic markers tightly linked to disease resistance genes. Molecular markers have been successfully utilized for breeding disease resistant plants. The effectiveness of marker-assisted selection largely depends on identification of markers tightly linked to the disease resistance locus. Segregation analysis and analysis of NILs are the most common strategies to identify molecular markers that can be used for MAS. Advances in DNA marker technology allowed the development of PCR-based markers such as random amplified polymorphic DNA (RAPD) (Williams et al. 1990), sequence characterized amplified regions (SCAR) (Paran and Michelmore 1993), and cleaved amplified polymorphic sequence (CAPS) (Konieczny and Ausubel 1993) that are relatively more convenient and economical than hybridization-based markers (e.g. RFLP) for high throughput assays. Due to a large difference between cleaved and uncleaved PCR products, CAPS markers can be easily scored on standard agarose gel electrophoresis (Glazebrook et al. 1998). In addition, CAPS markers overcome the limitations of RAPD methods such as lack of reproducibility and dominance scoring (Konieczny and Ausubel 1993). Recent studies demonstrated the usefulness of CAPS markers to map Arabidopsis genes (Konieczny and Ausubel 1993; Jarvis et al. 1994). Botella et al. (1997) applied the CAPS technique to resolve the map position of resistance like ESTs (R-ESTs) in *Arabidopsis*. De Jong et al. (1997) cloned and converted an amplified fragment length polymorphism (AFLP) marker linked to the *Nb* gene of potato, a dominant gene that confers resistance against potato virus X, into CAPS markers. Segregation analysis of 41 F_2 individuals placed these CAPS marker in the region of chromosome 5 where the *Nb* locus and other resistance genes are located. Other sequence-based DNA markers were also used to tag disease resistance genes in plants. In order to overcome the limitation of RAPD markers as genetic markers, SCARs were developed from previously identified RAPD markers in

lettuce (Paran and Michelmore 1993). Segregation analysis has been utilized to develop SCAR markers linked to *Verticillium* wilt resistance in tomato (Kawchuk et al. 1998), sequence tagged site (STS) marker for resistance to apple scab (Cheng et al. 1998), and AFLP markers for resistance to root knot nematodes in peach (Lu et al. 1998).

Mapping of a single resistance gene to a marker linkage map by traditional segregation analysis is time consuming and tedious. An alternative approach to rapidly identify markers tightly linked to disease resistance gene involves analysis of near-isogenic lines (NIL) (Muehlbauer et al. 1988; Young et al. 1988). The usefulness of NILs for identifying and mapping of markers linked to disease resistance genes has been demonstrated in several crop plants (Young et al. 1988; Paran et al. 1991; Martin et al. 1991; Mohler and Jahoor 1996). RFLP and RAPD markers linked to the downy mildew resistance genes in lettuce were identified using bulked segregant analysis (Michelmore et al. 1991) and near-isogenic lines (Paran et al. 1991).

Our objectives were to clone and map candidate disease resistance genes and to develop PCR-based marker for downy mildew resistance genes in sunflower. In this study, we present the cloning of CDR genes from sunflower using PCR based on heterologous gene sequence from several crop species and mapping of these clones as RFLP. Six near-isogenic lines, carrying downy mildew resistance, have been assayed to develop cleaved amplified polymorphic sequences (CAPS) markers closely linked to downy mildew resistance locus. The CAPS marker can be used in marker-assisted selection for downy mildew resistance.

MATERIALS AND METHODS

Plant material and DNA extraction

The DNA for PCR amplification of NBS-containing clones was extracted from the sunflower inbred line HA89 by a modified CTAB protocol described in Webb and Knapp (1990). Six near-isogenic lines (NILs) of oil seed sunflower (HA-335, HA-336, HA-337, HA-338, HA-339, and RHA-340) derived from the BC_2F_4 generation of the cross between HA89 and resistant wild relatives (*H. annuus* 423, *H. annuus* 432, *H. praecox* 417, *H. praecox* 419, *H. praecox* 424, and *H. argophyllus* 415, respectively) (Miller and Gulya 1988) were screened by Southern blot, SSCP, and CAPS analysis. The NILs used in this study contain *Pl6*, *Pl7*, and *Pl8* genes and are reported to be resistant to all known races of downy mildew (Miller and Gulya 1988; Miller and Gulya 1991; Viranyi and Gulya 1995).

Primers and PCR condition

Two pairs of overlapping degenerate oligonucleotide primers were designed based on conserved NBS regions in the tobacco N gene (Whitham et al. 1996), the *RPS2* gene (Bent et al. 1994), and the flax L^6 gene (Lawrence et al. 1995). The sequences of the primers were: N1 = 5'GGI GGI GTI GGI AAI ACI AC 3' (forward, based on P-loop); C1 = 5'(C,T)CT AGT TGT (A,G)A(C,T) (A,G,T)AT (A,G,T)A(C,T) (C,T)(C,T)T (A,G)C (reverse 1, kinase-3a); C2 = 5'IAG IGC IAG IGG IAG ICC 3' (reverse 2, membrane spanning region - GLPLAL). These primers were targeted to amplify a product of 300 bp (primer combination N1-C1) and 500 bp (primer combination N1-C2). The primers were synthesized at Central Services Lab of Oregon State University on an Applied Biosystems, model 394 DNA synthesizer (Foster City, California). Amplification of the NBS-

containing sequences was carried out in a 25 μl PCR reaction mixture containing 25ng genomic template DNA, 1 unit Taq polymerase (Gibco BRL), 1X PCR reaction buffer (Gibco BRL), 2.5mM $MgCl_2$, 0.1mM dNTP, 0.2 to 1μM of each primers. The mixture was subjected to initial denaturation for 3 minutes at 95°C followed by 40 cycles of denaturation at 94°C for 30 sec, reannealing at 50°C for 30 sec, and extension at 72°C for 45 sec. At the end of the cycles, a 10-minute final extension was allowed at 72°C on a DNA thermal cycler (Perkin-Elmer).

Cloning and Sequence Analysis

The heterogeneous PCR amplification products were separated into individual fragments by cloning using the TA cloning kit (Invitrogen) according to the manufacturer's protocol. Inserts were amplified from the vector by using T7 and M13 reverse primers. The PCR product was purified by QIAquick PCR purification kit (Qiagen) before they were subjected to SSCP analysis and sequencing. SSCP analysis was conducted as described earlier (Slabaugh et al. 1997), to test heterogeneity of PCR products and to classify clones based on SSCP band pattern. Briefly, 1-1.5 μl of each PCR reaction was added to 9 μl denaturing solution (95% formamide, 0.01M NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue), heated to 95°C for 3 minutes, then chilled on ice. Denatured samples were run on 0.5X MDE gels (AT Biochem, Malvern, PA) using 0.6X TBE running buffer. The gel was run at 2 watts for 12-16 hours at room temperature and stained with silver nitrate (Sanguinetti et al. 1994). The PCR products subjected to SSCP analysis were found to be mixtures of several loci. One to five clones representing each class were sequenced using an Applied Biosystems 373 or 377 automated sequencer (Foster City, California) at the Central Services Lab of Oregon State University. Sequence alignment and phylogenetic analysis were made by using PILEUP and other programs in the Genetics Computer Group (GCG) software. Sunflower CDR clones were aligned

with corresponding regions in the *Arabidopsis RPS2*, the tobacco *N*, the flax L^6 gene as well as six lettuce NBS sequences (kindly provided by R.W. Michelmore, UC Davis, CA). Percent similarity was computed using the GCG program.

Southern Blot analysis of NILs

Seeds for six near-isogenic lines (NILs) of sunflower (*Helianthus annuus* L.) containing resistance genes to races 2, 3, and 4 of downy mildew (*Plasmopara halstedii*) in HA-89 background (Miller and Gulya 1988) were obtained from Jerry Miller (USDA-ARS, Fargo, ND). Ten μg genomic DNA was digested overnight with *EcoRI*, *EcoRV* and *HindIII* using 4 units of enzyme per μg of DNA according to manufacturer's instructions (Gibco BRL). Separation of digested DNA fragments was performed on 1% agarose gel run overnight in TBE buffer. DNA was denatured (1.5M NaCl, 0.5M NaOH) and neutralized (1.5M NaCl, 0.5M Tris-HCl pH 7.5, 10mM EDTA) before being blotted on to Hybond-N (Amersham) or MSI (Micron Separations Inc.) nylon membranes according to standard procedure (Sambrook et al. 1989). Radioactive labeling of DNA probes was carried out by random primer method (Feinberg and Volgelstein, 1983) using Ready-To-Go DNA labeling beads (Pharmacia Biotech) or Oligo labeling buffer (OLB) as described (Berry et al. 1995). Prehybridization (6X SSPE, 0.1% SDS, 5X Denhardt's solution) was followed by hybridization at 60°C in hybridization buffer (6X SSPE, 0.1% SDS). Blots were rinsed in wash buffer (2X SSC, 0.1% SDS) and then washed twice for 30 minutes at 60°C. Membranes were finally exposed to X-OMAT AR film (Kodak) at -80°C. Southern procedure for mapping RFLP loci was done according to Berry et al. (1995) and Jan et al. (1998).

Disease Evaluation

Inoculation and greenhouse screening procedures were performed as described (Miller and Gulya, 1991). Briefly, twenty seedlings of each F_3 progeny were inoculated with Race 1 of downy mildew by a whole-seedling immersion technique. Germinated seeds with radicles of about 1 to 2 cm in length were immersed in an inoculum suspension containing 20 000 to 30 000 sporangia per ml in distilled water for 3 hours. The inoculated seedlings were planted in flats containing a 1:1 (v/v) mixture of perlite and sand. The seedlings were grown in a greenhouse for 12 days with temperatures between 20 and 25°C and a 16-hour photoperiod. The seedlings were then transferred to a chamber with 100% relative humidity for 16 h at 18°C. Susceptibility of seedlings was assessed by the presence of sporulation on the first true leaves and/or cotyledons. Downy mildew resistance was scored as dominant trait based on expected segregation ratio and chi square test. Individuals with ambiguous segregation ratio were scored as missing.

Mapping CDR clones

The mapping population in this study consists of 180 F_2 individuals derived from the cross between HA370 and HA372. The F1 plants were grown in Corvallis, Oregon whereas the F_2 plants were grown in Argentina. Segregation analysis of the CDR clones and 64 previously mapped Zeneca probes (Berry et al. 1996) was performed on 180 F_2 individuals whereas 44 USDA RFLP probes (Jan et al. 1998) were mapped on a subset of the same mapping population consisting of 91 individuals. Mapmaker 3.0 (Lander et al. 1987) and/or G-MENDEL (Holloway and Knapp, 1993) was used employing the Kosambi mapping function at a minimum LOD score of 3.0 and recombination value of 0.35.

Development of CAPS markers

The association of the clone HR-4W2 with the downy mildew resistance, as revealed by linkage analysis of RFLP markers, led us to develop PCR-based marker by utilizing the HR-4W2 sequence. The primer pair SFDM-L and SFDM-R were used to amplify a 362 bp NBS sequences specific to HR-4W2, from downy mildew resistant and susceptible sunflower inbred lines. Representative clones from each class were aligned and primers were designed to amplify the HR-4W2 clone specifically. The sequences of SFDM-L and SFDM-R are: ATG CGG AAA TCT CTC ACC and GAC AGC CTC GTC TTG TGA, respectively. Five different restriction endonuclease (*AluI*, *BstUI*, *NlaIII*, *Sau3AI*, and *Tsp509I* (NEB) were selected by mapping all possible restriction sites in the 362 bp fragment amplified from HA89 (susceptible, recurrent parent) by using Primer Premier computer program (Premier Biosoft International, Palo Alto, CA). Fifteen µl of the PCR product was digested with 2-2.5 units of each restriction enzymes for about 5 hours according to the manufacturer's recommendation. Since *Tsp509I* was the only restriction enzyme that detected polymorphism between resistant and susceptible lines, it was used to produce segregation data on 125 F_2 individuals. Restriction fragments were separated on 2% 3:1 Nusieve GTG agarose (FMC) in 1X TBE buffer and stained with Ethidium Bromide.

RESULTS AND DISCUSSION

PCR products

The primer pair N1-C1 yielded a single band of about 325 bp whereas primer pair N1-C3 resulted in a single band of about 500 bp on a 1% agarose gel. Since NBS is known to be a motif featured in various plant genes, both amplification products were expected to contain heterogeneous fragments. Heterogeneity of

PCR products was confirmed by subjecting both PCR products to SSCP analysis. The SSCP gel showed approximately nine different species from primer pair N1-C1 and five species from primer pair N1-C3 where one pair of SSCP band represents one allele (data not shown).

Cloning and analysis

A total of 38 clones derived from N1-C1 and 69 clones from N1-C3 product were assayed by SSCP and grouped into tentative classes based on their mobility. One to several clones from each tentative classes were sequenced for further characterization. A total of 32 N1-C1 type and 15 N1-C3 type clones were sequenced. Translation of sequences into polypeptides showed 21 clones (twenty from N1-C1, one from N1-C3 primer pair) with open reading frames uninterrupted by stop codons. The rest of the sequences seemed to be pseudogenes, as they contained frequent stop codons. GENBANK database search using BLAST showed that the sunflower CDR sequences were similar to previously identified NBS sequences of soybean (Kanazin et al. 1996) and potato (Leister et al. 1996). In addition, the sunflower sequences exhibited significant similarity to corresponding regions of cloned plant disease resistance genes such as *RPS2*, *N*, L^6, *M*, *RPP1* or *RPP5* all of which belong to the NBS-LRR structural classes (Parker and Coleman 1997; Baker et al. 1997).

Sequence alignment and grouping

Classification of the 21 selected clones based on amino acid sequence similarity computed by 'olddistances' function of the GCG program produced 11 unique classes (Table 4.1). Class 1 contained the largest number of members followed by class 3. Amino acid sequence similarity within a class range from 95-99%. Seven classes contained a single member whereas two classes

consisted of two members each. Clones were considered to belong to the same class if their similarity is above 85%. Alignment of deduced amino acid sequences of eleven sunflower (HR-), six lettuce (Ls-) NBS clones along with the NBS regions of *RPS2* (position 184 to 352), *N* (position 218 to 388), and L^6 (position 267 to 440) was made using the PILEUP function of the GCG (Fig. 4.1). In addition to the kinase-1a and the kinase-3a motifs, which were used as primer sites, the kinase-2 domain (LIVLDD) showed significant conservation in all sequences and was located at the same position as in *RPS2* and N genes. This finding is in agreement with previous studies in sunflower (Gentzbittel et al. 1998), soybean (Kanazin et al. 1996; Yu et al. 1996), and potato (Leister et al. 1996).

Comparison of representatives of each of the 11 classes with *RPS2*, *N*, or L^6 showed that clones from seven groups were more similar to *N* and L^6 (44-58%) than *RPS2* (25 – 36%) whereas clones from the other four groups were more similar to *RPS2* (36 – 43%) than *N* and L^6 (27 - 39%) (Table 4.1). Amino acid sequence similarity between sunflower NBS clones is presented in Table 4.2. Comparison of sunflower sequences to nine RGA clones of soybean (Kanazin et al. 1996) revealed 20-60% amino acid sequence similarity whereas similarity to six RLG clones of lettuce ranged from 13-72%. The sunflower clone HR-1W53 showed 60-70% similarity to two lettuce clones whereas the lettuce clone Ls-Rlg-4 showed 50-70% similarity to many sunflower clones. Figure 4.2 shows a dendrogram based on sequence similarity of various NBS/CDR clones.

Mapping CDR clones

Map position of sunflower CDR clones on four linkage groups of RFLP linkage map was presented in Figure 4.6. This map was constructed by using 108 RFLP markers segregating in 179 F_2 individuals. We attempted to map 17 clones of which only 7 were polymorphic. The 10 monomorphic clones could be mapped in another mapping population.

Figure 4.1. Alignment of deduced amino acid sequences of 11 sunflower (HR-) NBS clones, with, corresponding regions in At-*RPS2* (residues 184 to 352), Nt-N (position 218 to 388), Lu-L6 (position 265 to 378) and lettuce (Ls-) NBS sequences. White letters on a black background show identity and black letters on a grey background show similarity. Similar amino acids: V, I, L, M; F, W, Y; D,E; K, R; A, C, S, T; H, N.

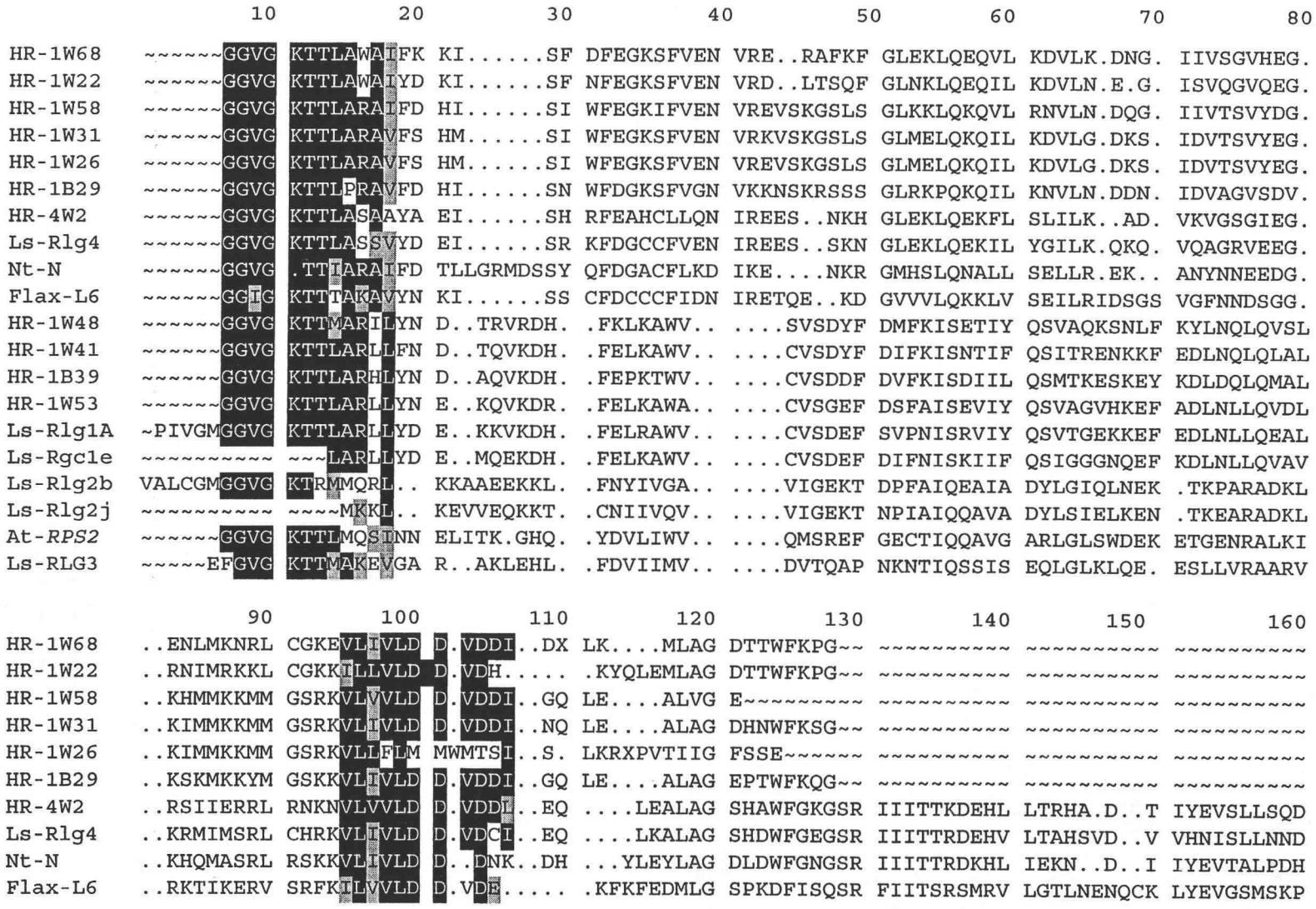
10 20 30 40 50 60 70 80
HR-1W68 ~~~~~~GGVG KTTLAWAIFK KI.....SF DFEGKSFVEN VRE..RAFKF GLEKLQEQVL KDVLK.DNG. IIVSGVHEG.
HR-1W22 ~~~~~~GGVG KTTLAWAIYD KI.....SF NFEGKSFVEN VRD..LTSQF GLNKLQEQIL KDVLN.E.G. ISVQGVQEG.
HR-1W58 ~~~~~~GGVG KTTLARAIFD HI.....SI WFEGKIFVEN VREVSKGSLS GLKKLQKQVL RNVLN.DQG. IIVTSVYDG.
HR-1W31 ~~~~~~GGVG KTTLARAVFS HM.....SI WFEGKSFVEN VRKVSKGSLS GLMELQKQIL KDVLG.DKS. IDVTSVYEG.
HR-1W26 ~~~~~~GGVG KTTLARAVFS HM.....SI WFEGKSFVEN VREVSKGSLS GLMELQKQIL KDVLG.DKS. IDVTSVYEG.
HR-1B29 ~~~~~~GGVG KTTLPRAVFD HI.....SN WFDGKSFVGN VKKNSKRSSS GLRKPQKQIL KNVLN.DDN. IDVAGVSDV.
HR-4W2 ~~~~~~GGVG KTTLASAAYA EI.....SH RFEAHCLLQN IREES..NKH GLEKLQEKFL SLILK..AD. VKVGSGIEG.
Ls-Rlg4 ~~~~~~GGVG KTTLASSVYD EI.....SR KFDGCCFVEN IREES..SKN GLEKLQEKIL YGILK.QKQ. VQAGRVEEG.
Nt-N ~~~~~~GGVG .TTIARAIFD TLLGRMDSSY QFDGACFLKD IKE....NKR GMHSLQNALL SELLR.EK.. ANYNNEEDG.
Flax-L6 ~~~~~~GGIG KTTTAKAVYN KI.....SS CFDCCCFIDN IRETQE..KD GVVVLQKKLV SEILRIDSGS VGFNNDSGG.
HR-1W48 ~~~~~~GGVG KTTMARILYN D..TRVRDH. .FKLKAWV.. ....SVSDYF DMFKISETIY QSVAQKSNLF KYLNQLQVSL
HR-1W41 ~~~~~~GGVG KTTLARLLFN D..TQVKDH. .FELKAWV.. ....CVSDYF DIFKISNTIF QSITRENKKF EDLNQLQLAL
HR-1B39 ~~~~~~GGVG KTTLARHLYN D..AQVKDH. .FEPKTWV.. ....CVSDDF DVFKISDIIL QSMTKESKEY KDLDQLQMAL
HR-1W53 ~~~~~~GGVG KTTLARLLYN E..KQVKDR. .FELKAWA.. ....CVSGEF DSFAISEVIY QSVAGVHKEF ADLNLLQVDL
Ls-Rlg1A ~PIVGMGGVG KTTLARLLYD E..KKVKDH. .FELRAWV.. ....CVSDEF SVPNISRVIY QSVTGEKKEF EDLNLLQEAL
Ls-Rgc1e ~~~~~~~~~~ ~~~LARLLYD E..MQEKDH. .FELKAWV.. ....CVSDEF DIFNISKIIF QSIGGGNQEF KDLNLLQVAV
Ls-Rlg2b VALCGMGGVG KTRMMQRL.. KKAAEEKKL. .FNYIVGA.. ....VIGEKT DPFAIQEAIA DYLGIQLNEK .TKPARADKL
Ls-Rlg2j ~~~~~~~~~~ ~~~~MKKL.. KEVVEQKKT. .CNIIVQV.. ....VIGEKT NPIAIQQAVA DYLSIELKEN .TKEARADKL
At-*RPS2* ~~~~~~GGVG KTTLMQSINN ELITK.GHQ. .YDVLIWV.. ....QMSREF GECTIQQAVG ARLGLSWDEK ETGENRALKI
Ls-RLG3 ~~~~~EFGVG KTTMAKEVGA R..AKLEHL. .FDVIIMV.. ....DVTQAP NKNTIQSSIS EQLGLKLQE. ESLLVRAARV
90 100 110 120 130 140 150 160
HR-1W68 ..ENLMKNRL CGKEVLIVLD D.VDDI..DX LK....MLAG DTTWFKPG~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
HR-1W22 ..RNIMRKKL CGKKILLVLD D.VDH..... .KYQLEMLAG DTTWFKPG~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
HR-1W58 ..KHMMKKMM GSRKVLVVLD D.VDDI..GQ LE....ALVG E~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
HR-1W31 ..KIMMKKMM GSRKVLIVLD D.VDDI..NQ LE....ALAG DHNWFKSG~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
HR-1W26 ..KIMMKKMM GSRKVLLFLM MWMTSI..S. LKRXPVTIIG FSSE~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
HR-1B29 ..KSKMKKYM GSKKVLIVLD D.VDDI..GQ LE....ALAG EPTWFKQG~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
HR-4W2 ..RSIIERRL RNKNVLVVLD D.VDDL..EQ ....LEALAG SHAWFGKGSR IIITTKDEHL LTRHA.D..T IYEVSLLSQD
Ls-Rlg4 ..KRMIMSRL CHRKVLIVLD D.VDCI..EQ ....LKALAG SHDWFGEGSR IIITTRDEHV LTAHSVD..V VHNISLLNND
Nt-N ..KHQMASRL RSKKVLIVLD D..DNK..DH ...YLEYLAG DLDWFGNGSR IIITTRDKHL IEKN..D..I IYEVTALPDH
Flax-L6 ..RKTIKERV SRFKILVVLD D.VDE..... .KFKFEDMLG SPKDFISQSR FIITSRSMRV LGTLNENQCK LYEVGSMSKP

Figure 4.1

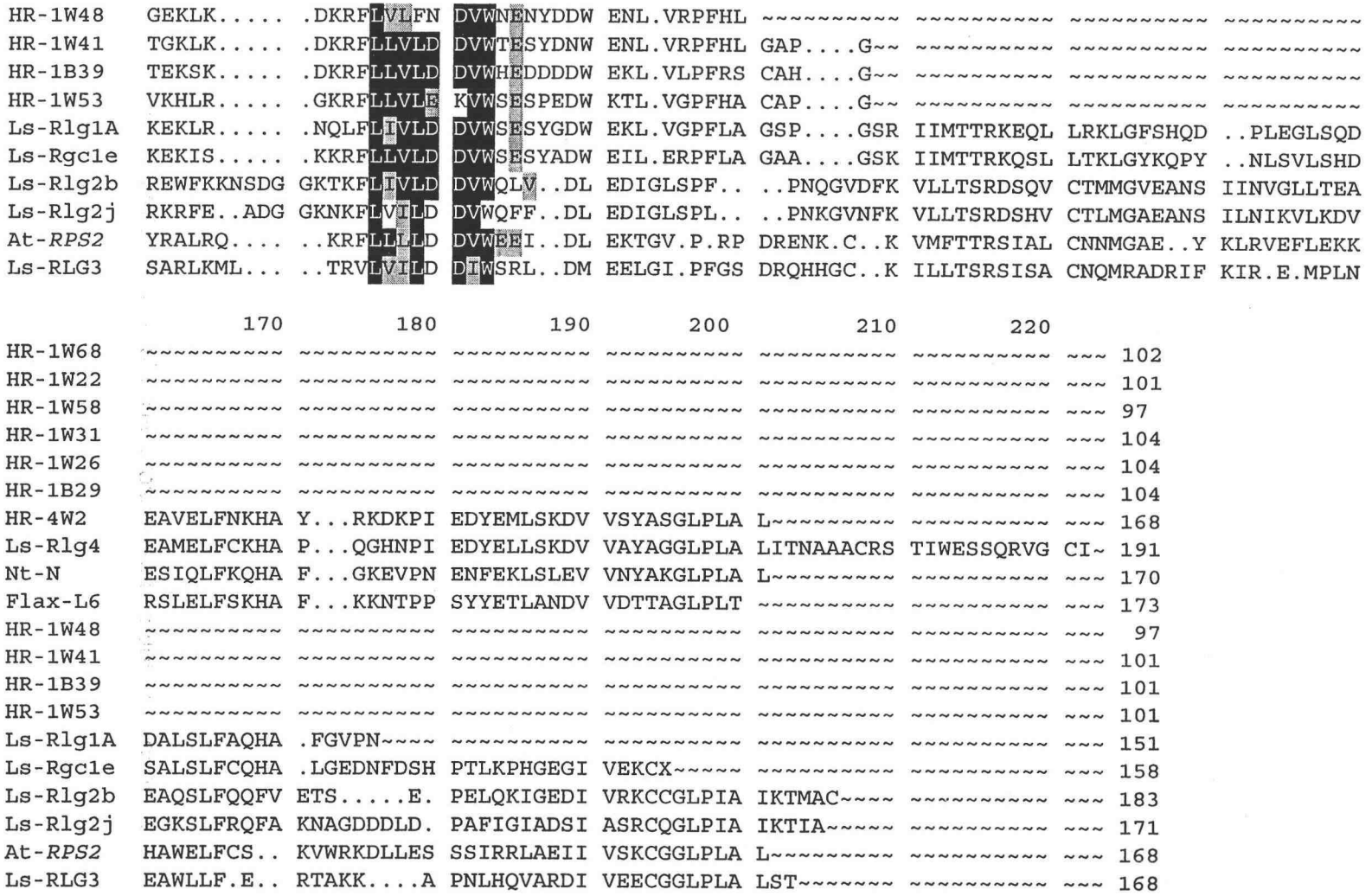

Figure 4.1 Continued

Table 4.1. Classification of sunflower CDR clones based on percent amino acid similarity, the relationship of these clones to tobacco *N*, flax L^6 and *Arabidopsis RPS2* genes, and their map position. Clones marked with "*" are class representatives for comparison with *N*, L^6, and *RPS2*. Map position of each clone on sunflower RFLP linkage map is shown in the last column with NP indicating clones that are not polymorphic.

Class	Clones	# AA	*N*	L^6	*RPS2*	Map position
1	HR-1W68*	102	49	45	28	NP
	HR-1B7	102				NP
	HR-1W4	102				NP
	HR-1B23	102				NP
	HR-1W67	102				-
2	HR-1W22*	101	54	46	26	LG15(2Loci)
3	HR-1B6	102	49	44	28	-
	HR-1W43	103				NP
	HR-1W31*	104				NP
	HR-1B1	104				NP
	HR-1W72	104				NP
4	HR-1W26*	104	50	44	28	LG13
5	HR-1W58*	97	44	40	25	NP
	HR-1W42	104				-
6	HR-1B29*	104	47	40	26	NP
7	HR-4W2*	168	58	48	36	LG8
8	HR-1W48*	98	30	34	38	LG7,13
	HR-1W23	101				LG13
9	HR-1W41*	101	39	36	38	LG13
10	HR-1B39*	101	36	35	43	-
11	HR-1W53*	101	32	27	36	LG13

The 7 polymorphic clones were placed on 4 linkage groups and identified a total of nine loci (Fig. 4.6). Comparison of this map with previous sunflower linkage map (Berry et al. 1996), showed fairly consistent loci order and distance. The clone HR-4W2 was found to be linked to *PI1*, the locus for downy mildew resistance, at a distance of 5.8 cM. Subsequently, the clone HR-4W2 was converted into CAPS marker and mapped. This clone showed greater than 94% amino acid identity and similarity to clone Ha-NBS (Gentzbittel et al. 1998). Ha-NBS probe co-segregated with three loci assigned to linkage group 1 in the

vicinity where the *PI* loci have been located (Mouzeyar et al. 1995; Vear et al. 1997; Roeckel-Drevet et al. 1996).

Table 4.2. Amino acid similarities (%) between partial sequences of sunflower NBS clones.

	HR-1W68	HR-1W22	HR-1W58	HR-1W31	HR-1W26	HR-1B29	HR-4W2	HR-1W48	HR-1W41	HR-1B39	HR-1W53
HR-1W68	100	80	68	66	54	63	54	35	36	38	33
HR-1W22		100	66	65	56	66	49	29	33	33	32
HR-1W58			100	86	75	79	54	31	35	35	34
HR-1W31				100	80	74	51	29	34	34	35
HR-1W26					100	59	39	27	34	29	31
HR-1B29						100	49	26	31	29	32
HR-4W2							100	35	37	40	35
HR-1W48								100	74	63	62
HR-1W41									100	74	69
HR-1B39										100	64
HR-1W53											100

Two probes (HR-1W48 and HR-1W22) each identified two loci. The probe HR-1W48 mapped to linkage group 7 and 13 whereas HR-1W22 identified two loci one of which mapped to linkage group 15 and the other one was not linked to any group. Five clones were located on linkage group 13 with two of these being located at the same position. Several studies have shown that candidate disease resistance genes tend to cluster in plant genomes and frequently map near known disease resistance loci (Aarts et al. 1998; Collins et al. 1998; Kanazin et al. 1996; Leister et al. 1996; Shen et al. 1998). Distribution and organization of these candidate gene sequences correspond to clustering of genes controlling resistance to downy mildew found in sunflower (Gentzbittel et

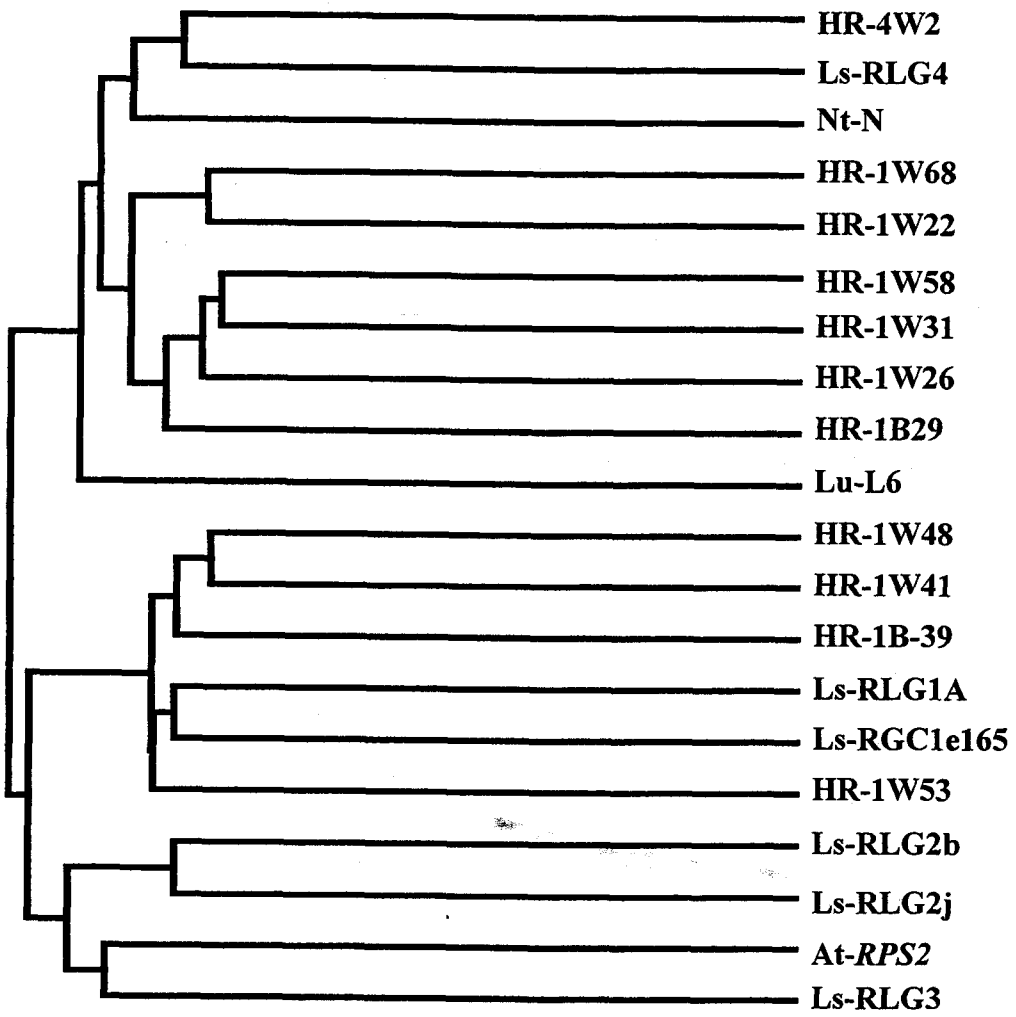

Figure 4.2. Phylogenetic tree based on alignment of amino acid sequences of sunflower CDR gene clones (HR-) along with lettuce (Ls-), *N*, *RPS2*, and L^6 by using Phylip program.

al. 1998; Roeckel-Drevet et al. 1996; Vear et al. 1997) as well as in lettuce (Witsenboer et al. 1995, Meyers et al. 1998; Shen et al. 1998).

HR-1B39 and HR-1W48 mapped to the same position on linkage group 13. HR-1W23 mapped close to the HR-1B39 and HR-1W48 cluster (2.2 cM). The amino acid sequence similarity of HR-1B39 and HR-1W48 was 63% whereas HR-1B39 and HR-1W48 exhibited 38% and 35% amino acid sequence similarity

to HR-1W23 (Table 4.1, class 1). This finding is similar to the observation of Leister et al. (1998) that highly divergent rice NBS-LRR probes map to the same genetic locus. They designated them mixed R gene homologue clusters (RHC). On the other hand, two sunflower NBS clones, HR-1W48 and HR-1W22, each detected two loci on different linkage groups suggesting that evolutionary events such as chromosomal rearrangements, involving duplication and deletion, have taken place in the parental sunflower lines. Our finding is in agreement with previous studies in which detection of multiple loci by a single clone has been reported. In *Arabidopsis* two resistance-EST (R-EST) clones each mapped to two chromosomes (Botella et al. 1997) whereas, in another study in *Arabidopsis,* two clones were found to be positionally linked to three different markers on three different chromosomes (Speulman et al. 1998). A single NBS-LRR probe that detected four loci on different chromosomes has also been reported in rice (Leister et al. 1998). In potato, while most probes detected between one and four loci, one probe revealed ten loci on 7 of 12 chromosomes (Leister et al. 1996). The present study and previous findings confirm the hypothesis that the presence of CDR fragments at multiple loci could be the result of duplication as well as intra- or inter-chromosomal crossover events on the ancestral sequence.

Southern analysis of NILs

We assayed six NIL derivatives of HA89 carrying *Pl* gene by using CDR clones as an RFLP probe. Genomic DNA was digested with *EcoRI*, *EcoRV*, and *HindIII* restriction enzymes. All tested probes detected multiple bands suggesting the presence of multiple copies of these sequences in the sunflower genome. However only HR-4W2 showed polymorphism between lines carrying *Pl* locus and those lacking it (Fig. 4.3). Polymorphism was exhibited in *EcoRV* digested DNA (Fig. 4.3A) by a 2.3 kb fragment that is present in downy mildew resistant lines (except RHA 340), but absent in HA 89 and HA 372 (susceptible lines). Similarly, *HindIII* digested DNA showed polymorphism at a 1.0 kb

fragment which is present in R lines (except RHA 340) and absent in susceptible lines (Fig. 4.3B). The absence of these fragments in RHA 340 suggests that this line might have a different allele. The probe HR-4W2 was amplified by a primer combination spanning the p-loop and membrane spanning region giving a product size of about 500 bp. Amino acid sequence similarity between HR-4W2 and the rest of the clones ranged from 26-54%. Those clones that are monomorphic in this set of NILs might be useful in another set of NILs for other important sunflower diseases, such as rust, phomopsis, anthracnose, and verticillium wilt.

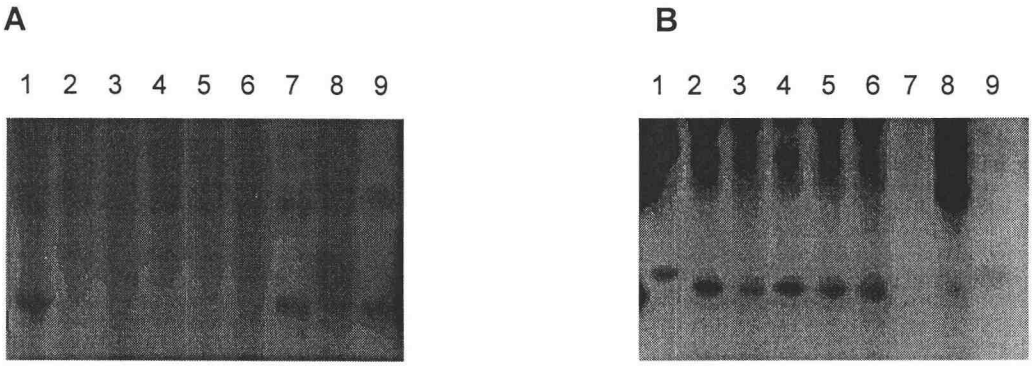

Figure 4.3. Southern blot analysis of sunflower NILs. Genomic DNA digested with *EcoRV* (A), and *HindIII* (B) and probed with the CDR clone HR-4W2. 1=HA89 (susceptible) 2=HA335 3=HA336 4=HA337 5= HA338 6=HA339 7=RHA340 8=HA370 9=HA372 (susceptible).

SSCP analysis of NILs

A pair of primers (SFDM-L and SFDM-R) designed to amplify specifically a region of 362 bp contained in HR-4W2 sequence were used. The hypothesis is that there is a difference in NBS sequence between resistant and susceptible lines. Visualization of PCR products on 1% agarose gel stained with ethidium bromide could not detect any polymorphism between NILs, HA 370, and HA 372.

However, SSCP analysis revealed polymorphism among these lines (Fig. 4.4). The pattern of the bands and number of alleles (2 bands/allele) are similar in resistant lines. Even though it is difficult to determine the precise number of alleles from this study, difference in band mobility was observed between resistant and susceptible lines.

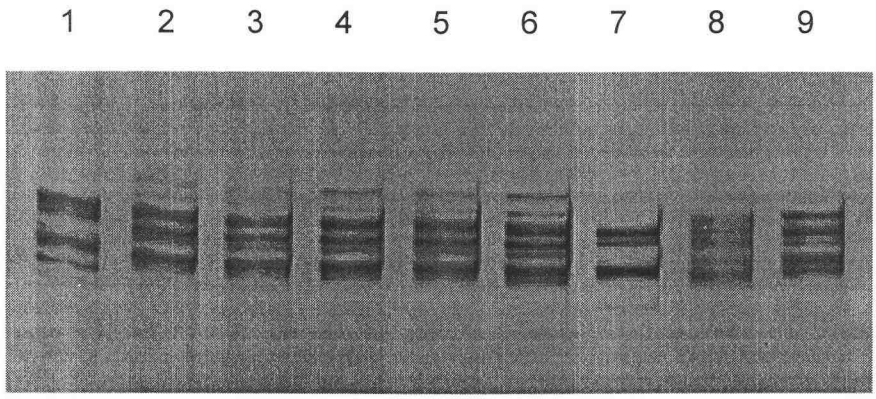

Figure 4.4. SSCP analysis of sunflower NILs. 1=HA89 (susceptible) 2=HA335 3=HA336 4=HA337 5= HA338 6=HA339 7=RHA340 8=HA370 9=HA372 (susceptible)

CAPS analysis of NILs and development of marker for downy mildew resistance

Of the five restriction enzymes tested, only *Tsp509I* revealed polymorphic band between resistant and susceptible lines. Digestion with *Tsp509I* is expected to give 3 fragments of size 85, 93, and 184 base pairs. While susceptible lines (HA89 and HA 372) showed the 3 expected fragment sizes, resistant lines contained an additional 269 bp fragment as well as uncut, 362 bp fragment (Fig. 5). We hypothesized that R lines might contain multiple NBS sequences, some of which lack one or both possible *Tsp509I* recognition sites, resulting in the 269 bp and the 362 bp fragments, respectively.

The 269 bp fragment consistently appeared in all downy mildew resistant lines including HA370, NILs, and donor wild *Helianthus* species. This result led us to map this CAPS marker in the HA370 x HA372 mapping population. The CAPS marker, HR-4W2, was assayed on a progeny of 125 F_2 individuals and showed only four mismatches with *Pl1* phenotype data. Mapping of HR-4W2 along with 118 RFLP markers revealed that HR-4W2 was closely linked to the *Pl1* locus (3.7 cM) (Fig. 4.6).

One of the problems associated with homology cloning is that many NBS containing genes may not be related to resistance. However, once clones associated with disease resistance are identified by segregation analysis or NIL analysis (Muehlbauer et al. 1988; Young et al. 1988), further characterization of the clone can be performed by screening a cDNA library or by rapid amplification of cDNA ends (RACE). Regardless of amino acid sequence similarity to R genes, only a fraction of clones might be functionally involved in disease resistance. Several of the clones used as an RFLP probe to screen NILs were not able to detect polymorphism in the present study. Ohmori et al. (1998) used degenerate primers based on sequences containing LRRs and NBS. Even though these clones showed high similarities with corresponding R genes, they could not detect polymorphism between susceptible and resistant NILs. In our study, out of more than 40 clones sequenced, only 21 were found to be uninterrupted with frequent stop codons suggesting that some of the clones may belong to pseudogenes.

PCR-based homology cloning of CDRs contributes to a better understanding of the molecular basis of evolution of disease resistance genes. This has been demonstrated in a recent study that elucidated rearrangement of NBS-LRR genes in the genomes of rice, barley, and foxtail millet (Leister et al. 1998). The evidence for rearrangement of NBS-LRR genes was the occurrence of intraspecific copy number variation, the existence of mixed R gene homologs (MRH), the absence of interspecific cross-hybridization for several NBS-LRR probes, and the frequent non syntenic map location when there is interspecific cross-hybridization. The use of NBS-LRR clones for comparative mapping has

also been suggested. For example, barley probes mapped in rice. Barley and rice probes could be located on the foxtail millet map by RFLP analysis (Leister et al. 1998).

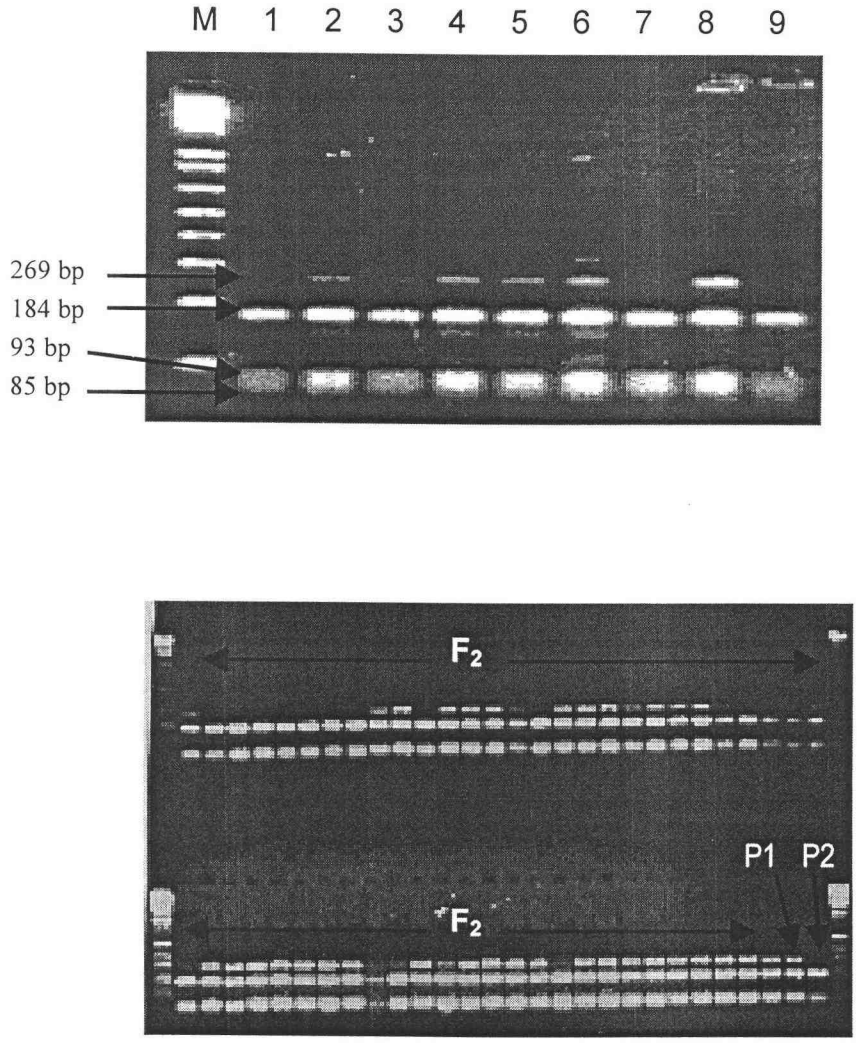

Figure 4.5. CAPS analysis of sunflower NILs (panel A) and F_2 segregating population (panel B). 1=HA89 (susceptible) 2=HA335 3=HA336 4=HA337 5= HA338 6=HA339 7=RHA340 8=HA370 9=HA372 (susceptible).

Mapping of the HR-4W2 probe placed it on linkage group 8 at a distance of 3.7 cM from the *Pl1* locus. SSCP and Southern analysis of NILs confirmed the association of HR-4W2 with downy mildew resistance. Furthermore, HR-4W2 showed 94% amino acid sequence identity with Ha-NBS (Gentzbittel et al. 1998). The latter mapped to the *Pl* region on linkage group 1 of the sunflower consensus linkage map (Mouzeyar et al. 1995; Roeckel-Drevet et al. 1996; Vear et al. 1997).

Isolation of candidate disease resistance genes [also known as resistance gene analogs (RGA), resistance gene homologs (RGH), or resistance gene candidates (RGC)] from diverse plant species, including lettuce (Shen et al. 1998), *Arabidopsis* (Aarts et al. 1998; Botella et al. 1997; Speulman et al. 1998), soybean (Kanazin et al. 1996; Yu et al. 1996), potato (Leister et al. 1996), wheat (Feuillet et al. 1997), tomato (Ohmori et al. 1998), barley, rice (Leister et al. 1998), and sunflower (Gentzbittel et al. 1998), consistently showed that these candidate genes are closely associated with mapped disease resistance genes, exist in multigene families, and are clustered in the genome. Such homology cloning was further exploited to isolate full-length cDNAs and ultimately led to the cloning of LRK-10, a gene conferring resistance to leaf rust of wheat (Feuillet et al. 1997). Isolation of a full-length cDNA of Ha-NBS is in progress (Gentzbittel et al. 1998). In the present study, we developed CAPS marker tightly linked with downy mildew resistance based on the sequence in HR-4W2. One of the advantages of CAPS marker is that they are expected to be co-dominant, in contrast to the dominant inheritance of RAPD markers (Konieczny and Ausubel 1993). In our study, however, we were only able to score the CAPS marker as a dominant (present/absent) marker. Scoring of this marker as a co-dominant locus was not possible due to, perhaps, the presence of multiple NBS sequences that have varying number of recognition sites. Previous studies indicated that resistance to several races of downy mildew mapped to the same chromosomal region in sunflower (Roeckel-Drevet et al. 1996; Vear et al. 1997). Further studies are required to confirm that HR-4W2 marker is linked to the *Pl* gene cluster. This study provides an easily assayable, PCR-based marker that can be

utilized for sunflower molecular breeding aimed at the development of disease resistant cultivars.

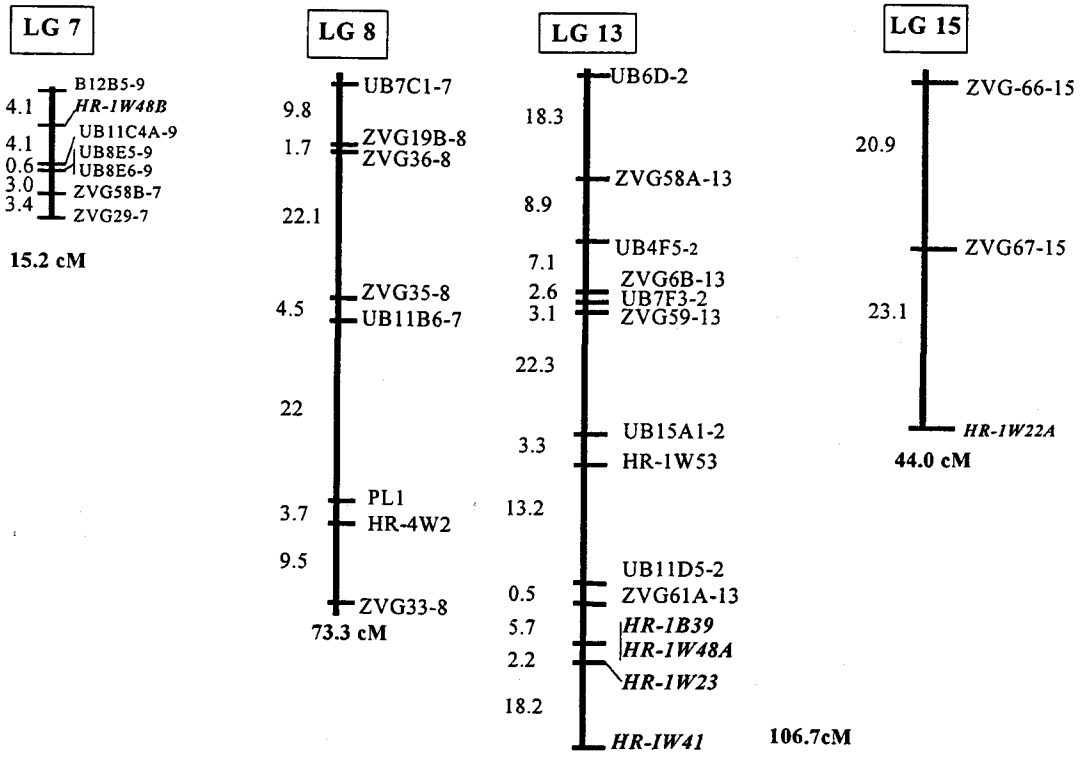

Figure 4.6. RFLP-based linkage map of sunflower CDR clones mapped in 180 F_2 plants derived from the cross HA370 x HA372. Four of seventeen linkage groups (shown at the top) that comprise CDR clones and previously mapped RFLP probes (locus names on the right and distance in centimorgan on the left) are shown. Marker names shown in bold italics and with an "HR" prefix refer to sunflower CDR clones. Letters following the locus name indicate probes that detected multiple loci.

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CHAPTER 5

CONCLUSION

As compared to other oil crops, the number of mapped public DNA markers in sunflower is limited, many gaps persist in the map and the density of mapped public markers need to be increased. The objectives of this study were to generate genetic mapping resources for sunflower molecular breeding and to utilize the markers for various genetic analyses. The first study, development of SSR markers, was undertaken to help alleviate the problem of low DNA marker polymorphism in sunflower. Despite their apparent advantages, SSR markers have not been widely used in sunflower because of limited number of SSR markers described so far. Screening of genomic DNA library with 5 TNRs and 2 DNR oligonucleotide probes yielded 313 putatively positive clones of which 109 contain SSRs. Primers were designed for 96 clones to facilitate multiplexing by varying the lengths of target sequences and forcing the primers to work under a narrow range of PCR conditions. A total of 120 primers sets, including 24 previously published SSR markers, were screened for variability across 22 sunflower genotypes. Out of the 44 polymorphic SSR markers tested on all genotypes, 41 were polymorphic among wild species whereas 23 were polymorphic among elite inbred lines. Overall, the number of alleles ranged from 1 to 13 and heterozygosities varied from 0.0 to 0.92. The mean number of alleles per locus and heterozygosities in elite inbred lines were 1.86 and 0.21, respectively. Number of alleles per locus and heterozygosities were slightly higher among the wild species than with in the elite lines. The level of polymorphism exhibited by TNRs was less than MNR and DNR. Cluster analysis and multidimensional scaling clearly separated elite lines from wild species. The SSR markers developed in the present study were not as hypervariable as expected. The heterozygosities detected by this markers were less than the heterozygosities reported for RFLPs based diversity analysis of sunflower inbred lines (Berry et al. 1994; Gentzbittel et al. 1994; Zhang et al. 1995). As most of

the SSR markers were polymorphic between inbred lines and wild species, they could be utilized for marker-assisted backcrossing for the introgression of desirable genes such as disease resistance. Since only few SSRs were found to be polymorphic in one or another existing mapping population their utility for mapping is not very high. However, different mapping populations can be used to maximize the number of mapped SSR markers (Senior et al. 1996).

Development of SSR markers is tedious, expensive, and time consuming. Screening of nearly nine hundred thousand clones yielded only 44 useful markers in the present study. Future work on SSR marker development for sunflower should be designed to improve efficiency by using libraries enriched for various repeats including DNRs and TNRs. Other strategies to improve the proportion of useful SSR markers include (i) pre-digestion of genomic DNA with methylation sensitive enzymes to represent the single copy DNA (Struss and Plieske 1998; Roder et al. 1998a), (ii) the use of physically sheared DNA (Chen et al. 1997), and (iii) the use of a cDNA library (Milbourne et al. 1998).

Twenty-seven SSR markers showed a banding pattern that was difficult to score. The scoring of these markers was complicated due to stutter bands, gene duplication and heterozygosities of the wild species. Resolution of these SSR markers can be improved by optimizing the PCR condition for each primer pairs individually as has been done previously (Whitton et al. 1997). Redesigning and testing of several possible primer pairs has yielded unequivocally scorable bands in maize (Smith et al. 1997) and wheat (Bryan et al. 1997). Recently, Stephenson et al. (1998) reported recovery, by redesigning of the primers, of 12 SSR markers from previously discarded sequences in wheat. Similar approaches can be followed to increase the utility of SSR markers developed in our study.

Even though a relatively dense sunflower RFLP map was constructed recently, the number of mapped public DNA markers is still limited and separate sets of RFLP probes were used in different mapping populations (Berry et al. 1995, 1996, 1997; Gentzbittel et al. 1995; Jan et al. 1998). The aim of the second study (chapter 3) was to develop an integrated RFLP-AFLP linkage map

of sunflower using RFLP probes from two different sources (Berry et al. 1996; Jan et al. 1998) and AFLP markers generated by 42 primer combinations. The map was built using 180 HA 370 x HA372 F_2 progeny. The polymorphism rates for RFLP and AFLP were 82% and 20%, respectively between HA370 and HA372. Four hundred and one loci (105 RFLP and 296 AFLP loci) were mapped into 17 linkage groups covering 1326 cM with a mean spacing of 3.3 cM between markers. This map integrates 14 out of 17 linkage groups from two previously published RFLP maps. The RFLP markers were well spaced and well distributed throughout the genome. Some linkage groups were sparsely populated with common markers. There were gaps of 35 or more cM in two linkage groups. This integrated map can serve as a framework map for the sunflower research community. Existing gaps can be filled by mapping more RFLP, AFLP, or SSR markers.

AFLPs provide an efficient, high throughput molecular marker for sunflower genome analysis and molecular breeding. They are particularly important tools in studies where scoring as dominant markers is sufficient. Such studies include mapping in backcross, doubled haploid, or recombinant inbred populations, and marker-assisted backcross breeding (Knapp et al. 1999). Furthermore, the utility of AFLPs as co-dominant markers can be realized as the hardware and software for co-dominant scoring AFLPs become available to most laboratories. Presently, RFLPs are the most widely used molecular markers for diversity analysis and construction of genetic linkage maps in sunflower. The present map combines the advantage of both RFLP and AFLP markers for sunflower molecular breeding and lays the groundwork for building high resolution linkage maps that also include sequence-based markers such as CAPSs, SCARs, and SSRs. Development of di-nucleotide SSR markers is underway in our lab. Mapping of SSR markers in other plants showed that SSRs are evenly distributed throughout the genome (Akkaya et al. 1992; Cho et al. 1998; Roder et al. 1998a, 1998b).

We used degenerate oligonucleotide primers based on conserved sequences in disease resistance genes from other species to PCR amplify CDR gene

fragments from sunflower (*Helianthus annuus* L.). Sequence analysis of 21 clones yielded 11 unique groups with majority of the classes showing more similarity to the tobacco N gene and the flax L^6 gene (44-58%) than to the *Arabidopsis RPS2* gene. BLAST search of the GENBANK database revealed that the sunflower CDR clones were homologous to previously cloned plant disease resistance genes such as *RPS2*, *N*, L^6, *M*, *RPP1*, and *RPP5*. Seven clones mapped to four linkage groups and identified nine loci. Two clones each produced two polymorphic loci suggesting chromosomal rearrangements such as duplication of resistance genes in sunflower.

We identified CDR genes closely linked to a downy mildew resistance gene and utilized this clone to develop PCR-based molecular marker. Cleaved amplified polymorphic sequence (CAPS) marker, linked to downy mildew resistance at 3.7 cM, was derived from the clone HR-4W2. Single strand conformational polymorphism (SSCP) and Southern analysis of NILs also confirmed the association of HR-4W2 with downy mildew resistance. This CAPS marker might be useful for marker-assisted selection for downy mildew resistance in sunflower. Available genetic information on downy mildew resistance genes in sunflower suggests that genes conditioning resistance to different races often map to the same region of a chromosome (Roeckel-Drevet et al. 1996; Vear et al. 1997). Resistance genes in other plant species such as lettuce were also found to be clustered and belong to multigene families (Witsenboer et al. 1995, Meyers et al. 1998).

CDRs or resistance gene analogs (RGAs) are frequently found linked to disease resistance genes in plants with many mapped resistance genes (Kanazin et al. 1996; Leister et al. 1996, 1998; Speulman et al. 1998; Yu et al. 1996, Meyers et al. 1998). Based on this finding, we speculate that some of the candidate genes we isolated may be linked to as yet unmapped clusters of resistance genes in sunflower. The CAPS marker developed for downy mildew resistance gene provides an opportunity for rapid screening of large population using a small amount of DNA. It allows marker-assisted selection, which accelerates the process of introgression of resistance genes from wild species to

cultivated species, reduces linkage drag, and facilitates pyramiding of resistance genes.

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