TOWARDS CLONING THE LEAF RUST RESISTANCE GENE *RPH5*

By

JAFAR MAMMADOV

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHYLOSOPHY

In

CROP AND SOIL ENVIRONMENTAL SCIENCES

Dr. M.A. Saghai Maroof, Chairman

Dr. G.R. Buss

Dr. A. Esen

Dr. C.A. Griffey

Dr. J.G. Jelesko

02 August, 2004

Blacksburg, Virginia

Keywords: barley, leaf rust, *Rph5*, molecular mapping, marker-assisted selection, gene pyramiding, rice, Expressed Sequence Tag (EST), high resolution map, synteny, comparative mapping, Resistance Gene Analog (RGA), physical mapping, Bacterial Artificial Chromosome (BAC), contig

Towards Cloning The Leaf Rust Resistance Gene *Rph5*

By

Jafar Mammadov

(Committee Chair: M.A. Saghai Maroof)

Department of Crop and Soil Environmental Sciences

Virginia Polytechnic Institute and State University

ABSTRACT

Leaf rust caused by *Puccinia hordei* is an important disease of barley (*Hordeum vulgare*) in many regions of the world. Yield losses up to 62% have been reported in susceptible cultivars. The *Rph*5 gene confers resistance to the most prevalent races (8 and 30) of barley leaf rust in the United States. Therefore, the molecular mapping of *Rph*5 is of great interest. Genetic studies were performed by analysis of 93 and 91 F_2 plants derived from the crosses 'Bowman' (*rph*5) × 'Magnif 102' (*Rph*5) and 'Moore' (*rph*5) × Virginia 92-42-46 (*Rph*5), respectively. Linkage analysis positioned the *Rph*5 locus to the extreme telomeric region of the short arm of barley chromosome 3H at 0.2 cM proximal to RFLP marker *VT1* and 0.5 cM distal from RFLP marker *C970* in the Bowman × Magnif 102 population. Synteny between rice chromosome 1 and barley chromosome 3 was employed to saturate the region within the sub-centimorgan region around *Rph5* using sequence-tagged site (STS) markers that were developed based on barley expressed sequence tags (ESTs) syntenic to the phage (P1)-derived artificial chromosome (PAC) clones comprising distal region of the rice chromosome 1S. Five rice PAC clones were used as queries to blastn 370,258 barley ESTs. Ninety four non-redundant EST sequences were identified from the EST database and used as templates to design 174 pairs of primer combinations. As a result, 10 EST-based STS markers were incorporated into the 'Bowman' × 'Magnif 102' high-resolution map of the *Rph5* region. More importantly, six markers, including five EST-derived STS sequences, co-segregate with *Rph5*. Genes, represented by these markers, are putative candidates for *Rph5*. Results of this study demonstrate the usefulness of rice genomic resources for efficient deployment of barley EST resources for marker saturation of targeted barley genomic region.

DEDICATION

To my father, Akif, father in law, Chinghiz, and grandmother, Najiba. They will forever live in my dreams

ACKNOWLEDGMENTS

- I would like to sincerely thank my advisor, Dr. Saghai Maroof for being an exceptional mentor during my graduate study at Virginia Tech.
- I am thankful to my advisory committee members Drs. Asim Esen, Glenn R. Buss, John G. Jelesko and Carl A. Griffey for providing expert guidance during my graduate work and revising my dissertation.
- Special thank goes to Dr. Ruslan M. Biyashev for colossal help in the lab and valuable advices throughout my research.
- I am very grateful to the people who spent their time and effort to assist me in the laboratory and greenhouse. These include Dr. A.J. Hayes, Dr. S.-C. Jeong, Dr. B. Steffenson, and W. Brooks.
- I am thankful to all my friends for their support and inspiration.
- Last but not least, I would like to thank my mother, Izabella, for her continuous love and support. I am very grateful to my mother in law, Saida, for taking care of my family during my first year of graduate study at Virginia Tech, without her I would not be able to survive. Finally, I would like to thank my amazingly wonderful wife, Afet, and children, Najiba and Ilkin, for their love, patience, support and belief.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

History of barley crop

Barley is one of the earliest domesticated plants. The most ancient remains of wild barley [*Hordeum spontaneum*] date back to about 17,000 years B.C. (Kislev et al. 1992). Its history as a crop began in the Fertile Crescent, an area that today includes parts of Iran, Turkey, Syria and Jordan. From its origin in Middle Eastern civilization, barley spread to the Mediterranean Basin. Based on archaeological research, during the sixth and fifth millenniums B.C., cultivated barley was found in the Aegean region and subsequently in all other regions of the eastern part of the Mediterranean Basin (Zohary and Hopf 1993) and later in the sites of the Nile Delta (Darby et al. 1977). At the same time barley rapidly expanded in the eastern direction that is documented from early remains of this crop found in the Caucasus and Transcaucasus regions (Lisitsina 1984) and even in the highlands of the Indian subcontinent (Costantini 1984). During the fourth millennium B.C. barley reached the western parts of the Mediterranean Basin (Hopf 1991). The pathway of barley expansion from the Aegean region turned to the north moving upward along the riverbeds of the Danube, throughout the Balkan region, upward along the Dniester from Ukraine into Poland. During the third millennium, cultivated barley reached Central and Northern Europe (Körber-Grohne 1987). In Asia, namely China, cultivated barley appeared only during the second half of the second millennium B.C. (Ho 1977). Much more recent in time and well documented is the spread of cultivated barley into the Americas and Oceania. Settlers following the Spanish conquerors introduced barley seeds into Mesoamerica and the southern parts of the United States (Poehlman 1959).

Major uses of the barley crop

Animal feed

About 85 percent of today's world barley production is used for feeding animals. Primarily, grain supplies carbohydrates and protein in the ration. Protein content varies from 10 to 15 percent depending on the climatic and soil conditions and is generally comparable to protein content of wheat grown in a similar environment. In areas, where corn cannot be grown successfully due to short growing seasons, cool temperatures or deficiency in rainfall, barley often is grown as the principal feed grain. The demand for livestock feed accounts for the intensity of barley cultivation in areas such as western and northern Europe, Russia, California, the Northern Plains in the USA and western Canada (Fischbeck 2002).

Malt

Malt production is the second largest use of barley. Mainly, malt from barley is used in the brewing industry. Despite the decrease in beer consumption in the major beer-consuming European countries, world production of beer grows and approaches 1.3 billion liters of beer per year that requires 18 million tons of barley (Fischbeck 2002). This phenomenon can be explained by rising beer consumption in other European countries and in parts of Asia and South America (Fischbeck 2002).

Food

Barley is largely used for human consumption in regions with climate conditions that are unfavorable for other cereals. These regions include Tibet, Ethiopia and Peru. Apart from these, barley food consumption has lost most of its earlier position in human nutrition, at least in industrialized countries (Fischbeck 2002).

Shares of barley in today's cereal production

Recently (1996 to 1998) about 65 million ha per year were devoted to barley cultivation worldwide and occupied approximately 9.1 percent of the world's cereal

acreage (FAO 1998). Since the early decades of the twentieth century, barley ranked fourth in total cereal production behind wheat, rice and maize. However, a close look at the geographical distribution of barley cultivation reveals a very characteristic degree of unbalance. For example, Europe contributes 60 percent to the world's barley production, while production in North America has about an equal share as Asia, despite substantial differences in barley acreage. For instance, in Syria and Iraq, barley occupies more than 40 percent of the cereal acreage. These countries include large parts of the Fertile Crescent region. The large barley acreage in this region is based on high degree of adaptation of barley landraces (Ceccarelli et al. 1995) that still dominate barley production within the area due to the drought-inflicted climate and salinity-prone soil conditions. Another region in which barley occupies the prime position in cereal culture extends from Ireland across Scotland to the northern countries of Scandinavia, including Estonia.

In contrast, in the US, barley is mainly produced in the Northern Plains and Pacific Northwest, where the climate conditions are favorable for malting barley. In total, 27 states in the USA are involved in barley production, and the largest producers of this important crop are North Dakota (28.5%), Idaho (17.8%), Montana (15.6%), Washington (9.9%) and Minnesota (5.0%), which produce more than 76% of the total barley countrywide (Statistical Highlights of U.S. Agriculture 2001-2002). In contrast to those five states, Virginia barley production is not large-scale. It is mostly oriented towards production of feed barley for grain. In 2001 Virginia producers harvested 45,000 acres (18,225 ha) of winter feed-barley for grain. In 2001 grain yields across the state averaged 80 bu ac^{-1} (4300 kg ha^{-1}). Total grain production in 2001 was 3.6 million bushels (78,367 metric tons) (Rohrer et al. 2001).

Application of genomics tools to barley improvement

Introduction

People using conventional methods of plant breeding have improved cultivated crops, including barley, by increasing their yield, improving their quality and making them resistant to devastating diseases and pests and tolerant to stresses. For each crop, specific breeding objectives and strategies towards improvement had been applied and

advanced. However, a majority of these methods are labor- and time-consuming; and therefore conventional plant breeding was supplemented by the new biotechniques (Bourlag 1997). A rapidly developing science during the last twenty years, genomics and its techniques, particularly DNA Marker Technology (DMT), became an extremely important tool in plant breeding, with potential for significant contributions to crop improvement in terms of reduction of time necessary to produce crop cultivars with desirable traits. DMT is based on new types of genetic markers: DNA molecular markers. The evolution of genetic markers encompasses several periods (Liu 1997). The first genetic markers were morphological and cytological ones. They were popular up to late 1950s. In the 1960s they were supplanted by biochemical genetic markers such as isozymes. First DNA molecular markers, namely Restriction Fragment Length Polymorphism (RFLP), arose after discovering restriction enzymes and developing Southern blotting techniques in 1975. Finally, after discovering the thermostable enzyme Taq polymerase and inventing the PCR machine in 1980s, a new era in the history of genetic markers began - the era of PCR-based molecular markers such as Simple Sequence Repeats (SSR), Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment-Length Polymorphism (AFLP), Sequence Tagged Site (STS), Simple Sequence Length Polymorphism (SSLP) and Single Nucleotide Polymorphism (SNP).

The use of molecular markers is based on DNA sequence polymorphisms (difference), which can serve as genetic loci. Depending on the nature of polymorphism, the methods of detection can include the use of restriction enzymes, nucleic acid hybridization or DNA sequence amplification. However, regardless of detection method, the primary goal is that the monitored polymorphism determines a specific region on a particular chromosome that is limited to a trait of interest (Beckman and Osborn 1992).

Molecular markers

DNA sequence differences are very useful markers, because they are abundant and easy to characterize precisely. Molecular markers can be developed by different techniques. However, they may vary in their degree of reliability, ease of use and cost of assay and the nature of polymorphism they detect. Below is a brief description of the most popular molecular markers.

RFLP was one of the first DNA markers developed. Polymorphism, revealed by RFLP, is a result of difference in molecular weight of the fragments of host DNA, caused by simple point mutation, a nucleotide insertion/deletion or transposition events at the region where a restriction enzyme cuts (Botstein et al. 1980; Burr 1994). RFLP technology is very reliable and can be used for accurate scoring of genotypes. Moreover, RFLPs are co-dominant and allowing the detection of two or more allelic states of a given locus. Additionally, it does not require DNA sequencing and is gel-based. The detection and visualization of RFLP markers are carried out by techniques called Southern blotting and autoradiography, respectively (Snyder and Champness 1997). However, conventional RFLP techniques are difficult to automate and that makes analysis very slow and time-consuming. Furthermore, RFLP analysis is very expensive (Kohert 1994). Using RFLP markers, linkage maps for many crops have been developed, including barley, wheat, rye, oats, sugarcane (GrainGenes database, http://wheat.pw.usda.gov/index.shtml) and rice (Kurata et al.1994).

The above-mentioned disadvantages of RFLPs have been more or less eliminated with the development of PCR-based markers such as SSR, RAPD, STS and AFLP. For instance**, RAPD** techniques, which are based on the amplification of random DNA sequences by a single and short oligonucleotide primer, are inexpensive, fast, easy to perform and do not require radioactive visualization. However, one of the main drawbacks of RAPD analysis is its inconsistency, which is conditioned by the sensitivity of the short primers to the annealing conditions (Burr 1994). Moreover, they are dominant markers. However, Paran and Michelmore (1993) converted them to **Sequence Characterized Amplified Regions** (SCARs), which are inherited in a co-dominant manner.

SSRs, or microsattelites, are short motifs (2-5 bases) that are repeated multiple times and are flanked by unique DNA sequence (Hearne et al. 1992). These unique DNA sequences are used to design primers to amplify the different number of repeat units in different genotypes. Polymorphism is detected as a length difference in the amplified product. Wide use of SSRs in genetic mapping is conditioned by high degree of polymorphism (Saghai Maroof et al. 1994) and abundance, high frequency in diverse genome, ease of PCR assay and ease of distribution among laboratories. However, not

many primer sequences for detecting SSRs were initially developed in plants (Liu et al. 1996). Recently SSR markers have been developed for plant species, including soybean, rice, barley, maize, Arabidopsis and grape (Powel et al. 1996).

Another efficient PCR-based marker, called AFLP, was developed in the mid 1990s (Vos et al. 1995). AFLPs combine the restriction site recognition element of RFLP with the exponential amplification aspects of PCR markers. It operates on the same principle as a RAPD, but the primer consists of a longer fixed portion (~ 15 bp) and shorter random portion (2-4bp). The long portion conditions the primer stability and the short random portion means it will amplify many loci. Polymorphism is detected as the presence/absence of a band (Vuylsteke et al.1999).

STS is a short stretch of sequence that can be PCR-amplified (Olson et al. 1989). The primers for STS are designed based on the DNA sequence of RFLP, genomic DNA or cDNA clones. However, RFLP clones are not the only source for generating the STS markers. Expressed Sequence Tags (EST) can also be used to design STS primers. Since STS has a known sequences, they are very important markers for conversion of genetic map into a physical one. Based on sequence information of STS, specific genes can also be isolated.

SNP represents sites, where two sequences differ by a single base as a result of a point mutation. SNPs are very popular, because they are highly abundant in the genome (Cho et al. 1999; Picoult-Newberg et al. 1999; Griffin and Smith 2000). SNPs have been characterized in crop plants such as beet, maize and soybean at a frequency of 1/130 bp, 1/61 bp and 1/610 bp, respectively (Schneider et al. 2001; Rafalski 2002a, 2002b). Most SNPs, actually about two of every three SNPs, involve the replacement of cytosine (C) with thymine (T). SNPs occur every 100 to 300 bases along the human genome. SNPs are stable from an evolutionary standpoint - not changing much from generation to generation - making them easier to follow in population studies, and their inheritance is much higher than SSRs and AFLPs (Kwok et al. 1996). That is why, they are especially useful for association studies and ideally suited for the generation of high-density genetic maps (Cho et al. 1999; Nairz et al. 2002). SNPs can be detected either by oligonucleotide hybridization, oligonucleotide ligation, primer extension, DNA sequencing, PCR primer mismatch, pyrosequencing or heteroduplex assays (Wallace et al. 1979; Newton et al.

1989; Wu and Wallance 1989; Syvanen et al. 1990; Ronaghi et al. 1998; Oefner and Underhill 1998; Giordano et al. 1999; Pastinen et al. 2000). In most cases, these methods require initial determination of the target sequence within a set of different genotypes, making them quite expensive to implement for the detection of polymorphism. Another disadvantage of SNP is its biallelic nature (Landegren et al. 1988).

EST is a DNA sequence that corresponds to a reverse translated mRNA. They represent single pass, partial sequences from cDNA clones and have been extensively used for gene discovery and mapping in a wide range of organisms. Rapidly growing EST databases allow detecting the regions showing sequence similarity in functionally related gene products from distantly related organisms. It is possible to assign putative functions for a large portion of anonymous cDNA clones. Importantly, EST databases are available for many economically important crops (http://www.ncbi.nlm.nih.gov) including cereals: *Triticum aestivum* (wheat) 549,926, *Zea mays* (maize) 391,417, *Hordeum vulgare subsp. vulgare* (barley) 352,924 and *Oryza sativa* (rice) 283,935 (Summary by Organism - February 27, 2004). These databases are publically available and free to use. EST clones have been extensively used as molecular markers for the construction of high-density genetic linkage maps of rice and maize (Harushima et al. 1998; Davis et al. 1999) and for a physical map of rice (Kurata et al. 1997). In addition, sequence data can be used to study gene families (Cooke et al. 1997; Epple et al. 1997) and to develop SNPs (Cho et al. 1999). Along with applications in structural and comparative genomics, ESTs are very important for functional genomics. They are the core resource for the analysis of gene expression with the help of high-density arrays, as demonstrated for *Arabidopsis* (Schena et al. 1995; Girke et al. 2000; Schenk et al. 2000).

Molecular mapping in barley

Molecular markers very quickly found their application in genetic research projects for the improvement of cultivated crops, including barley. They have been involved in tagging and mapping the agronomically important genes. Use of molecular techniques as diagnostic tools to assist the conventional breeding process demands the construction of linkage maps. Molecular mapping of the barley genome has been facilitated by the development of molecular markers, doubled haploid (DH) lines, the

availability of numerous mutants and cytogenetic stocks, particularly the barley-wheat addition lines, and the recent development of large insert libraries (e.g., Bacterial Artificial Chromosome library).

In 1988 Kleinhofs, Chao, and Sharp created the first RFLP map for the barley chromosome 6 (1988). After two years Shin et al. (1990) published a partial map of the whole barley genome. These two initiatives served as a starting point towards the further molecular mapping of the barley genome. Later in the 1990s, numerous and high-density molecular linkage maps of the barley genome were constructed (Graner et al. 1991; Kelinhofs et al. 1993; Qi et al. 1998). Additionally, various studies focusing in specific chromosomes, regions, genes, or traits have generated a number of additional maps (Hinze et al. 1991; Barua et al. 1993; Devos et al. 1993; Giese et al. 1994; Becker et al. 1995; Komatsuda et al. 1995; Laurie et al. 1995; Bezant et al. 1996; Laurie et al. 1996; Schonfeld et al. 1996; Ellis et al. 1997). Finally, worldwide mapping efforts have located more than 2,000 different molecular markers in the Steptoe × Morex and Igri × Franka maps, which have been the most comprehensive genetic maps so far (Kleinhofs and Graner 2001). Importantly, more than 100 common markers were placed in both maps. These findings allow the scientists to create the so-called consensus maps. The main idea of the consensus maps is to combine several genetic linkage maps into one based on the order of the common markers. Several consensus maps were created by Langridge et al. (1995) and Qi et al. (1996). Due to numerous consensus map efforts, the North American Barley Genome Mapping Project (NABGMP) introduced the Bin Map concept to barley (Kleinhofs and Graner 2001). Briefly, using the Steptoe × Morex (SM) map as a base, the barley genome was segmented into 10 cM Bins. Genes and markers mapped in different genetic maps were placed in the corresponding Bins. The SM map contains 60, 64, 58, 41, 50, 49, and 62 unique loci assigned to chromosomes 1 through 7, respectively. Additional 235 markers co-segregate with the 384 unique loci. A total of 952 different molecular markers or genes have been located to individual Bins on the seven chromosomes. The total number of different markers or genes that have been placed to the SM map chromosome Bins is 1,571. In the Bin maps, a majority of the markers are unique RFLP markers that have been mapped in barley. It also contains very few of the morphological/physiological genes, and practically none of the hundreds of RAPD or

AFLP markers. The description of individual markers and the maps are accessible at http://barleygenomics.wsu.edu.

Marker-assisted selection of disease resistance cultivars in barley

Marker-assisted selection

Genetic linkage maps highly saturated with DNA molecular markers have been widely used in mapping, or tagging, disease resistance genes (R genes). Basically, gene tagging refers to locating genes of interest through linkage to molecular markers. A molecular marker that is closely linked to the gene of interest serves as a "tag" that can be utilized for the selection of the gene in breeding programs. Consequently, gene tagging constitutes the basis of marker-assisted selection (MAS).

It has been almost 80 years since Sax (1923) demonstrated the potential value of genetic markers, namely morphological, in the selection of agronomic traits associated with them. Particularly, he showed the association of seed size with seed coat pigmentation in beans. However, inherited morphological markers are very rare and in most cases irrelevant to breeding germplasm, marker-assisted plant breeding remained of theoretical interest until the development of DNA-based molecular markers in the late 1970s. For the first time, researchers began to identify the large number of markers dispersed throughout the genome of species and use of markers to detect associations with traits of interest, thus allowing MAS to finally become a reality. In classical genetic improvement programs, selection is carried out based on observable phenotypes but without knowing which genes are actually being selected. The development of molecular markers was therefore greeted with great enthusiasm as it was seen as a major breakthrough promising to overcome this key limitation. In fact, MAS represents enormous potential. Theoretical studies have demonstrated that MAS has potential to be more effective than conventional phenotypic selection. According to Knapp (1998), in order to guarantee selection of one or two superior genotypes, a breeder using phenotypic selection must test 2.0 to 16.7 times more progeny than a breeder using MAS. Unlike phenotypic selection, MAS is faster and more reliable. For example, during a single day, a breeder can test hundreds of samples using agarose or polyacrylamide gel

electrophoresis (PAGE). Young (1999) considers MAS useful, when it is difficult to score the phenotype of trait. That is why, it is especially promising in breeding for agronomic traits such as resistance to pathogens, insects and nematodes; tolerance to abiotic stresses and, some quality parameters (Melchinger 1990). The reliability of MAS is especially important in breeding for disease resistance, because breeders can eliminate the risk of selecting pseudo-resistant samples, which are, in fact, susceptible plants escaping infection (Melchinger 1990). However, the success of MAS strongly depends on the availability of tightly linked molecular markers. According to Dekkers (2003), there are two types of relationship between a molecular marker and a gene of interest that make MAS very successful and efficient. (1) The molecular marker is located within the gene of interest. The author refers to this situation as gene-assisted selection (GAS). This is the most favorable case. On the other hand, it is very difficult to find this kind of markers, because researcher has, first, to isolate and sequence a gene of interest and then based on this sequence to develop a PCR-based marker, which can be employed for MAS. (2) Another relationship between a molecular marker and a gene of interest is when marker is in linkage disequilibrium (LD) with a gene. LD is the tendency of certain combinations of alleles to be inherited together. LD can be found when markers and genes of interest are physically very close to each other (Dekkers et al. 2003).

Because of universal nature of DNA markers, MAS, in theory can be applied to any agriculturally important species and can be applied to support existing conventional breeding programs (Dekkers and Hospital 2002).

However, despite all potentials of MAS, there are few reports available, where a practicing plant breeder successfully used MAS leading to release of a commercial variety. Particularly in barley, one of the most notable cases is the development of efficient MAS scheme for screening genotypes carrying *rym4* and *rym5* genes conferring resistance to barley yellow mosaic virus (BaYMV). Initially, Tuvesson et al. (1998) developed STS marker derived from the RFLP probe MWG838 that allowed screening for *rym4* gene in the early stages of the breeding cycle. Scottish Crop Research Institute (SCRI) developed an SSR marker closely linked to the *rym4* and *rym5* genes (Thomas 2003). The marker was of great value because it could also distinguish between the two resistance genes as well as the susceptible phenotype. SCRI used this marker to evaluate

over 100 barley genotypes of known reaction to BaYMV and correctly predicted the phenotype of each. Currently, many European plant breeders are using this marker in MAS schemes for resistance to BaYMV (Thomas 2003). Additionally, in order to speed up detection methods, SCRI has developed an SNP assay that can be multiplexed on the Pyrosequencing platform to select lines predicted to carry either *rym4* or *rym5* (Thomas 2003). Apart from MAS schemes for resistance to BaYMV, SCRI has other SSR markers that can be used to select for the *mlo11* and *Rh2* resistance genes to powdery mildew and scald of barley, respectively (Thomas 2003).

A majority of studies advocating MAS have ignored factors that can limit development and application of this technique. One of them is the relatively high expense related to molecular marker assays (Gupta et al. 2002). Use of molecular markers is quite costly, because standard procedures in molecular marker analysis are multi-step, require well-trained staff, technical support, lab space, and radioisotope permits. All the above-mentioned issues have to be considered and ways to alleviate them are needed. One of the ways to decrease the expense of MAS is to use markers that are amenable to automation. If several years ago, the molecular marker of choice was SSR, currently, marker technology has changed. In particular, "SNPs are starting to remove any effective limitation on marker discovery, in an even more spectacular way than the development of microsatellite technology has already done" (Koebner 2003). Currently, the large private sector, particularly the maize industy, and major breeding companies (in the USA primarily Pioneer Hi-Bred, Syngenta and Monsanto; in Europe, in addition to these, KWS and Limagrain) automate the whole process of marker genotyping, and increasingly rely on SNPs as a technology platform. For example, in "Monsanto's US operation, the past five years have seen the development of thousands of new marker assays, a 17-fold increase in the acquisition rate of marker data, and a decrease in unit data point cost of 75%" (Koebner 2003). However, the cost of an average SNP assay still is high. As a comparison, in human genomics, wide-scale usage of SNP technology can be feasible in case of ten-fold reduction of the current rate of $0.10 per assay (Roses 2002).

Another factor that should be considered is the number of genes involved in a MAS program. Mackill et al. (1999) discussed the population size requirement based on the number of genes involved in MAS. For instance, with only four or five loci being

selected, the population size and the number of F_2 seeds needed for MAS will be considerable and any further addition will lead to an exponential increase. According to the authors, only the most important traits or loci should be identified and selected through MAS program. Furthermore, Gupta et al. (2002) argue that use of molecular markers becomes prohibitive due to factors including the size of segregating populations, the number of replications in a trial, the number of field trials that are needed to study genotype × environment interaction, and finally the number of QTL-associated molecular markers that need to be used simultaneously in the same population.

The special issue is the efficiency of MAS for the traits governed by QTLs. In their recent review, Dekkers and Hospital (2002) argued that "as theoretical and experimental results of QTL detection have accumulated, the initial enthusiasm for the potential genetic gains allowed by molecular genetics has been tempered by evidence for limits to the precision of the estimates of QTL effects" and that "overall, there are still few reports of successful MAS experiments or applications". According to Koebner (2003), "in situations where QTLs are themselves unreliable, either because of epistasis or due to genotype × environmental interactions (so that the effect is environmental-dependent), MAS directed at QTL variation is also unreliable; but where such interactions are insignificant, genetic progress is predictable and MAS, if economically justifiable, is advantageous".

The efficiency of MAS strongly depends on physical location of the marker and tagged target gene or QTL within the chromosome. In the regions of the genome, where recombination is restricted, markers can appear to be genetically closely linked to the gene of interest but in reality separated by a considerable physical distance. Barua et al. (1993) identified markers closely linked to a major gene for scald resistance on chromosome 3H. However, these markers failed to identify genotypes carrying scald resistance genes. This situation can be explained by the location of scald resistance gene near the centromere of chromosome 3H, where recombination is suppressed. Consequently, "there is a need to consider the location of the target region in relation to the physical map before embarking upon an MAS scheme" (Thomas 2003).

Meanwhile, there is considerable divergence between different crop species with respect to their applications of MAS. Koebner (2003) stressed the relatively fast uptake of

MAS in maize compared with wheat and barley, "arguing that it largely reflects the breeding structure, where maize breeding is dominated by a small number of large private companies that produce F_1 hybrids, a system allowing protection from farm-saved seed and competitor use, while for the other major cereal species breeding is carried out primarily by public sector organizations and most varieties are inbred pure breeding lines, a system allowing less protection over the released varieties".

In his "A cautiously optimistic vision for marker-assisted breeding", Young (1999) wrote: "even though marker-assisted selection now plays a prominent role in the field of plant breeding, examples of successful, practical outcomes are rare. It is clear that DNA markers hold great promise, but realizing that promise remains elusive". However, five years passed and "situation with MAS is starting to crystallize" (Koebner 2003). Koebner (2003) argues that "technology itself is no longer limiting". In a year to come marker availability will not be an issue anymore, because SNP "will soon represent a source of plentiful within-gene markers and are set to be developed for all the major cereals" (Koebner 2003). Due to genomics revolution, biological assays have gotten miniaturized and automated leading to reduction of the assay price and consequently, the penetration of MAS into commercial cereal breeding. According to Koebner (2003), for maize "this stage is already being reached. But for wheat and barley, MAS use is likely to remain less central to the breeding process and be deployed only for specific purposes, including

- the accelerated selection of a few traits that are difficult to manage via conventional phenotypic selection
- maintenance of recessive alleles in backcrossing programs
- pyramiding of disease resistance genes
- guiding the choice of parents to be used in crossing programs"

Gene pyramiding

MAS likely will be very useful in pyramiding genes conferring disease resistance. Since pathogens and insects can eventually overcome resistance conditioned by a single race-specific gene, the primary goal of a breeder is to incorporate different multiple resistance genes into one genotype. This approach is called gene pyramiding. Gene

pyramiding can be very difficult or impossible using conventional breeding methods primarily due to epistasis, where the effect of one gene masks the effects of other genes, particularly when a breeding line already has one or more effective genes for the traits of interest. So far, rice has been the only crop, where gene pyramiding has been successfully employed through MAS. The remarkable examples of gene pyramiding include bacterial blight (BB) resistance, blast resistance and gall midge resistance. Huang et al. (1997) brilliantly showed the value of MAS in pyramiding four BB resistance genes including *Xa4*, *xa5*, *xa13* and *Xa21*. Since the effect of two recessive genes *xa5* and *xa13* were masked by dominant genes *Xa4* and *Xa21*, correspondingly, it was difficult to select plants carrying both dominant and recessive genes by conventional methods alone. Only the molecular marker RG136, tightly linked to the recessive gene *xa13*, enabled the selection of genotypes containing both recessive and dominant genes. Similar research with BB resistance gene pyramiding was done by Yoshimura et al. (1996), Sanchez et al. (2000) and Singh et al. (2001). Hittalmani et al. (2000) fine mapped three genes (*PiI*, *Piz-5*, *Pita*) for blast resistance in rice and then pyramided those using MAS.

Pyramiding epistatic resistance genes through conventional breeding has been difficult. Miklas et al. (2000) combined three genes conferring resistance to bean common mosaic virus in common bean, namely, $bc\text{-}1^2$, $bc\text{-}2^2$ and $bc\text{-}3^3$. Interestingly, both $bc\text{-}2^2$ and $bc\text{-}3^3$ were epistatic to $bc\text{-}1^2$ and it was practically impossible to select genotypes containing all three genes based on phenotypic data alone. However, after the identification of a RAPD marker tightly linked to the gene $bc\text{-}1^2$, it became possible to pyramid all three genes.

Molecular mapping of disease resistance genes in barley

Potential acceleration in the development of disease resistant cultivars in barley using marker-assisted selection has encouraged plant molecular geneticists to tag disease resistance genes with molecular markers. Thomas (2002) [in Slafer et al. (2002)] summarized the long-term efforts of barley R gene mappers and included information about molecular markers that could be used in breeding for resistance to all economically important diseases (Table 1.1). The table does not contain information about barley leaf rust, because it will be discussed later in the experimental part of the dissertation.

High-resolution maps

High-resolution, or fine map, is a genetic map, which is based on a large mapping population, size of which can be 1000 and more individuals. A large mapping population allows for identification of more recombinants that can give valuable information about the real distance between markers and a gene of interest. Such information is an important prerequisite for map-based cloning. High-resolution maps have been created for several barley genomic regions. The first fine maps were constructed to locate the genes conferring morphological traits such as liguleless (*lig*) (Konishi 1981), and waxyness *(wax)* (Rosichan et al. 1979) as well as the disease resistance locus *mlo* (Jorgensen and Jensen 1979). In 1994, DeScenzo et al. conducted the first molecular marker-based high-resolution mapping of *Hor1/Mla/Hor2* region on barley chromosome 5HS. Since then molecular-based fine maps have been produced for the disease resistance loci *Rpg1* (Brueggeman et al. 2002), *Mlg* (Kurth et al. 2001), *rpg4* (Druka et al. 2000), *Mla* (Schwarz et al. 1999) *Rar1* (Lahaye et al.1998) and *Mlo* (Simons et al. 1997). Additionally, apart from single disease resistance genes, fine maps have been constructed to map QTLs conferring traits such as malting quality (Han et al. 1997) and head shattering in a six-rowed barley (Kandemir et al. 2000).

Map-based cloning of barley R genes

MAS and gene pyramiding are only methods to accelerate the development of disease resistant cultivars. Plant transformation is the other very promising method to create disease resistant cultivars by direct introduction of a gene into a desirable background. However, in order to make a gene available for transformation, it has to be cloned. Map-based, or positional cloning strategy provides a promising method for gene isolation based on its phenotype and genomic location. The first step in map-based cloning is to generate a genetic map for the genomic region containing the gene of interest. A genetic map for the specific genomic region is commonly obtained by resolving the order of a number of markers tightly flanking the target gene. The main idea is to find molecular markers closely linked and flanking the gene of interest. The next step is physical mapping, i.e., mapping of the distance between the markers flanking the gene of interest, where the distance is measured in base pairs. Physical mapping includes

several stages. The major requirement for physical mapping is the availability of large insert genomic libraries such as Bacterial Artificial Chromosome (BAC) or Yeast Artificial Chromosome (YAC). The use of YAC libraries has been limited because of the high frequency of chimeric and unstable clones. In contrast, BAC vectors from the mini—F plasmid have much higher cloning efficiencies, improved fidelity and greater ease of handling (Shizuya et al. 1992). Due to BAC clone stability and ease of use, the BAC cloning system has emerged as the system of choice for the construction of large insert genomic DNA libraries. Plant BAC libraries have been used for a number of structural genomic applications such as map-based cloning of disease resistance genes (Song et al. 1995), physical mapping (Yang et al. 1998) and examining genomic structure (Chen et al. 1997). The first stage in physical mapping is screening a BAC library with the bracketing markers for the gene of interest to identify BAC clones with DNA segment identical to flanking markers. The second stage is the construction of BAC contig, spanning the region between the markers flanking the gene of interest. If the identified BAC clones do not overlap, the development of contig becomes impossible meaning that physical distance between two flanking markers is too large. In order to find the "missing" BAC clones for contig construction, it is necessary to undertake a third stage of physical mapping: chromosome walking or chromosomal landing. Chromosome walking includes the isolation of the ends of BAC clones followed by the screening of BAC library with them. Chromosomal landing starts with identifying tightly linked molecular markers within the sub-cM region. The DNA markers are then used to screen a library and isolate (or land on) the clone containing the gene (Tanksley et al. 1995). The clones identified by chromosome walking/chromosomal landing allow us to complete the contig and get physical distance between the markers flanking the region.

Map-based cloning has been very successful in species with a small genome size such as *Arabidopsis thaliana* and rice (Bent et al. 1994; Grant et al. 1995, Mindrinos et al. 1994; Parker et al. 1997; Yoshimura et al. 1996; Song et al. 1995; Jander et al. 2002; Yamanouchi et al. 2002). Also, this technique has been applied for isolation of genes from plants with large genomes, including lettuce (Meyers et al. 1998), potato (Bendahmane et al. 1997), tomato (Martin et al. 1993), apple (Patocchi et al. 1999), pepper (Tai et al. 1999) and sugar beet (Cai et al. 1997). With respect to barley, so far

four genes have been cloned by map-based cloning: two genes conferring resistance to powdery mildew (*Erysiphe graminis*) *mlo* (Simons et al. 1997; Buschges et al. 1997), *Mla* (Wei et al. 1999) and particularly *Mla6* (Halterman et al. 2001); *Rar1* (Shirasu et al. 1999), gene mediating resistance to powdery mildew in barley and participating in signal transduction leading to synthesis of Reactive Oxygen Species (ROS) such as hydrogen peroxide (H_2O_2) and *Rpg1*- gene conferring resistance to barley stem rust (*Puccinia graminis*) (Brueggeman et al. 2002). In addition, another member of the *Rpg* family, *rpg4,* has been physically mapped (Druka et al. 2000).

Plant bioinformatics and barley genomics

The growth in the number and scale of genome programs in crop plants including barley in the last two decades has led to the creation of public plant bioinformatics resources, accessible over the Internet. In general, bioinformatics is a research approach that includes the engineering of information systems (such as the creation of databases), the development of analytic methods (such as data-mining tools to extract biologically significant patterns in sequence or other data), and the creation of computation-based, predictive models that use multiple types of data to understand how plant systems operate. Bioinformatics enables the investigators to access, integrate, analyze, and compare large datasets, and is becaming vital to genomics research.

Barley genomics resources also have been growing due to several ongoing huge genome projects. Below is a summary of the genome databases, where barley scientists can find valuable information for their research projects in structural, comparative and functional genomics.

UK CropNet

http://ukcrop.net/barley.html

The UK genome database program was established in 1996 with the creation of the UK Crop Plant Bioinformatics Network (UK CropNet). UK CropNet is a BBSRC-funded collaboration between the Nottingham Arabidopsis Stock Centre (NASC), the John Innes Centre (JIC), the Institute of Grassland and Environmental Research (IGER) and the Scottish Crop Research Institute (SCRI). The network was established to

maintain the data coming from the UK plant genome programs, to develop databases and other bioinformatics tools with a comparative genomic focus, and to collaborate closely with US counterparts. UK CropNet curates the Arabidopsis Genome Resource (AGR), BarleyDB, BrassicaDB, CerealsDB (a UK node of the US GrainGenes database), FoggDB (forage grasses), MilletGenes and SpudBase (potato) databases and the comparative databases ComapDB (a comparative mapping database) and CropSeqDB (Crop Sequence Database).

The cereals research group at the Scottish Crop Research Institute is making a major contribution towards understanding the structure and evolution of the barley genome. This work is generating a large amount of linkage, genotypic, and phenotypic information, which has been recorded and stored at BarleyDB (http://ukcrop.net/barley.html). Barley DB has been expanded considerably through incorporation of the barley maps, traits, and sequences from public databases. In addition, there are details of sequences and primers for almost 480 SSR markers, 370 of which have linkage map locations. The SSR primers and their details are available as an Excel spreadsheet that users can download from the SCRI. In addition to the linkage maps, there are also over 78,000 barley DNA and over 230 barley protein sequences in the database.

Gramene

http://www.gramene.org/about/index.html

Gramene is a comprehensive information system for grass genomes, centered on rice, the model grass species. Extensive work over the past two decades has shown remarkably consistent conservation of gene order within large segments of linkage groups in rice, maize, sorghum, barley, wheat, rye, sugarcane and other agriculturally important grasses.

A substantial body of data supports the notion that the rice genome is substantially collinear at both large and short scales with other crop grasses, opening the possibility of using rice synteny relationships to rapidly isolate and characterize homologues in maize, wheat, barley and sorghum. Gramene has been built upon data

derived from public projects in genomics and EST sequencing, protein structure and function analysis, genetic and physical mapping, interpretation of biochemical pathways, gene and QTL localization and descriptions of phenotypic characters and mutations. Gramene is an extension of the RiceGenes database.

GrainGenes

http://wheat.pw.usda.gov/index.shtml

GrainGenes is a genetic database for Triticeae, oats, and sugarcane, being assembled as part of the United States Department of Agriculture, National Agricultural Library's Plant Genome Program. GrainGenes is a compilation of molecular and phenotypic information on wheat, barley, rye, triticale, and oats. Barley scientists can find various useful kinds of information related to genetic and cytogenetic maps, genomic probes, nucleotide sequences, genes, alleles and gene products, associated phenotypes, quantitative traits and QTLs, genotypes and pedigrees of cultivars, genetic stocks, and other germplasms, pathologies and the corresponding pathogens, insects, and abiotic stresses, addresses and research interests of colleagues, and relevant bibliographic citations.

The Institute for Genomic Research

http://www.tigr.org/tdb/tgi/hvgi/GenInfo.html

The Institute for Genomic Research (TIGR) was founded in 1992 as a not-for-profit research institute whose primary research interests are in structural, functional and comparative analysis of genomes and gene products from a wide variety of organisms including viruses, eubacteria (both pathogens and non-pathogens, archaea) (the so-called third domain of life), and eukaryotes (plants, animals, fungi and protists such as the malarial parasite). TIGR's Genome Projects are a collection of curated databases containing DNA and protein sequence, gene expression, cellular role, protein family, and taxonomic data for microbes, plants and humans.

TIGR’s Barley Gene Index (HvGI) integrates research data from international Barley EST sequencing and gene research projects. The ultimate goal of the HvGI is to represent a non-redundant view of all barley genes and data on their expression patterns,

cellular roles, functions, and evolutionary relationships. According to Release 8.0 (January 9, 2004), there are 21, 981 Temporary Contigs (TC) and 27,041 singleton ESTs deposited at HvGI; and all data are freely available to researchers at nonprofit institutions using it for non-commercial purposes.

National Center for Biotechnology Information (NCBI)

http://www.ncbi.nlm.nih.gov

NCBI was established in 1988 as a national resource for molecular biology information. NCBI's nucleotide and protein sequence databases receive genome data from sequencing projects from around the world and serve as the basis for bioinformatics research. Different types of barley sequences are widely represented at various NCBI's databases such as GenBank, EST database (dbEST), GSS database (dbGSS), STS database and UniGene database. Hereafter, all information related to the number of barley sequences deposited in the above-mentioned databases are as of March 5, 2004.

- **GenBank** is an annotated collection of all publicly available nucleotide and amino acid sequences. In total 389,788 and 3,015 barley nucleotide and protein sequences are being stored at GenBank.
- **dbEST** - A collection of expressed sequence tags, or short, single-pass sequence reads from mRNA (cDNA). Approximately, 356,848 EST sequences have been entered so far.
- **dbGSS -** A database of genome survey sequences, or short, single-pass genomic sequences. This database includes the following type of data: random "single pass read" genome survey sequences, cosmid/BAC/YAC end sequences, exon trapped genomic sequences, Alu PCR sequences, and transposon-tagged sequences. To date, 146 GSS have been entered into database.
- **dbSTS -** is an NCBI resource that contains sequence and mapping data on short genomic landmark sequences or Sequence Tagged Sites. About 224 STS barley sequences can be retrieved from this database.

To conclude, the role of Bioinformatics in plant molecular biology is increasing. It is not surprising, why co-chair of the Interagency Working Group (IWG) for Plant Genomes, the group that establishes NPGI funding and research priorities, Mary Clutter

indicated in her speech at the Plant and Animal Genomics XI Conference held in San Diego earlier last year that “each proposal submitted for funding will be required to have informatics component”. To get a sense of exactly what type of informatics work the agencies are looking for in new proposals, one need only look at the specifications spelled out by the National Science Foundation for its Plant Genome Research Program. The NSF tells researchers applying for grants: “This year's competition focuses on functional genomics, the identification of functions of a pathway or a cluster of genes at a genomic scale, and new informatics tools to disseminate, access, and analyze massive dispersed data sets”.

This increased emphasis on plant bioinformatics is not confined to the United States, where the federal funding for the NPGI is about $100 million a year. Earlier this year, the Australian government set aside $18 million to build the National Center for Plant Functional Genomics (http://www.bio-itworld.com/news/031003_report2163.html).

Table 1.1. List of major genes and molecular markers with potential to be used in barley breeding [Thomas in Slafer et al. (2002)].

Gene	Marker Type	Chromosome	Distance from R gene, cM	Reference
Resistance to powdery mildew				
Mla	RFLP	1(7H)	<1	Schuller et al. 1992
Mla6	RFLP	1(7H)	<5	Graner et al. 1991
Mla25-28	RFLP	1(7H)	<1	Jahoor and Fischbeck 1993
Mla29	RFLP	1(7H)	<1	Kintzios et al. 1995
Mla32	RFLP	1(7H)	<1	Kintzios et al. 1995
Mlf	RFLP	7(5H)	5.3	Schonfeld et al. 1996
Mlg	RFLP	4(4H)	~1	Grog et al. 1993
Mlj	RFLP	5(1H)	3.5	Schonfeld et al. 1996
MILa	RFLP	2(2H)	1	Giese et al. 1993
MILa	RFLP	2(2H)	3	Hilbers et al. 1992
mlo	RFLP	4(4H)	~1	Hinze et al. 1991
mlo	RAPD	4(4H)	1.6	Manninen et al. 1997
MITR	RFLP	5(1H)	5	Falak et al. 1999
Resistance to net blotch				
Pt,,a	STS	3(3H)	0.8	Graner et al. 1996
Resistance to leaf stripe				
Rdg1a	RFLP	2(2H)	0.2	Thomsen et al. 1997
Resistance to scald				
Rh	RFLP	3(3H)	0	Graner and Tekauz 1996
Rrs13	RFLP	6(6H)	7.3	Abbot et al. 1995
Rh2	RFLP	7(5H)	0	Schweizer et al. 1995
Resistance to stem rust				
Rpg1	RFLP	7(5H)	0.3	Kilian et al. 1994
rpg4	RAPD	5(1H)	0.8	Borovkova et al. 1995
Resistance to Barley Yellow Mosaic Virus (BaYMV)				
rym3	RFLP	5(1H)	7.2	Saeki et al. 1999
rym4	RFLP	3(3H)	1.2	Graner and Bauer 1993
rym4	STS	3(3H)	1.2	Bauer and Graner 1995
rym4	RAPD	3(3H)	3.2	Weyen et al. 1996
rym5	SSR	3(3H)	1.3	Graner et al. 1999
rym9	RFLP	4(4H)	0	Bauer et al. 1997
rym11	RFLP	4(4H)	0	Bauer et al. 1997
Resistance to BaMMV				
rmm7	RFLP	1(7H)	0	Graner et al. 1999
Resistance to Barley Yellow Dwarf Virus (BYDV)				
Yd2	RFLP	3(3H)	0	Collins et al. 1996
Yd2	STS	3(3H)	0.7	Paltridge et al. 1998

References

Abbott, D.C., Lagudah, E.S., and Brown, A.H.D. 1995. Identification of RFLPs flanking a scald resistance gene on barley chromosome 6. Journal of Heredity, **86**: 152-154.

Barua, U.M., Chalmers, K.J., Thomas, W.T.B., Hackett, C.A., Lea, V., Jack, P., Forster, B.P., Waugh, R., and Powell, W. 1993. Molecular mapping of genes determining height, time to heading, and growth habit in barley (*Hordeum vulgare*). Genome, **36**: 1080-1087.

Bauer, E. and Graner, A. 1995. Basic and applied aspects of the genetic analysis of the *ym4* virus resistance locus in barley. Agronomie, **15**: 469-473.

Bauer, E., Weyen, J., Schiemann, A., Granre, A., and Ordon, F. 1997. Molecular mapping of novel resistance genes against barley mild mosaic virus (BaMMV). Theor. Appl. Genet. **95**: 1263-1269.

Becker, J., Vos, P., Kuiper, M., Salamini, F., and Heun, M. 1995. Combined mapping of AFLP and RFLP markers in barley. Mol. Gen. Genet. **249**: 65-73.

Beckman, J.S. and Osborn, T.C. 1992. Plant genomes: methods for genetic and physical mapping. Kluwer Academic Publishers, London, pp. 37-69.

Bendahmane A., Kanyuka, K., and Baulcombe, D.C. 1997. High-resolution genetical and physical mapping of the *Rx* gene for extreme resistance to potato virus X in tetraploid potato. Theor. Appl. Genet. **95**: 153-162.

Bent, A.F., Kunkel, B.N., Dahlbeck, D., Brown, K.L., Schmidt, R.K., Giraudat, J., Leung, J.L., and Staskawicz, B.J. 1994. *RPS2* of *Arabidopsis thaliana*: A leucine-rich repeats class of plant disease resistance gene. Science, **265**: 1856-1860.

Bezant, J., Laurie, D., Pratchett, N., and Chojecki, J. 1996. Marker regression mapping of QTL controlling flowering time and plant height in a spring barley (*Hordeum vulgare L.*) cross. Heredity, **77**: 64-73.

Borovkova, I.G., Jin, Y. and Steffenson, B.J. 1998. Chromosomal location and genetic relationship of leaf rust resistance genes *Rph9* and *Rph12* in barley. Phytopathology, 88: 76-80.

Botstein, D., White, R.L., Skolnick, M., and Davis, R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. **32**: 314-331.

Bourlag, N.E. 1997. Feeding a world of 10 billion people: the miracle ahead. Plant Tissue Culture and Biotechnology, **3**: 119-127.

Brueggeman, R., Rostoks, N., Kudrna, D., Kilian, A., Han, F., Chen, J., Druka, A., Steffenson, B., and Kleinhofs, A. 2002. The barley stem rust-resistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinases. Proc. Natl. Acad. Sci. U.S.A. **99**: 9328-9333.

Burr, B. 1994. Some concepts and new methods for molecular mapping in plants. *In* DNA-based markers in plants. *Edited by* R.L. Phillips and I.K. Vasil. Kluwer Press, Amsterdam, pp. 1–7.

Buschges, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., van Dalen, R., van der Lee, T., Diergaarde, P., Groenendijk, J. et al. 1997. The barley Mlo gene: A novel control element of plant pathogen resistance. Cell, **88**: 695-705.

Cai, D., Klein, M., Kifle, S., Harloff, H.J., Sandal, N.N., Marcker, K.A., Klein-Lankhorst, R.M., Salentijn, E.M.J., Lange, W., Stiekema, W.J., Wyss, U., Grundler, F.M.J., and C. Jung. 1997. Positional cloning of a gene for nematode resistance in sugar beet. Science, **275**: 832-834.

Ceccarelli, S., Grando, S., and van Leur, I.A.G. 1995. Barley landraces offer new breeding options for stress environments. Diversity, **11**: 112-113.

Chen, M., SanMiguel, P., De Olivera, A.C., Woo, S.-S., Zhang, H., Wing, R.A., and Bennetzen, J.L. 1997. Microcolinearity in sh2-homologous regions of the maize, rice and sorghum genomes. Proc. Natl. Acad. Sci. U.S.A. **94**: 3431-3435.

Cho, R. J., Mindrinos, M., Richards, D. R., Sapolsky, R. J., Anderson, M., Drenkard, E., Dewdney, J., Reuber, T. L., Stammers, M., Federspiel, N., Theologis, A., Yang, W. H., Hubbell, E., Au, M., Chung, E. Y., Lashkari, D., Lemieux, B., Dean, C., Lipshutz, R. J., Ausubel, F. M., Davis, R. W., and Oefner, P. J. 1999. Genome-wide mapping with biallelic markers in *Arabidopsis thaliana.* Nat. Genet. **23**: 203-7.

Collins, N.C., Paltridge, N.G., Ford, C.M. and Symons, R.H. 1996. The *Yd2* gene for barley yellow dwarf virus resistance maps close to the centromere on the long arm of barley chromosome 3. Theor. Appl. Genet. **92**: 858-864.

Cooke, R., Raynal, M., Laudie, M., and Delseny, M. 1997. Identification of members of gene families in *Arabidopsis thaliana* by contig construction from partial cDNA sequences: 106 genes encoding 50 cytoplasmic ribosomal proteins. Plant J. **11**: 1127–1140.

Costantini, L. 1984. The beginning of agriculture in the Kalchi plain: The evidence of Mehrgarts. South Asian Archaeology 1981. Proceedings of the 6th International Conferences Association of South Asian Archeologists in Western Europe. *Edited by* B. Allchin. Cambridge University Press. Cambridge, pp. 29-33.

Darby, W.J., Ghaliounugi, P., and Grivetti, L.1977. Food: The gift of Osiris, Academic Press, London, pp 23-36.

Davis, G.L., M.D. McMullen, C. Baysdorfer, T. Musket, D. Grant, M. Staebell, G. Xu, M. Polacco, L. Koster, S. Melia-Hancock, K. Houchins, S. Chao and E.H. Coe. 1999. A maize map standard with sequenced core markers, grass genome reference points and 932 expressed sequence tagged site ESTs in a 1,736-locus map. Genetics, **152**: 1137–1172.

Dekkers, J.C.M. and Hospital, F. 2002. The use of molecular genetics in the improvement of agricultural populations. Nature Reviews: Genetics, **3**: 22-32.

Dekkers, J.C.M. 2003. Commercial application of marker and gene-assisted selection in livestock: strategies and lessons. In Molecular marker assisted selection as a potential tool for genetic improvement of crops, forest, livestock and fush in developing countries. [Online] Available from http://www.fao.org/biotech/Conf10.htm.

DeScenzo, R.A., Wise, R.P., and Mahadevappa, M. 1994 High resolution mapping of the *Hor1/Mla/Hor2* region on chromosome 5S in barley. Molecular Plant-Microbe Interactions, **7**: 657-665.

DeScenzo, R.A., and Wise, R.P. 1996. Variation in the ratio of physical to genetic distance in intervals adjacent to the *Mla* locus on barley chromosome 1H. Mol. Gen. Genet. **251**: 472-482.

Devos, K.M., Millan, T., and Gale, M.D. 1993. Comparative RFLP maps of homoelogous group 2 chromosomes of wheat, rye and barley. Theor. Appl. Genet. **85**: 784-792.

Druka, A., Kudrna D., Han F., Kilian, A., Steffenson, B., Frisch, D., Tomkins, J., Wing, R., and Kleinhofs, A. 2000. Physical mapping of the barley stem rust resistance gene *rpg4*. Mol. Gen. Genet. **264**: 283-290.

Ellis, R.P., Forster, B.P., Waugh, R., and Bonar, N. 1997. Mapping physiological traits in barley. The New Physiologist, **137**:149.

Epple, P, Apel, K., and Bohlmann, H. 1997. ESTs reveal a multigene family for plant defensins in *Arabidopsis thalian*a. FEBS. Lett. **400**:168–172.

Falak, I., Falk D.E., Tinker, N.A., and Mather, D.E. 1999. Resistance to powdery mildew in a doubled haploid barley population and its association with marker loci. Euphytica, **107**: 185-192.

FAO, 1998. Production Yearbook. Volume 52. Rome: FAO, pp.16-19.

Fischbeck, G. 2002. Contribution of barley to agriculture: a brief overview. *In* Barley Science. *Edited by* G.A. Slafe, J.L.Molina-Cano, R. Savin, J.L. Araus, I. Romagosa. Food Product Press, New Yok, London, Oxford, pp. 11-12.

Giese, H., Holm-Jensen, A.G., Jensen, H.P., and Jemsen, J. 1993. Localization of the laevigatum powdery mildew resistnce gene to barley chromosome 2 by the use of RFLP markers. Theor. Appl. Genet. **85**: 897-900.

Giese, H., Holm-Jensen, A.G., Mathiassen, H., Kjaer, B., Rasmussen, S.K., Bay, H., and Jensen, J. 1994. Distribution of RAPD markers on a linkage map of barley. Hereditas, **120**: 267-273.

Giordano, M, Oefner, P.J., Underhill, P.A., Cavalli-Sforza, L., Tosi, R., and Richiardi, P.M. (1999). Identification by denaturing high-performance liquid chromatography of numerous polymorphisms in a candidate region for multiple sclerosis susceptibility. Genomics, **56**: 247 .253.

Girke, T., Todd, J., Ruuska, S., White, J., Benning, C., and Ohlrogge, J. 2000. Microarray analysis of developing *Arabidopsis* seeds. Plant Physiol. **124**:1 570–1581.

Graner, A., and Bauer, E. 1993. RFLP mapping of the *ym4* virus resistance gene in barley. Theor. Appl. Genet. **86**: 689-693.

Graner, A., Foroughi-Wehr, B., and Tekauz, A. 1996. RFLP mapping of a gene in barley conferring resistance to net blotch (*Pyrenophora teres*). Euphytica. **91**: 229-234.

Graner, A., Jahor, A., Schondelmaier, J., Siedler, H., Pillen, K., Fischbeck, G., Wenzel, G., and Herrman, R.G. 1991. Construction of an RFLP map of barley. Theor. Appl. Genet. **83**: 250-256.

Graner, A., and Tekauz, A. 1996. RFLP mapping in barley of a dominant gene conferring resistance to scald (*Rhynochosporium secalis*). Theor. Appl. Genet. **93**: 421-425.

Grant, M.R., Godiard, L., Straube, E., Ashfield, T., Lewald, J., Sattler, A., Innes, R.W., and Dangle, J.L. 1995. Structure of the *Arabidopsis RPM1* gene enabling dual specificity disease resistance. Science, **269**: 843-846.

Griffin, T.J., and Smith, L.M. 2000. Single nucleotide polymorphism analysis by MALDI-TOF mass spectrometry. Trends Biotechnol. **18**: 77-84.

Grog, R., Hollrucher, K., and Schultze-Lefert, P. 1993. Functional analysis and RFLP-mediated mapping of the *Mlg* resistance locus in barley. Plant Journal, **3**: 857-866.

Gupta, P.K., Varshney, R.K., and Prasad, M. 2002. Molecular markers: principles and methodology. *In* Molecular Techniques in Crop Improvement. *Edited by* S.J. Mohan, D.S. Brar and B.S. Ahloowalia. Kluwer Press, Amsterdam, pp. 10-42.

Halterman, D., Zhou, F., Wei, F., Wise, R.P., and Schulze-Lefert P. 2001. The *MLA6* coiled-coil, NBS-LRR protein confers AvrMla6-dependent resistance specificity to *Blumeria graminis* f. sp. *hordei* in barley and wheat. Plant Journal, **25**: 335-348.

Han, F., Ullrich, S. E., Kleinhofs, A., Jones, B. L., Hayes, P. M., and Wesenberg, D. M. 1997. Fine structure mapping of the barley chromosome-1 centromere region containing malting-quality QTLs. Theor. Appl. Genet. **95**: 903-910.

Harushima, Y., Yano, M., Shomura, A., Sato, M., Shimano, T., Kuboki, Y., Harushima, Y., Yano, M., Shomura, A., Sato, M., Shimano, T., Kuboki, Y., Yamamoto, K., Nagamura, Y., Kurata, N., Khush, G.S., and Sasaki, T. 1998. A high-density rice genetic linkage map with 2275 markers using a single F_2 population. Genetics, **148**: 479–494.

Hearne, C.M., Ghosh, S., and Todd, J.A. 1992. Microsatellites for linkage analysis of genetic traits. Trends Genet. **8**: 288-294.

Hilbers, S., Fischbeck, G., and Jahoor, A. 1992. Localization of the laevigatum resistance gene *Mla* against powdery mildew in the barley genome by the use of RFLP markers. Plant Breeding, **109**: 335-338.

Hinze, K., Thompson, R.D., Ritter, E., Salamini, F., and Schulze-Lefert, P. 1991. Restriction fragment length polymorphism-mediated targeting of the *mlo*-resistance locus in barley (*Hordeum vulgare*). Proc. Natl. Acad. Sci. U.S.A. **88**: 3691-3695.

Hittalmani, S., Parco, A., Mew, T.V., Zeigler, R.S., and Huang, N. 2000. Fine mapping and DNA marker-assisted pyramiding of the three major genes for blast resistance in rice. Thoer. Appl. Genet. **100**: 1121-1128.

Ho, P.T. 1997. The indigenous origins of Chinese agriculture. *In* Origins of agriculture. *Edited by* C.A. Reed. Mouton, The Hague, pp. 413-418.

Hopf, M. 1991. South and Southwest Europe. *In* Progress in Old World paleoethnobotany. *Edited by* W. van Zeist, K. Wasilikowa, and K.E. Behre. Balkema, Rotterdam, pp. 241-277.

Huang, N., Angeles, R.R., Domingo, J., Magpantay, G., Singh, S., Zhang, G., Kumaravadivel, N., Bennet, J. and Khush, G.S. 1997. Pyramiding of bacterial blight resistance genes in rice: marker-assisted selection using RFLP and PCR. Theor. Appl. Genet. **95**: 313-320.

Jahoor, A., and Fischbeck, G. 1993. Identification of new genes for mildew resistance of barley at the *Mla* locus in lines derived from *Hordeum spontaneum*. Plant Breeding, **110**: 116-122.

Jander, G., Norris, S.R.; Rounsley, S.D., Bush, D.F., Levin, I.M., and Last, R.L. 2002. *Arabidopsis* Map-Based Cloning in the Post-Genome Era Plant Physiol. **129**: 440-450.

Jorgensen, J.H., and Jensen, H.P. 1979. Inter-allelic recombination in the *mlo* locus in barley. Barley Genet. Newsl. **9**: 37-39.

Kandemir, N., Kudrna, D.A., Ullrich, S.E., Kleinhofs, A. 2000. Molecular marker assisted genetic analysis of head shattering in six-rowed barley. Theor. Appl. Genet. **101**: 203-210.

Kilian, A., Steffenson, B.J., Maroof, M.A.S., and Kleinhofs, A. 1994. RFLP markers linked to the durable stem rust resistance gene *Rpg1* in barley. Mol. Plant Microbe Interact. **7**: 298-301.

Kintzios, S., Jahoor, A., and Fischbeck, G. 1995. Powdery mildew resistance genes *Mla29* and *Mla32* in *H. spontaneum* derived winter barley lines. Plant Breeding, **114**: 265-266.

Kislev, M.E., Nadel, D., and Carmi, I. 1992. Grain and fruit diet 19,000 years old at Ohalo II, Sea of Galilee. Israel. Rev. Paleobot. Palynol. **73**: 161-166.

Kleinhofs, A., Chao, S., and Sharp, P.J. 1988. Mapping of nitrate reductase genes in barley and wheat. *In* T.E. Miller and R.M.D. Koebner. Proceeding of the 7th International Wheat Genetics Symposium, Bath, Bath Press, pp. 541-546.

Kleinhofs, A., and Graner, A. 2001. An integrated map of the barley genome. *In* DNA-Based Markers in Plants, Second Edition. *Edited by* R.L. Philips and I. Vasil. Kluwer Academic Publishers, Dordrecht, pp. 65-77.

Kleinhofs, A., Kilian, A., Saghai Maroof, M.A., Biyashev, R.M., Hayes, P.M. et al. 1993. A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*). Theor. Appl. Genet. **86**: 705-712.

Knapp, S. J. 1998. Marker-assisted selection as a strategy for increasing the probability of selecting superior genotypes. Crop Sci. **38**: 1164-1174.

Koebner, R. 2003. MAS in cereals: Green for maize, amber for rice, still red for wheat and barley. [online]. Available from http://www.fao.org/biotech/docs/Koebner.pdf.

Kohert, G. 1994. RFLP technology. *In* DNA-based markers in plants. Kluwer Academic Publishers, Dordrecht/Boston/London, pp. 24-35.

Körber-Grohne, U. 1987. Nutzpflanzen in Deutschland. Stuttgart: Thesis, pp. 48-52.

Komatsuda, T., Taguchi-Shiobara, F., Oka, S., Takaiwa, F., Annaka, T., and Jacobsen, H.J. 1995. Transfer and mapping of the shoot-differentiation locus *Shd1* in barley chromosome 2. Genome, **38**: 1009-1014.

Konishi, T. 1981. Reverse mutation and interallelic resombination at the liguleless locus of barley. *In* Barley Genetics IV. *Edited by* R.N.H. Whitehouse. 4th International barley genetics Symposium, Edinburgh, pp. 838-845.

Kurata, N., Nagamura, Y., and Yamamoto, K. 1994. A 300 kilobase interval genetic map of rice including 883 expressed sequences. Nature Genet. **8**: 365-372.

Kurata, N., Umehara, Y., Tanoue, H. and Sasaki, T. 1997. Physical mapping of the rice genome with YAC clones. Plant Mol. Biol. **35**: 101–113.

Kurth, J., Kolsch, R., Simons, V., and Schulze-Lefert, P. 2001. A high-resolution genetic map and a diagnostic RFLP marker for the *Mlg* resistance locus to powdery mildew in barley. Theor. Appl. Genet. **102**: 53-60.

Kwok, P.Y., Deng, Q., Zakeri, H., Taylor, S.L., and Nickerson, D.A. 1996. Increasing the information content of STS-based genome maps: identifying polymorphisms in mapped STSs. Genomics, **31**: 123-126.

Lahaye, T., Hartmann, S., Töpsch, S., Freialdenhoven, A., Yano, M., Schulze-Lefert, P. 1998. High-resolution genetic and physical mapping of the *Rar1* locus in barley. Theor. Appl. Genet. **97**: 526-534.

Landegren, U., Kaiser, R., Sanders, J., and Hood, L. 1988. Ligase-mediated gene detection technique. Science, **241**: 1077-1080.

Langridge, P., Karakousis, A., Collins, N., Kretschmer, J., and Manning, S. 1995. A consensus linkage map of barley. Molecular Breeding, **1**: 389-395.

Laurie, D.A., Pratchett, N., Allen, R.L., and Hantke, S.S. 1996. RFLP mapping of barley homeotic mutant lax-a. Theor. Appl. Genet. **93**: 81-85.

Laurie, D.A, Pratchett, N., Bezant, J., and Snape, J.W. 1995. RFLP mapping of five major genes and eight quantitative trait loci controlling flowering time in a winter × spring barley (*Hordeum vulgare* L.) cross. Genome, **38**: 575-585.

Lisitsina, G.N. 1984. The Caucasus- A center of ancient farming in Eurasia. *In* Plants and ancient man. *Edited by* W van Zeist and W.A. Casparie. Balkema, Rotterdam, pp. 285-292.

Liu, B.L. 1997 Statistical genomics: linkage, mapping, and QTL analysis. CRC Press, Boca Raton, 691.

Liu, Z.-W., Biyashev, R. M., and Saghai Maroof, M. A. 1996. Development of simple sequence repeat DNA markers and their integration into barley linkage map. Theor. Appl. Genet. **93**: 869.

Manninen, O.M., Turpeinen, T., and Nissila, E. 1997. Identification of RAPD markers closely linked to the *mlo*-locus in barley. Plant Breeding, **116**: 461-464.

Mackill, D.J., Nguyen, H.T., and Zhang, J.X. 1999. Use of molecular markers in plant improvement programs for rainfed lowland rice. Field Crops Res. **64**: 117-185.

Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganal, M.W., Sivey, R., Wu, T., Earle, E.D., and Tanksley, S.D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science, **262**: 1432-1436.

Melchinger, A.H., 1990. Use of molecular markers in breeding for oligogenic disease resistance. Plant Breed Z. Pflansenzuch, **104**: 1-19.

Meyers, B.C., Chin, D.B., Shen, K.A, Sivaramakrishnan, S., Lavelle, D.O., Zhang, Z., and Michelmore, R.W. 1998. The major resistance gene cluster in lettuce is highly duplicated and spans several megabases. Plant Cell, **10**: 1817-1832.

Miklas, P.N., Strausbaugh, C.A., Larsen, R.C., and Forster, 2000. NL-3 (K) - a more virulent strain of NL-3 and its interaction with *bc-3*. Annu. Rep. Bean Improv. Coop. **43**: 168–169.

Mindrinos, M., Katagiri, F., Yu, G.-L., and Ausubel, F.M. 1994. The *Arabidopsis thaliana* disease resistance gene *RPS2* encodes a protein containing a nucleotide-binding site and leucine-rich repeats. Cell, **78**: 1089-1099.

Nairz, K., Stocker, H., Schindelholz, B., and Hafen, E. 2002. High-resolution SNP mapping by denaturing HPLC. Proc. Natl. Acad. Sci. U.S.A. **99**: 10575-10580.

Newton, C.R, Graham, A., Heptinstall, L.E., Powell, S.J., Summers, C., Kalshekar, N., Smith, J.C., and Markham, A.F. 1989. Analysis of any point mutation in DNA: the amplification refractory mutation system (ARMS). Nucleic Acids Res. **17**: 2503-2516.

Oefner, P.J., and Underhill, P.A. 1998. DNA mutation detection using denaturing high performance liquid chromatography (DHPLC). In Current Protocols in Human Genetics. Wiley & Sons, New York, Supplement **19**: 7.10.1-7.10.12.

Olson, M., Hood, L., Cantor, C., and Botstein, D. 1989. A common language for physical mapping of the human genome. Science, **245**: 1434-1435.

Paltridge, N.G., Collonis, N.C., Bendahmane, A., and Symons, R.H. 1998. Development of YLM, a codominant PCR marker closely linked to the *Yd2* gene for resistance to barley yellow dwarf disease. Theor. Appl. Genet. **96**: 1170-1177.

Paran, I., and Michelmore, RW. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor. Appl. Genet. **89**: 632-638.

Parker, J.E., Coleman, M.J., Szabo, V., Frost, L.N., Schimdt, R., van der Biezen, E.A., Moores, T., Dean, C., Daniles, M.J., and Jones, J.D.G. 1997. The *Arabidopsis* downy mildew resistance gene *RPP5* shares similarity to the toll and interleukin-1 receptors with *N* and *L6*. Plant Cell, **9**: 879-894.

Patocchi, A., Vinatzer, B.A., Gianfranceschi, L., Tartarini, S., Zhang, H.B., Sansavini, S., and Gessler, C., 1999. Construction of a 550 kb BAC contig spanning the genomic region containing the apple scab resistance gene *Vf*. Mol. Gen. Genet. **262**: 884-891.

Pastinen, T., Raitio, M., Lindroos, K., Tainola, P., Peltonen, L., Syvanen, A.C. 2000. A system for specific, high-throughput genotyping by allele-specific primer extension on microarrays. Genet. Res. **10**: 1031 .1042.

Picoult-Newberg, L., Ideker, T.E., Pohl, M.G., Taylor, S.L., Donaldson, M.A., Nickerson, D.A., Boyce-Jacino, M. 1999. Mining SNPs from EST databases. Genome Res. **9**: 167-147.

Poehlman, J.M. 1959. Breeding field crops. H. Holl, New York, pp. 22-50, 150-173.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., and Rafalski, A. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Molecular Breeding, **2**: 225-238.

Qi, X., Stam, P., and Lindhout, P. 1998. Use of locus-specific AFLP markers to construct a high-density molecular map in barley. Theor. Appl. Genet. **96**: 376-384.

Rafalski, J.A. 2002a. Novel genetic mapping tools in plants: SNPs and LD-based approaches. Plant Sci. **162**: 329-333.

Rafalski, J.A. 2002b. Application of single nucleotide polymorphisms in crop genetics. Curr. Opin. Plant. Biol. **5**: 94-100.

Rohrer, W. L., Pridgen, T.H., Griffey, C.A., Brooks, W.S., Vaughn, M.E., and Brann, D.E. Winter Barley Production and Research in 2001. 2001. Barley Newsletter. Volume 45. [Online] Available from http://wheat.pw.usda.gov/ggpages/BarleyNewsletter/45/Barley_Production.html.

Ronaghi, M., Uhlen, M., and Nyren, P. 1998. A sequencing method based on real-time pyrophosphate. Science, **281**: 363-365.

Roses, A.D. 2002. Pharmacogenetics place in modern medical science and practice. Life Sci. 70:1471-1480.

Rosichan, J., Nilan, R.A, Arenaz, P., and Kleinhofs, A. 1979. Intragenic recombination at the waxy locus in *Hordeum vulgare*. Barley Genet. Newsl. **9**: 79-85.

Saeki, K., Miyazaki, C., Horota, N., Saito, A., Ito, K., and Konishi, T. 1999. RFLP mapping of BaYMV resistance gene *rym3* in barley (*Hordeum vulgare*). Theor Appl. Genet. **99**: 727-732.

Sanchez, A.C., Brar, D.S., Huang, N., Li, Z., and Khush, G.S. 2000. Sequence tagged site marker-assisted selection for three bacterial blight resistance genes in rice. Crop Sci. **40**: 792-797.

Saghai Maroof, M.A., Biyashev, R.M., Yang, G., Zhang, Q., and Allard, R.W. 1994. Extraordinarily polymorphic microsatellite DNA in barley: species diversity, chromosomal locations, and population dynamics. Proc. Natl. Acad. Sci. U.S.A. **91**: 5466-5470.

Sax, K. 1923. The association of size differences with seed-coat pattern and pigmentation in *Phaseolus vulgaris*. Genetics, **8**: 552-560.

Schena, M., Shalon, D., Davis, R.W., and Brown, P.O. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science, **270**: 467–470.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., and Manners, J.M. 2000. Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proc. Natl. Acad. Sci. U.S.A. **97**: 11655–11660.

Schonfeld, M., Ragni, A., Fischbeck, G., and Jahoor, A. 1996. RFLP mapping of three new loci for resistance genes to powdery mildew (*Erysiphe graminis f. sp. Hordei*) in barley. Theor. Appl. Genet. **93**: 48-56.

Schuller, C., Backes, G., Fischbeck, G., and Jahoor, A. 1992. RFLP markers to identify the alleles at the *Mla* locus conferring powdery mildew resistance in barley. Theor. Appl. Genet. **84**: 330-338.

Schweizer, G.F., Baumer, M., Daniel, G., Rugel, H., and Rider, M.S. 1995. RFLP markers linked to scald (*Rhynchosporium secalis*) resistance gene *Rh2* in barley. Theor. Appl. Genet. **90**: 920-924.

Schwarz, G., Michalek, W., Mohler, V., Wenzel, G., and Jahoor, A. 1999. Chromosome landing at the *Mla* locus in barley (*Hordeum vulgare* L.) by means of high-resolution mapping with AFLP markers. Theor. Appl. Genet. **98**: 521-530.

Schneider, S., Roessli, D., and Excofier L. 2001 Arlequin, In Genetics and Biometry Laboratory. University of Geneva, Geneva, Switzerland.

Shin, J.S., Chao, S., Corpuz, L., and Blake, T.K. 1990. A partial map of the barley genome incorporating restriction fragment length polymorphism, polymerase chain reaction, enzymes, and morphological marker loci. Genome, **23**: 803-810.

Shirasu, K., Lahaye, T., Tan, M.-W., Zhou, F., Azevedo, C., and Schulze-Lefert, P. 1999. A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *C. elegans*. Cell, **99**: 355-366.

Shizuya, H., Birren, B., Kim, U.-J., Mancino, V., Slepak, T., Tachiiri, T. and Simon, M. 1992. Cloning and stable maintenance of 300 kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc. Natl. Acad. Sci. U.S.A. **89**: 8794-8797.

Simons, G., van der Lee, T., Diergaarde, P., van Daelen, R., Groenendijk, J., Frijters, A., Buschges, R., Hollricher, K., Topsch, S., Schulze-Lefert, P., Salamini, F., Zabeau, M., and Vos, P. 1997. AFLP-based fine mapping of the *Mlo* gene to a 30-kb DNA segment of the barley genome. Genomics, **44**: 61-70.

Singh, S., Sidhu, J.S., Huang, N., Vikal, Y., Li, Z., Brar, D.S., Dhaliwal, H.S. and Khush, G.S. 2001. Pyramiding three bacterial blight resistance genes (*xa5*, *xa13* and *Xa21*) using marker-assisted selection into indica rice cultivar PR106. Theor. Appl. Genet. **102**: 1011-1015.

Snyder, L., and Champness, W. 1997. Molecular genetics of bacteria. ASM Press, Washington, D.C., pp 37-45.

Song, W.-Y., Wang, G.-L., Chen, L.-L., Kim, H.-S., Pi, L.-Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.-X., Zhu, L.-H., Fauquet, C., and Roland, P. A receptor kinase like protein encoded by rice disease resistance gene, *Xa21*. Science, **270**: 1804-1806.

Statistical Highlights of U.S. Agriculture 2001-2002. [Online]. Available from http://www.usda.gov/nass/pubs/stathigh/2002/stathi2002.pdf.

Syvanen, A.C., Aalto-Setala, K., Harju, L., Kontula, K. and Soderlund, H. 1990. A primer-guided nucleotide incorporation assay in the genotyping of Apolipoprotein E. Genomics, **8**: 684-692.

Tai, T., Dahlbeck, D., Stall, R.E., Peleman, J., and Staskawicz, B.J. 1999. High-resolution genetic and physical mapping of the region containing the *Bs2* resistance gene of pepper. Theor. Appl. Genet. **99**: 1201-1206.

Tanksley, S.D., Ganal, M.N., and Martin, G.B. 1995. Chromosomal landing: a paradigm for map-based cloning in plants with a large genome. Trends Genet. **11**: 63-68.

Thomas, W.T.B.2002. Molecular marker-assisted versus conventional selection in barley breeding. *In* Barley science: recent advances from molecular biology to agronomy of yield and quality. *Edited by* Gustavo A. Slafer, José Luis Molina-Cano, Roxana Savin, José Luis Araus, Ignacio Romagosa. Food Product Press (New York, USA), an imprint of The Haworth Press, Inc., New York, pp. 177-205.

Thomas, W.T.B. 2003. Prospects for molecular breeding of barley. Ann. Appl. Biol. **142**: 1-12.

Thomsen, S.B., Jemsen, H.P., Jensen, J., Skou, J.P., and Jorgensen, J.H. 1997. Localization of a resistance gene and identification of sources of resistance to barley leaf stripe. Plant Breeding, **116**: 455-459.

Tuvensson, S., von Post, L., Ohlund, R., Hagberg, P., Graner, A., Svitashev, S., Schehr, M., and Elovsson, R. 1998. Molecular breeding for the BaMMV/BaYMV resistance gene *ym4* in winter barley. Plant Breeding, **117**: 19-22.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. Nucl. Acids Res. **23**: 4407-4414.

Vuylsteke, M., Mank, R., Antonise, R., Bastiaans, E., Senior, M.L., Stuber, C.W., Melchinger, A.E., Lübberstedt, T., Xia, X.C., Stam, P., Zabeau, M., and Kuiper, M. 1999. Two high-density AFLP linkage maps of *Zea mays* L.: analysis of distribution of AFLP markers. Theor. Appl. Genet. **99**: 921-935.

Wallace, R.B., Shaffer, J., Murphy, R.F., Bonner, J., Hirose, T., and Itakura, K. 1979. Hybridization of synthetic oligodeoxyribonucleotides to fX174 DNA: the effect of single base pair mismatch. Nucleic Acids Res. **6**: 3543-3557.

Wei, F., Gobelman-Werner, K., Morroll, S.M., Kurth, J., Mao, L., Wing, R., Leister, D., Schultze-Lefert, P., and Wise, R.P. 1999. The *Mla* (powdery mildew) resistance cluster is associated with three NBS-LRR gene families and suppressed recombination within a 240-kb DNA interval on chromosomal 5S (1HS) of barley. Genetics, **153**: 1929-1948.

Weyen, J., Bauer, E., Graner, A., Friedt, W., and Ordon, F. 1996. RAPD-mapping of the distal portion of chromosome 3 of barley, including the BaMMV/BaYMV resistance gene *ym4*. Plant Breeding, **115**: 285-287.

Wu, D.Y., and Wallance, R.B. 1989. The ligation amplification reaction (LAR)-amplification of specific DNA sequences using sequential rounds of template-dependent ligation. Genomics, **4**: 460-569.

Yamanouchi, U., Masahiro, Y., Hongxuan, L., Motoyuki, A., and Yamada, K. 2002. A rice spotted leaf gene, *Spl7*, encodes a heat stress transcription factor protein. Proc. Natl. Acad. Sci. U.S.A. **99**: 7530-7535.

Yang D., Sanchez, A., Khush, G.S., Zhu, Y., and Huang, N. 1998. Construction of BAC contig containing the *xa5* locus in rice. Theor. Appl. Genet. **97**: 1120-1124.

Yoshimura, S., Yoshimura, A., Iwata, N., McCough, S.R., Abenes, M.I., Baraoidan, M.R., Mew, T.W., and Nelson, R.J. 1996. Tagging and combining bacterial blight resistance genes in rice using RAPD and RFLP markers. Mol. Breed. **1**: 375-387.

Young, N.D. 1999. A cautiously optimistic vision for marker-assisted breeding. Molecular Breeding **5**: 505-510.

Zohary, D. and M. Hopf. 1993. Domestication of plants in the Old World. Oxford Science Publications, Clarendon Press, pp 33-64.

CHAPTER 2

MOLECULAR MAPPING OF THE LEAF RUST RESISTANCE GENE *RPH5* IN BARLEY

Abstract

Leaf rust caused by *Puccinia hordei* is an important disease of barley (*Hordeum vulgare*) in many regions of the world. Yield losses up to 62% have been reported in susceptible cultivars. The *Rph*5 gene confers resistance to the most prevalent races (8 and 30) of barley leaf rust in the United States. Therefore, the molecular mapping of *Rph*5 is of great interest. The objectives of this study were to map *Rph*5 and identify closely linked molecular markers. Genetic studies were performed by analysis of 93 and 91 F_2 plants derived from the crosses 'Bowman' (*rph*5) × 'Magnif 102' (*Rph*5) and 'Moore' (*rph*5) × Virginia 92-42-46 (*Rph*5), respectively. Bulk segregant analysis (BSA) using amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and simple sequence repeat (SSR) markers was conducted. Linkage analysis positioned the *Rph*5 locus to the extreme telomeric region of the short arm of barley chromosome 3H at 0.2 cM proximal to RFLP marker *VT1* and 0.5 cM distal from RFLP marker *C970* in the Bowman × Magnif 102 population. Map positions and the relative order of the markers were confirmed in the Moore × Virginia 92-42-46 population. RFLP analysis of the near isogenic line (NIL) Magnif 102/*8Bowman, the susceptible recurrent parent Bowman, and *Rph*5 donor Magnif 102, confirmed the close linkage of the markers *VT1*, *BCD907* and *CDO549* to *Rph5*. Results from this study will be useful for marker-assisted selection and gene pyramiding in programs breeding for leaf rust resistance and provide the basis for physical mapping and further cloning activities.

Keywords: barley, leaf rust, *Rph5*, molecular mapping, marker-assisted selection, gene pyramiding.

Introduction

Leaf rust caused by *Puccinia hordei* is generally considered the most important rust disease of barley on a worldwide basis. Severe yield losses have been observed in Australia (62%) (Coterill et al. 1992) and Europe (17-31%) (King and Polley 1976). In the United States, a 32% yield reduction was reported for susceptible cultivars under epidemic conditions in Virginia (Griffey et al. 1994).

Clifford (1985) listed two types of resistance against *P. hordei* in barley: partial resistance and race-specific resistance. Partial resistance is controlled by several to many genes and is generally considered more durable than the race-specific resistance (Qi et al. 2000; Kicherer et al. 2000). However, the quantitative expression of this trait and complex genetics make this type of resistance more difficult to use in breeding programs. Race-specific resistance is usually governed by single dominant genes. Although race-specific leaf rust resistance genes have not provided durable protection, they can be easily identified and transferred into appropriate germplasm (Parlevliet 1976). To date, 19 major race-specific genes (designated as *Rph*1 to *Rph*19) for leaf rust resistance have been identified (Franckowiak et al. 1997; Ivandic et al. 1998; Weerasena et al. 2004).

Development of disease resistant barley cultivars has been the most efficient way to control leaf rust (Mathre 1997; Zillinsky 1983). The pyramiding of multiple *Rph* genes is expected to increase the durability of leaf rust resistance in cultivars. Although virulence for *Rph*5 is widely prevalent in Europe (Parlevliet 1976) and South America (Brodny and Rivadeneira 1996; Fetch et al. 1998), it has not been identified in North America. Thus, *Rph*5 could be used to protect barley cultivars from leaf rust damage in North America. However, a more sound gene deployment strategy would be to use this gene in combination with other effective genes such as *Rph*3 and *Rph*9 (Brooks et al. 2000).

Most of the known barley leaf rust resistance genes have been described and mapped using morphological characters, biochemical markers, and cytogenetic stocks (Table 2.1). However, so far only five *Rph* genes have been mapped using molecular markers. Using molecular markers, *Rph2* was mapped to chromosome 5H (Borovkova et al. 1997). Two alleles at the *Rph*9 locus, *Rph*9.*i* and *Rph*9.*z* (formerly designated as

*Rph*12) were located on chromosome 5H using RFLP and sequence tagged site (STS) markers (Borovkova et al. 1997 and 1998). STS and cleaved amplified polymorphic sequence (CAPS) markers were employed to map *Rph*16 onto barley chromosome 2H (Ivandic et al. 1998). *Rph*7 was mapped onto the short arm of chromosome 3H by means of RFLP markers (Brunner et al. 2000; Graner et al. 2000). Park and Karakousis (2002) assigned *Rph19* to the chromosome 7H. Finally, *Rph6* (now designated as *Rph5.f*) and *Rph15* were mapped to the chromosome 3HS and 2HS (Zhong et al. 2003; Weeresena et al. 2004). The precise chromosomal position of *Rph*5 is not known, although the gene was assigned to chromosome 3H by trisomic analysis (Tan 1978; Tuleen and McDaniel 1971). Thus, the objectives of this study were to map *Rph*5 by means of molecular markers and develop closely linked markers for marker-assisted selection.

Materials and methods

Genetic materials

Two F_2 populations derived from crosses Bowman (PI 483237) × Magnif 102 (PI 337140) and Moore (CI 7251) × Virginia 92-42-46 (hereafter, referred to as BM and MV populations, respectively) and consisting of 93 and 91 individuals, respectively, were used for molecular mapping. Magnif 102 (Franckowiak et al. 1997) and Virginia 92-42-46 (Zwonitzer 1999) carry *Rph*5 and provide the genetic sources of resistance to leaf rust in this experiment. To confirm the close linkage between *Rph*5 and flanking markers, the near isogenic line (NIL) Magnif 102/*8Bowman, together with recurrent parent Bowman and *Rph5* donor Magnif 102, were used in this study. Seeds of the NIL were kindly provided by Dr. J. D. Franckowiak at North Dakota State University, Fargo.

Disease screening

Briefly, to determine infection type (disease reaction phenotype), F_2 plants from both populations were inoculated with race 8 as described by Brooks et al. (2000). To confirm the genotype for resistance in F_2 plants (i.e. whether *Rph5*/*Rph5*, *Rph5*/*rph5*, *rph5/rph5*), 50 seeds from each $F_{2:3}$ family were planted inoculated, and evaluated for their leaf rust reaction. A set of host differential lines including 'Sudan' (*Rph1*), 'Peruvian' (*Rph2*), 'Aim' (*Rph3*), 'Estate' (*Rph3*), 'Gold' (*Rph4*), 'Bolivia' (*Rph2 +Rph6*), 'Cebada Capa' (*Rph7*), 'Egypt 4' (*Rph8*), 'Hor 2596' (*Rph9.i*), 'Triumph' (*Rph9.z*), 'Clipper BC8' (*Rph10*), 'Clipper BC67' (*Rph11*), Berac*3/HS2986 (*Rph1*3), 'PI 531901-1' (*Rph14*) and Bowman*4/PI 3555447 (*Rph15*) were included as checks in the experiments. The virulence/avirulence formula of race 8 is *Rph1, 4, 8, 10, 11/Rph2, 3, 5, 2+6, 7, 9.i, 9.z, 13, 14, 15* (Griffey et al. 1994). Infection types were scored using the 0-4 scale of Levine and Cherewick (1952). Infection types of 0, 1, or 2 were considered indicative of host resistance, whereas infection types 3 or 4 were considered indicative of host susceptibility. Disease assessments were performed 10 to 14 days after inoculation. Infection types of F_2 progeny were compared with infection types of the parental lines and host-differentials to assure proper scoring and assignment into resistant and/or susceptible classes.

Molecular mapping

RFLP analysis

Genomic DNA from 91 MV individual F_2 plants and 93 BM $F_{2:3}$ families was processed for molecular marker analysis by Zwonitzer (1999). Briefly, DNA was extracted from freeze-dried leaf tissue as described by Saghai Maroof et al. (1984). For Bulk Segregant Analysis (BSA) (Michelmore et al. 1991), DNA samples from six MV F_2 individuals identified as homozygous resistant or homozygous susceptible, based on $F_{2:3}$ disease data, as well as six BM $F_{2:3}$ homozygous resistant and homozygous susceptible families were pooled to form resistant and susceptible bulks. Using these DNA samples, RFLP analysis was carried out as described by Saghai Maroof et al. (1984) and Biyashev et al. (1997). Briefly, 7 micrograms of DNA from the susceptible and resistant bulks, parental samples, NIL Magnif 102/*8Bowman, and 91 MV and 93 BM individual F_2 plants were digested with six restriction enzymes *Bam*HI, *Dra*I, *Eco*RI, *Hin*dIII, *Sst*I and *Xba*I according to the manufacturer's protocol (Gibco BRL, Rockville, MD). After digestion, DNA fragments were separated by electrophoresis on low melting 0.8% agarose gels overnight and Southern blotted onto Hybond nylon membrane (Amersham, Piscataway, NJ). Southern blots were hybridized with randomly primed ^{32}P-dCTP RFLP probes (Ambion, Austin, TX). Hybridizing bands were visualized by autoradiography on Kodak Xomat (New Haven, Conn).

Conversion of RFLP clones to PCR markers

Promising RFLP clones were converted into PCR markers in order to develop molecular markers that would be useful in marker assisted selection for *Rph*5. PCR markers were developed by designing the primers based on the insert sequence of the given RFLP clone. Information related to insert sequence of the RFLP clones was obtained either through GenBank database or if the sequence is not available in GenBank database, it was sequenced using an ABI377 DNA sequencer. The sequencing procedure was as follows: plasmid template was prepared using standard alkaline-lysis followed by purification using QiaexII (Qiagen, Valencia, CA). Dye-terminator cycle sequencing was done based on the manufacturer's protocols (Perkin Elmer, Foster City, CA.). Sequence

analysis, including primer design, was conducted using Lasergene software from DNAstar (Madison, Wis.). PCR amplification was carried out according to the published procedures (Liu et al. 1996; Ramsey et al. 2000).

AFLP analysis

To conduct AFLP analysis, we will follow the procedure described by Vos et al. (1995) and Maughan et al. (1996). Briefly, DNAs from resistant and susceptible bulks as well as resistant parents, Magnif and Virginia 92-42-46, and susceptible ones, Bowman and Moore, will be digested with the six cutter *Eco*RI and four cutter *Mse*I restriction enzymes. Adaptor sequences will be ligated to the restriction ends as previously described. An initial round of PCR will be conducted using *Mse*I +1 and *Eco*RI +1 selective primers, to amplify a subset of the digested and ligated fragments. Thirty cycles of a 25 μL PCR reaction using 1X buffer, 0.9 mM $MgCl_2$, 0.6 μM of each primer, 0.25 mM dNTP, 0.5 U of *Taq* polymerase (Gibco/ BRL) and 250 ng of template will be conducted with denaturation at 94°C for 60 s, primer annealing at 60°C for 30 s, and primer extension at 72 °C for 30 s. An aliquot of this reaction equivalent to 25 ng of DNA then will be used in a second round of amplification. In the second round of amplification, ^{32}P end-labeled *Eco*R +3 and *Mse*I +3 primers will be used. The rest of the PCR components will be essentially the same as the previous reaction. This second round of PCR will be conducted using touchdown conditions beginning at 65°C annealing and reducing by 1°C per cycle to 56°C, followed by 26 cycles at 56°C. All other cycling conditions will be as in the previous reactions. Second-round PCR products will be separated on a 7 M urea 6.5% polyacrylamide gel for 2 h at 60 W. The gel then will be transferred to 3MM paper, covered with plastic wrap, and exposed to Kodak film (New Haven, Conn) for 12-18 hours.

Conversion of AFLP markers to RFLPs

Conversion of AFLP markers to RFLP will be performed as described by Hayes and Saghai Maroof (2000). Briefly, AFLP markers found to be linked to *Rph5,* based on BSA, were cut from the gel and eluted in 200 μl of water incubated in a boiling bath as described by Upender et al. (1995). The eluate was amplified via PCR using the same +3

primers that generated the polymorphic product under the same conditions. The PCR product was then cloned into the pCR2.1-TOPO vector using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). To confirm the proper size of the cloned inserts, they were amplified with the original +3 primers under the same second-round PCR conditions. These labeled fragments then were run on 6.5% polyacrylamide gel along with labeled parental samples. Tentatively confirmed positive inserts were amplified for use as RFLP markers.

Sequence analysis

DNA fragments were sequenced using an ABI377 DNA sequencer. Plasmid template was prepared using standard alkaline-lysis method followed by purification with QiaexII (Qiagen Inc., Valencia, CA). Dye-terminator cycle sequencing was done based on the manufacturer's protocols (Perkin Elmer, Foster City, CA). Sequence analysis, including primer design was conducted using Lasergene software (DNASTAR, Madison, WI).

Linkage analysis

Disease and marker data was used to determine the chromosomal location of *Rph5*. For genetic mapping and linkage analysis the computer program MAPMAKER version 3.0 b was used (Lander et al. 1987). Linkage maps was constructed based on LOD threshold of 3.0 and maximum Haldane distance of 50 cM.

Results

In both crosses, the number of resistant and susceptible F_2 progeny approximated a 3:1 ratio indicating that a single dominant gene (*Rph5*) conferred resistance in Magnif 102 and Virginia 92-42-46 (Table 2). This result was confirmed by the 1:2:1 ratio of homozygous resistant, segregating, homozygous susceptible $F_{2:3}$ families (Table 2.2). Infection types of resistant parents and resistant progeny are summarized in Table 2.3. Trisomic analysis of Tuleen and McDaniel (1971) and Tan (1978) indicated that *Rph5* was located on barley chromosome 3H. Therefore, we selected previously reported RFLP and SSR markers from chromosome 3H for BSA. Six RFLP markers (*CDO549*,

BCD907, *C970*, *MWG2021, MWG848* and *TAG683*) in the BM population were mapped in the vicinity of the *Rph*5 locus (Fig. 2.1A). RFLP clone *MWG691*, originally mapped to telomeric region of the barley chromosome 3HS (Graner et al. 1994), was monomorphic in the BM population. In order to map *MWG691*, we converted it into a PCR-based marker. An insert fragment of 290 bp from the *MWG691* clone was sequenced. The sequence information was used to design a pair of primers (5'gatcacttggggccgtatgtgtta3' and 5'aattccgggtgagtgcctcttc 3') to PCR-amplify the DNA from parental forms and bulk segregants of both populations. As a PCR-based marker, *MWG691* revealed polymorphism between Bowman and Magnif 102. In the BM F_2 population, this marker segregated in a co-dominant fashion and was mapped 0.9 cM proximal to *Rph*5. Map positions and the relative order of the markers *CDO549*, *BCD907*, *MWG2021, MWG848* were confirmed on the MV population (Fig. 2.1B). In addition to the above-mentioned markers, two RFLP markers, *MWG2158* and *MWG2266,* were mapped 1.5 and 1.9 cM proximal to *Rph*5, respectively, in this population.

In order to identify more closely linked markers, AFLP analysis on parental lines and bulks from both populations was conducted. As a result, an AFLP fragment of 120 bp was detected with the primer combination *Eco*+ACA/*Mse*+AGG in both populations. This AFLP marker was converted to an RFLP probe (hereafter, referred to as *VT1*) and mapped 0.2 cM distal to *Rph*5 in both populations. Also, an AFLP (E06M10) fragment of 200 bp was detected with the primer combination *Eco*+AGA/*Mse*+ATA and mapped to the most telomeric region of barley chromosome 3HS 3.7 cM distal from *Rph*5 in the BM population. This DNA fragment was cloned and sequenced. A BLAST search detected high similarity with the wheat telomere-specific DNA fragment (GenBank accession # AF004950).

The close linkage of the RFLP markers flanking *Rph*5 was confirmed by RFLP analysis of NIL Magnif 102/*8Bowman, recurrent parent Bowman and the *Rph*5 donor Magnif 102 as well as the other known source of *Rph*5 'Quinn' (PI39401). The markers *VT1*, *BCD907* and *CDO549* detected DNA fragments of the same size in NIL Magnif 102/*8Bowman, Magnif 102 and Quinn, while a different size fragment was observed in Bowman. In total, 16 RFLPs, 4 SSRs, and one AFLP marker were placed on chromosome 3H in the BM population, and 15 RFLP and 5 SSR markers were mapped

on the same linkage group in the MV population. Established maps share 13 common markers, including 10 RFLPs and 3 microsatellites, and cover 172.7 and 105.8 cM of the barley chromosome 3H in BM and MV populations, respectively (Fig. 2.2).

Discussion

Using two segregating populations, the leaf rust resistance gene *Rph*5 was precisely mapped to the extreme telomeric region of chromosome 3HS using molecular markers. Mapping results were confirmed by NIL analysis. Several closely linked molecular markers were identified for *Rph*5. In the BM cross, the bracketing markers are *VT1* (at 0.2 cM distal) and *C970* (at 0.5 cM proximal), and in the MV cross, *VT1* (also at 0.2 cM distal) and *MWG2158* (at 1.2 cM proximal) (Fig. 2.1A & B)**.** The closely linked markers, identified in this study, may be useful as probes for detecting the barley lines carrying resistance alleles of *Rph*5. The other benefit derived from comprehensive mapping is the possibility of positional cloning of *Rph*5 in the future. One of the most crucial steps in positional cloning is the discovery of molecular markers bracketing the gene of interest as demonstrated here for *Rph*5. Map-based cloning has been successfully applied for several disease resistance genes in barley (Buschges et al. 1997; Wei et al. 1999, Brueggeman et al. 2002).

As was mentioned above, the *Rph*7 locus was also mapped to the extreme telomeric region of barley chromosome 3HS (Brunner et al. 2000; Graner et al. 2000). Interestingly, this part of chromosome 3HS does show an increased recombination rate that indicates a relatively high level of genetic activity in the region (Kunzel et al. 2000). The availability of molecular maps with common markers allowed comparison of map positions and estimation of relative locations of other loci. In this study, we compared three molecular maps of the *Rph*5 and *Rph*7 flanking regions: two of the maps were developed in this study and the third by Brunner et al. (2000). Based on the positions of common markers, we estimate that *Rph*5 is located about 6 cM distally from *Rph7* on barley chromosome 3HS (Fig. 2.3).

Another interesting finding is the positioning of the AFLP marker E06M10 on the extreme telomeric region of chromosome 3HS. It was mapped 3.3 cM distal to the RFLP markers *CDO549* and *BCD907* in the BM cross. Sequence analysis of the DNA fragment

detected by E06M10 revealed a high level of similarity with wheat telomere-associated DNA (GenBank accession # AF004950). In this regard, it is interesting to note that Kilian et al. (1999) generated marker Tel3S from a telomere-associated sequence of barley and mapped it to the most terminal region of barley chromosome 3HS, which is located ~ 4.5 cM away from the *MWG691/ABG316A* cluster. In our map, the distance between *MWG691* and E06M10 is approximately the same - 4.6 cM (Fig. 2.1A). This observation confirmed the position of marker Tel3S in the terminal region of barley chromosome 3HS.

Precise mapping of *Rph*5 has resulted in the identification of closely linked molecular markers that are potentially suitable for marker-assisted selection and pyramiding of genes confirming more durable resistance to leaf rust. Also, the results provide the basis for physical mapping and map-based cloning of *Rph*5 gene.

Table 2.1. Summary of described and mapped *Rph* genes in barley

Gene *	Number of alleles	Chromosome	Means of Positioning Locus	Reference
Rph1	1	2H	Trisomic analysis	Tuleen and McDaniel (1971); Tan (1978)
Rph2	12	5H	Molecular markers	Franckowiak et al. (1997); Borovkova et al. (1997)
Rph3	3	7H	Morphological markers	Jin et al. (1993)
Rph4	1	1H	Trisomic analysis	Tuleen and McDaniel (1971); Tan (1978)
Rph5	1	3H	Trisomic analysis	Tuleen and McDaniel (1971); Tan (1978)
Rph6	1	3H	Molecular markers	Zhong et al. (2003)
Rph7	2	3H	Molecular markers	Brunner et al. (2000); Graner et al. (2000)
Rph9.i	1	5H	Molecular markers	Borovkova et al. (1998)
Rph9.z†	1	5H	Molecular markers	Jin et al. (1993); Borovkova et al. (1998)
Rph10	1	3H	Isozymes	Feuerstein et al. (1990)
Rph11	1	6H	Isozymes	Feuerstein et al., (1990)
Rph15	1	2H	Molecular markers	Weeresena et al. (2004)
Rph16	1	2H	Molecular markers	Ivandic et al. (1998)

*Gene designations of *Rph*1 to *Rph*16 are from Franckowiak et al (1997). The leaf rust resistance gene mapped by
Borovkova et al. (1997) in Q21861 is thought to be *Rph*2 based on allelism tests with the known *Rph*2 sources of Peruvian, PI 531840, and PI 531841.

†Rph9.z (formerly designated as *Rph*12) is based on Borovkova et al. (1998)

Table 2.2. Segregation for leaf rust resistance in F_2 plants and $F_{2:3}$ families in MV and BM barley populations

Cross	Number of F_2 plants			Ratio	χ^2	P-value
	Resistant	Susceptible				
MV	71	20		3:1	0.44	0.506
BM	70	23		3:1	0.01	0.952
	Number of $F_{2:3}$ families					
	Homozygous resistant	Segregating	Homozygous susceptible			
MV	22	49	20	1:2:1	0.63	0.731
BM	22	47	24	1:2:1	0.10	0.953

Table 2.3. Infection types of barley parents to *Puccinia hordei* race 8

Line	Accession #	Infection type *
Moore	CI 7251	4
VA92-42-46		0;
MV resistant progeny		0;N
Bowman	PI 483237	33^-
Magnif 102	PI337140	0;
BM resistant progeny		0;N

* Infection type (IT) ratings are based on the 0-4 scale of Levine and Cherewick (1952). Plants with IT of 4 are extremely susceptible with large uredia. Plants with IT of 0; are resistant with necrotic flecks. Plants with IT of 0;N exhibit resistance with necrotic lesions. Plants with IT 33^- are moderately susceptible with combination of medium size (3) and small size (3^-) uredia.

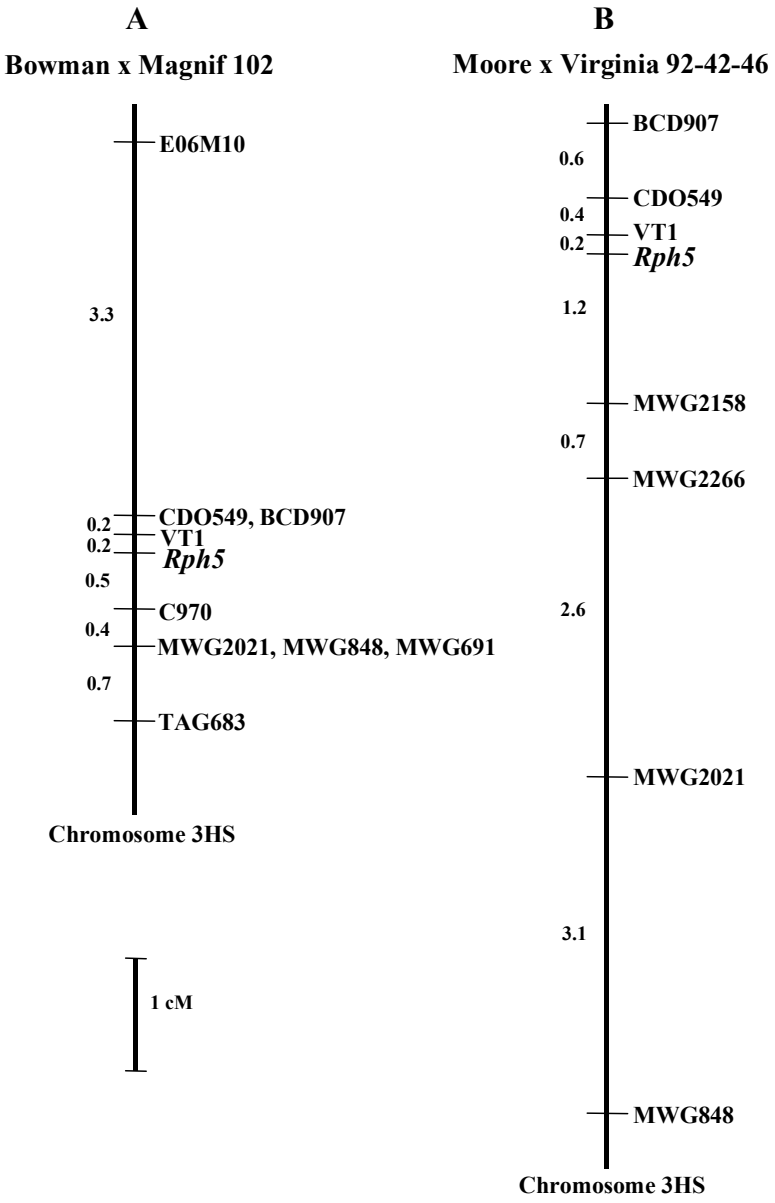


Figure 2.1. Partial molecular maps of barley chromosome 3H showing the genetic location of leaf rust resistance gene *Rph5*. Markers were mapped in two segregating populations: A. Bowman (*rph5*) ´ Magnif 102 (*Rph5*); B. Moore (*rph5*) ´ Virginia 92-42-46 (*Rph5*), respectively. Map distances are given in centimorgans (cM).

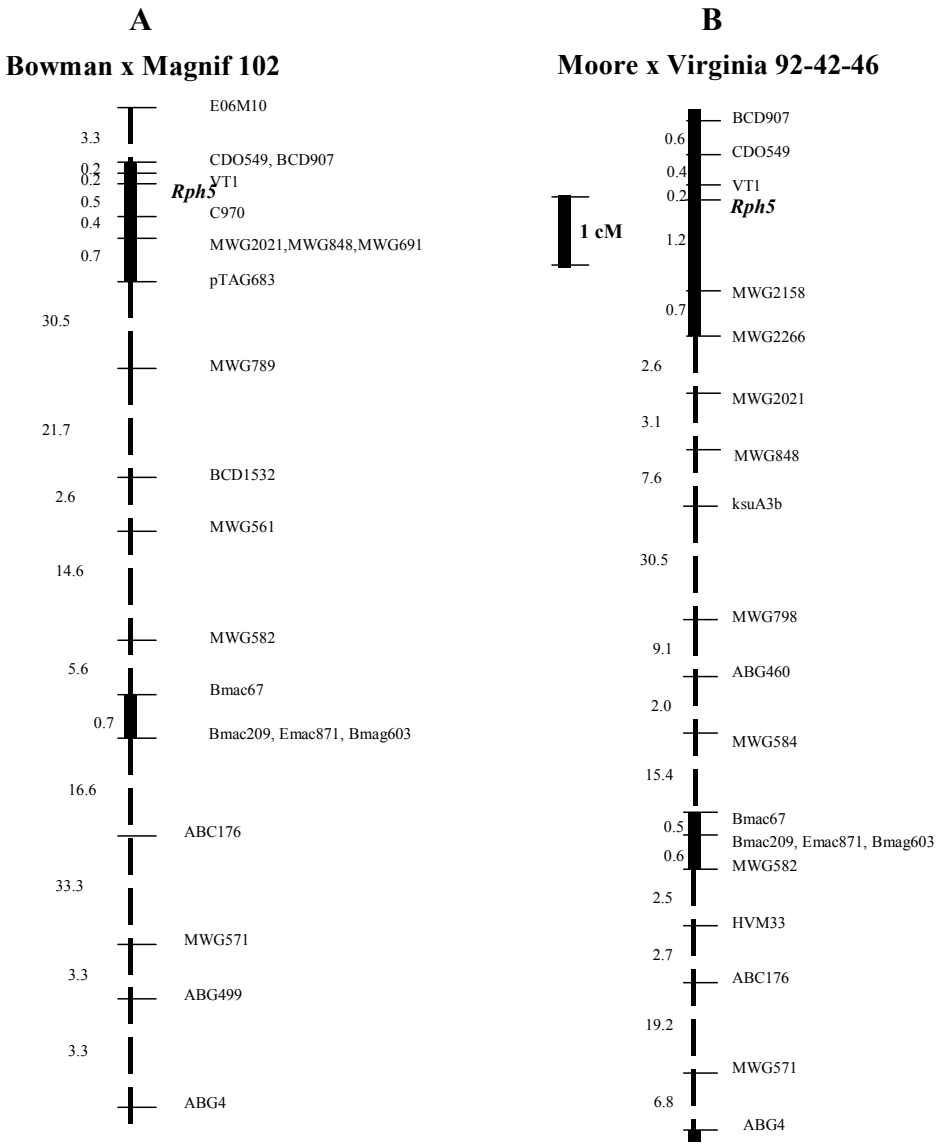


Figure 2.2. Detailed map of barley chromosome 3HS showing the genetic location of leaf rust resistance gene *Rph5*. Markers were mapped in two segregating populations: A. Bowman (*rph5*) ´ Magnif 102 (*Rph5*); B. Moore (*rph5*) ´ Virginia 92-42-46 (*Rph5*), respectively. Map distances are given in centimorgans (cM).

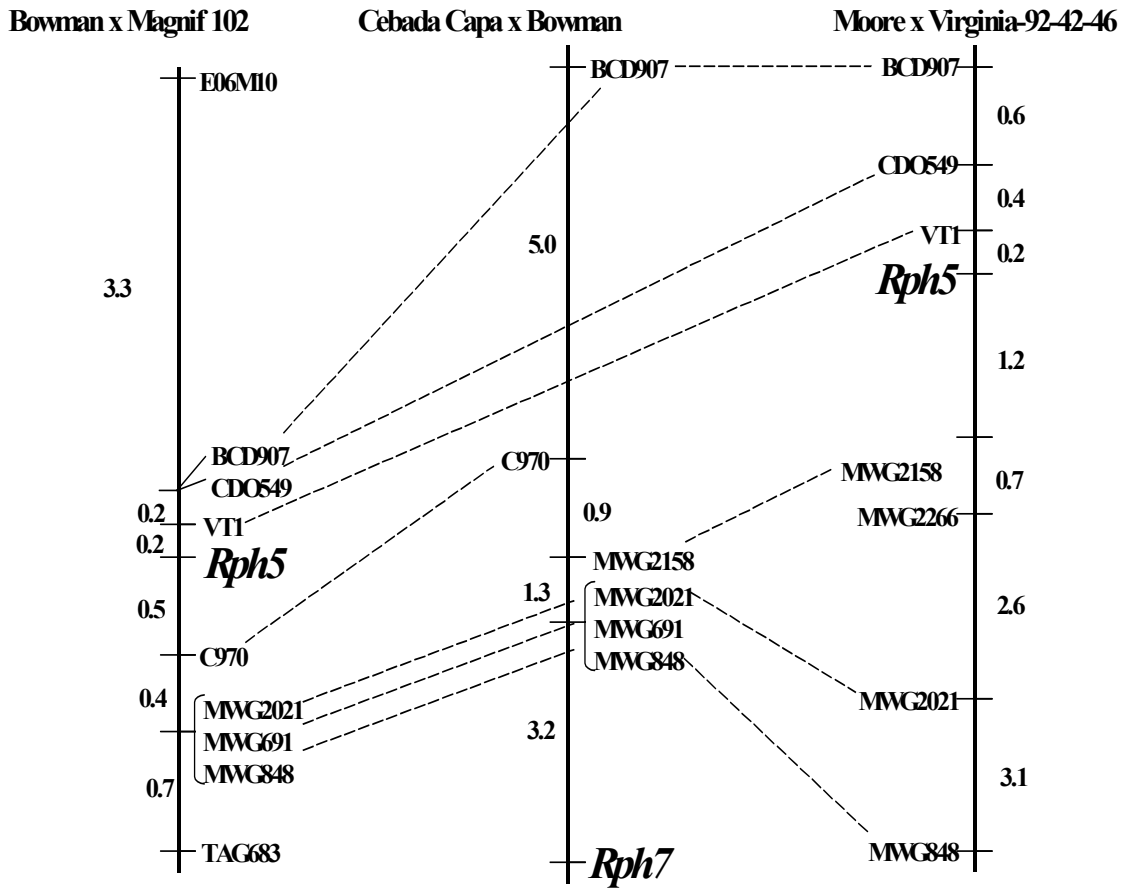


Figure 2.3. Estimation of the relative locations of *Rph5* and *Rph7* leaf rust resistance genes in barley based on comparison of three maps with common markers. Maps of Moore × Virginia 92-42-46 and Bowman × Magnif 102 are from this study, and Cebada Capa × Bowman map was published recently (Brunner et al. 2000)

References

Biyashev, R.M., Ragab, R.A., Maughan, P.J., and Saghai Maroof, M.A. 1997. Molecular mapping, chromosomal assignment, and genetic diversity analysis of phytochrome loci in barley (*Hordeum vulgare*). J. Heredity, **88**: 21-26.

Borovkova, I.G., Jin, Y., and Steffenson, B.J. 1998. Chromosomal location and genetic relationship of the leaf rust resistance genes *Rph*9 and *Rph*12 in barley. Phytopathology, **88**: 76-80.

Borovkova, I.G., Jin, Y., Steffenson, B.J., Kilian, A., Blake, T.K., and Kleinhofs, A. 1997. Identification and mapping of leaf rust resistance gene in barley line Q21861. Genome, **40**: 236-241.

Brodny, U., and Rivadeneira, M. 1996. Physiological specialization of *Puccinia hordei* in Israel and Ecuador: 1992 to 1994. Can. J. Plant Pathol. **18**: 375-378.

Brooks, W.S., Griffey, C.A., Steffenson, B.J., and Vivar, H.E. 2000. Genes governing resistance to *Puccinia hordei* in thirteen spring barley accessions. Phytopathology, **90**: 1131-1136.

Brueggeman, R., Rostoks, N., Kudrna, D., Kilian, A., Han, F., Chen, J., Druka, A., Steffenson, B., and Kleinhofs, A. 2002. The barley stem rust-resistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinases. Proc. Natl. Acad. Sci. U.S.A. **99**: 9328-9333.

Brunner, S., Keller, B., and Feuillet, C. 2000. Molecular mapping of the *Rph7*.g leaf rust resistance gene in barley (*Hordeum vulgare* L.). Theor. Appl. Genet. **101**: 783-788.

Buschges R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., van Daelen, R., van der Lee, T., Diergaarde, P., Groenendijk, J., Topsch, S., Vos, P., Salamini, F., and Schulze-Lefert, P. 1997. The barley *Mlo* gene: a novel control element of plant pathogen resistance. Cell, **88**: 695-705.

Clifford, B.C. 1985. Barley leaf rust. *In* Cereal Rust. Vol. 2, Diseases, Distribution, Epidemiology, and Control., *Edited by* A.P Roelfs and W.R. Bushnell. Academic Press, New York, pp. 173-205.

Cotterill, P.J., Rees, R.G., Platz, G.J., and Dill-Macky, R. 1992. Effects of leaf rust on selected Australian barleys. Aust. J. Exp. Agric. **32**: 747-751.

Coterill, P.J., and Vertigan, W.A. 1992. Detection of *Puccinia hordei* virulent on the *Pa*9 and 'Triumph' resistance genes in barley in Australia. Austr. Plant Pathol. **21**: 32-34.

Fetch, T.G., Steffenson, B.J., and Jin, Y. 1998. Worldwide virulence of *Puccinia hord*ei on barley. Phytopathology, **88**: 28.

Feuerstein, U., Brown, A.H.D., and Burdon, J.J. 1990. Linkage of rust resistance genes from wild barley (*Hordeum spontaneum*) with isozyme markers. Plant Breeding, **104**: 318-324.

Franckowiak, J.D., Jin, Y., and Steffenson, B.J. 1997. Recommended allele symbols for leaf rust resistance genes in barley. Barley Genet. Newsl. **27**: 36-44.

Graner, A., Bauer, E., Kellermann, A., Kirchner, S., Muraya, J.K., Jahoor, A., and Wenzel, G. 1994. Progress of RFLP-map construction in winter barley. Barley Genet. Newsl. **23**: 53-59.

Graner, A., Streng, S., Drescher, A., Jin, Y., Borovkova, I., and Steffenson, B.J. 2000. Molecular mapping of the leaf rust resistance gene *Rph*7 in barley. Plant Breeding, **119**: 389-392.

Griffey, C.A., Das, M.K., Baldwin, R.E., and Waldenmaier, C.M. 1994. Yield losses in winter barley resulting from a new race of *Puccinia hordei* in North America. Plant Dis. **78**: 256-260.

Hayes, A.J., and Saghai Maroof, M.A. 2000. Targeted resistance gene mapping in soybean using modified AFLPs. Theor. Appl. Genet. **100**: 1279-1283.

Ivandic, V., Walther, U., and Graner, A. 1998. Molecular mapping of a new gene in wild barley conferring complete resistance to leaf rust (*Puccinia hordei* Otth). Theor. Appl. Genet. **97**: 1235-1239.

Jin, Y., Statler, G.D., Franckowiak, J.D., and Steffenson, B.J. 1993. Linkage between leaf rust resistance genes and morphological markers in barley. Phytopathology, **83**: 230-233.

Kicherer, S., Backes, G., Walther, U., and Jahoor, A. 2000. Localizing QTLs for leaf rust resistance and agronomic traits in barley (*Hordeum vulgare L.*). Theor. Appl. Genet. **100**: 881-888.

Kilian, A., Kudra, D., and Kleinhofs, A. 1999. Genetic and molecular characterization of barley chromosome telomeres. Genome, **42**: 412-419.

King, K.E., and Polley, R.W. 1976. Observations on the epidemiology and effect on grain yield of brown rust in spring barley. Plant Pathol. **25**: 63-73.

Kunzel, G., Korzun, L., and Meister, A. 2000. Cytologically integrated physical restriction fragment length polymorphism maps for the barley genome based on translocation breakpoints. Genetics, **154**: 397-412.

Lander E.S., Green, P., Abrahamson, J., Barlow, A., Daly, J.M., Lincoln, S.E., and Newberg, L. 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics, **1**: 174-181.

Levine, M.N., and Cherewick, W.J. 1952. Studies on dwarf leaf rust of barley. U.S. Department of Agriculture Technical Bulletin. No. 1056, Washington D.C.

Liu, Z.-W., Biyashev, R.M., and Saghai Maroof, M.A.. 1996. Development of simple sequence repeat DNA and their integration into a barley linkage map. Theor. Appl. Genet. **93**: 869-876.

Mathre D.E. 1997. Diseases caused by fungi. *In* Compendium of Barley Diseases. *Edited by* D.E. Mathre. The American Phytopathological Society, St. Paul, MN, pp. 32-41.

Maughan, P.J., Saghai Maroof, M.A., Buss, G.R., and Huestis, G.M.. 1996. Amplified fragment length polymorphism (AFLP) in soybean: species diversity, inheritance, and near-isogenic line analysis. Theor. Appl. Genet. **96**: 331-338.

Michelmore, R.W., Paran, I., and Kesseli, R.V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. U.S.A. **88**: 9828-9832.

Parlevliet, J.E. 1976. The genetics of seedling resistance to leaf rust, *Puccinia hordei* Otth in some spring barley cultivars. Euphytica, **25**: 249-254.

Park, R.F., and Karakousis, A. 2002. Characterization and mapping of the gene *Rph19* conferring resistance to Puccinia hordei in the cultivar 'Reka I' and several Australian barleys. Plant Breeding, **121**: 232-236.

Qi, X., Fekadu, F., Sijtsma, D., Niks, R.E., Lindhout, P., and Stam, P. 2000. The evidence for abundance of QTLs for partial resistance to *Puccinia hordei* on the barley genome. Mol. Breed. **6**: 1-9.

Ramsay L., Macaulay, M., Degli Ivanissevich, S., MacLean, K., Cardle, L., Fuller, J., Edwards, K.J., Tuvesson, S., Morgante, M., Massarie, A., Maestri, E., Marmiroli, N., Sjakste, T., Ganalg, M., Powell, W., and Waugh, R. 2000. A simple sequence repeat-based linkage map of barley. Genetics, **156**: 1997-2005.

Saghai Maroof, M.A., Soliman, K.M., Jorgensen, R.A., and Allard, R.W. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. U.S.A. **81**: 8014-8018.

Tan, B.H. 1978. Verifying the genetic relationships between three leaf rust resistance genes in barley. Euphytica, **27**: 317-323.

Tuleen, N.A., and McDaniel, M.E. 1971. Location of genes *Pa* and *Pa5*. Barley Newsl. **15**: 106-107.

Upender, M.M., Raj, L., and Weir, M. 1995. Rapid method for elution and analysis of PCR products separated on high-resolution polyacrylamide gels. Biotechniques, **18**: 33-34.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. Nucl. Acids Res. **23**: 4407-4414.

Weerasena, J.S., Steffenson, B.J., and Falk, A.B. 2004. Conversion of an amplified fragment length polymorphism marker into a co-dominant marker in the mapping of the *Rph15* gene conferring resistance to barley leaf rust, *Puccinia hordei Otth*. Theor. Appl. Genet. **108**: 712-719.

Wei, F., Gobelman-Werner, K., Morroll, S.M., Kurth, J., Mao, L., Wing, R., Leister, D., Schultze-Lefert, P., and Wise, R.P. 1999. The *Mla* (powdery mildew) resistance cluster is associated with three NBS-LRR gene families and suppressed recombination within a 240-kb DNA interval on chromosome 5S (1HS) of barley. Genetics, **153**: 1929-1948.

Zilinsky, F.J. 1983. Common diseases of small grain cereals: a guide to identification.. The International Maize and Wheat Improvement Center, Mexico, pp. 11-16.

Zhong, S., Effertz, R.J., Jin, Y., Frankowiak, J.D., and Steffenson, B.J. 2003. Molecular mapping of the leaf rust resistance gene *Rph6* in barley and its linkage relationships with *Rph5* and *Rph7*. Phytopathology, **93**: 604-609.

Zwonitzer, J.C. 1999. Identification and mapping of a resistance gene to barley leaf rust (*Puccinia hordei* G. Otth). M.S. Thesis. Virginia Tech, Blacksburg, VA.

CHAPTER 3

HIGH-RESOLUTION MAPPING OF THE BARLEY LEAF RUST RESISTANCE GENE *RPH5* USING BARLEY ESTs AND SYNTENY WITH RICE

Abstract

The rapidly growing expressed sequence tag (EST) resources of species representing the *Poacea* family and availability of comprehensive sequence information for the rice (*Oryza sativa*) genome create an excellent opportunity for comparative genome analysis. Extensively reported synteny between rice chromosome 1 and barley (*Hordeum vulgare L.*) chromosome 3 has extremely useful application in saturation of chromosomal region around a target gene of the large-genome barley with conserved orthologous genes from the syntenic regions of the rice genome. Barley leaf rust (caused by *Puccinia hordei* G. Otth) resistance gene *Rph5* was previously mapped to the chromosome 3HS, which is syntenic with rice chromosome 1S. The objective of this study was to increase marker density within the sub-centimorgan region around *Rph5* using sequence-tagged site (STS) markers that were developed based on barley ESTs syntenic to the phage (P1)-derived artificial chromosome (PAC) clones comprising distal region of the rice chromosome 1S. Five rice PAC clones were used as queries to blastn 370,258 barley ESTs. Ninety four non-redundant EST sequences were identified from the EST database and used as templates to design 174 pairs of primer combinations. As a result, 10 EST-based STS markers were incorporated into the 'Bowman' × 'Magnif 102' high-resolution map of the *Rph5* region. More importantly, six markers, including five EST-derived STS sequences, co-segregate with *Rph5*. Genes, represented by these markers, are putative candidates for *Rph5*. Results of this study demonstrate the usefulness of rice genomic resources for efficient deployment of barley ESTs for marker saturation of targeted barley genomic region.

Keywords: barley, rice, leaf rust, STS, EST, high resolution map, synteny, comparative mapping

Introduction

Comparative genetic mapping in several economically important crops, particularly rice and barley (Saghai Maroof et al. 1996), discovered the conservation of gene or marker order (collinearity) within large portions of the genomes of these two species due to low numbers of chromosomal rearrangements during their evolution. According to Moore (1995), individual rice chromosome arms or complete chromosomes can be assembled like "Lego" blocks to reconstitute the chromosomes of large-genome grass species. Comparison of sequences from putatively syntenic loci in rice and other grass species demonstrated that sequence conservation between the species is mainly restricted to coding regions (Avramova et al. 1996; Tikhonov et al. 1999; Dubcovsky et al. 2001). Extensively reported synteny between rice chromosome 1 and barley chromosome 3, has extremely useful applications in saturation of the chromosomal region around a target gene of the large-genome barley (Smilde et al. 2001) with conserved orthologous genes from syntenic regions of the rice genome (Bennetzen and Freeling 1993). From this point of view, comprehensive datasets of 370,258 barley expressed sequence tags (EST), which represent portions of the coding regions (summary dbEST release June 2003, http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html) are rich resources for genome mapping.

Draft genome sequences of two rice subspecies *Oryza sativa ssp. indica* (Yu et al. 2002) and *Oryza sativa ssp. japonica* (Goff et al. 2002) and high-quality sequences of rice chromosomes 1 (Sasaki et al. 2002) and 4 (Feng et al. 2002) have been recently reported. In contrast to rice, the large-genome barley is unlikely to become the target of full genome sequencing in the near future. This is reflected by the limited availability of complete sequences of genomic bacterial artificial chromosome clones (BAC) of barley, which resulted from attempts of targeted gene isolation and studies of microcollinearity among grass species (Büschges et al. 1997; Lahaye et al. 1998; Wei et al. 1999; Druka et al. 2000; Collins et al. 2001; Dubcovsky et al. 2001). Hence, available rice genomic sequences as well as barley EST sequences, serve as excellent resources for "gene-based marker saturation of a syntenic target region and may accelerate attempts of synteny-

based positional cloning of agronomically important genes from large-genome cereal species" (Perovic et al. 2004).

Few studies have focused specifically on synteny-based marker saturation around genes of interest in the barley genome. Using synteny between rice chromosome 4 and barley chromosome 2, Schmierer et al. (2003) mapped 26 barley ESTs to the major Fusarium head blight QTL region. These barley ESTs were chosen for mapping purposes based on their homology to the rice BAC clones comprising rice chromosome 4. Perovic et al (2004) integrated 11 barley ESTs into an *rph16* (barley leaf rust resistance gene) high-resolution map using synteny between barley chromosome 2 and rice chromosomes 4 and 7L (Dunford et al. 2002). However, in both studies, the target regions for marker saturation were quite large, 20 cM and 40 cM, respectively.

The objective of this study was to increase marker density within the sub-centimorgan region around barley leaf rust resistance gene *Rph5* using sequence-tagged site (STS) markers developed based on barley ESTs syntenic to BAC and PAC clones comprising rice chromosome 1. Gene *Rph5* confers resistance to the most prevalent barley leaf rust races 8 and 30 in the United States. Although virulence for *Rph*5 is widely prevalent in Europe (Parlevliet 1976) and South America (Brodny and Rivadeneira 1996; Fetch et al. 1998), it has not been identified in North America. Thus, *Rph*5 could be used to protect barley cultivars from leaf rust damage in North America. *Rph5* was recently mapped onto the extreme telomeric region of the short arm of barley chromosome 3H at 0.2 cM proximal to the RFLP marker VT1 and 0.5 cM distal from the RFLP marker C970 in a 'Bowman' (*rph5*) × 'Magnif 102' (*Rph5*) population (Mammadov et al. 2003). In this study an initial F_2 population of 93 individuals, developed from a Bowman × Magnif 102 cross, was expanded to 400 individuals. Rice clone C970, mapped to the syntenous region in rice chromosome 1, was used as an anchor marker for saturation and construction of a high-resolution map around the *Rph5* locus.

Materials and methods

Genetic materials

An F_2 population derived from the cross 'Bowman' (PI 483237) × 'Magnif 102' (PI 337140) was used for high-resolution mapping of *Rph5*. Magnif 102 (Franckowiak et

al. 1997) is a donor of *Rph5* and provides the genetic source of resistance to leaf rust in this experiment. The new F_2 mapping population (400 individuals) was developed by combining the original F_2 (93 individuals) population (Mammadov et al. 2003) with an additional 307 F_2 individuals. The 400 F_2 individuals were used for high-resolution mapping of *Rph5*.

Disease screening

Disease screening was done by Dr. B. Steffenson at the University of Minnesota St. Paul. Briefly, 15 F_3 seeds per F_2 plant were planted in the greenhouse and inoculated with race 8 as described by Jin et al. (1996). The symptoms were rated according to the 0-4 scale of Levine and Cherewick (1952). Infection types of 0, 1, or 2 were considered indicative of host resistance, whereas infection types 3 or 4 were considered indicative of host susceptibility. Disease assessments were performed 10 to 14 days after inoculation. Infection types of F_2 progeny were compared with infection types of the parental lines and host-differentials to assure proper scoring and assignment into resistant and/or susceptible classes.

Development of barley STS markers

The entire sequences of 5 rice PAC [phage (P1)-derived artificial chromosome] clones syntenic to the *Rph5* region (see Results section) were used as queries in a blastn (Altschul et al. 1997) search to screen 370,258 barley ESTs stored in the EST database (dbEST) of the NCBI. Barley ESTs with an expected (E) value equal to or less than $1e^{-10}$ were selected for construction of temporary contigs (TCs) using SeqMan tool of the Lasergene software (DNASTAR, Madison, WI). Furthermore, consensus sequences of TCs as well as sequences of the singletons were used as a template to design EST-specific primers using the Primer Select tool of the Lasergene software. Simple sequence repeats (SSRs) and indels (insertion and deletions) were targeted for primer design. If SSRs and indels were absent, the entire consensus sequences of the TCs were used as queries to blastn the GenBank NR database. The query and the best non-barley hit were, then, aligned using Megalign tool of the Lasergene software. If alignment showed large gaps between the two sequences, primers were designed to flank those gaps. PCR

amplification was carried out according to published procedures (Saghai Maroof et al. 1994; Liu et al. 1996; Ramsay et al. 2000).

Mapping of other molecular markers to the *Rph5* region

In addition to barley ESTs, we tested 28 wheat STS markers that were kindly provided by Dr. J.A. Anderson from the University of Minnesota at St. Paul in our Bowman × Magnif 102 mapping population. These STS markers were mapped by Liu and Anderson (2003) to the wheat 3BS chromosome to tag major wheat QTL for Fusarium head blight resistance. Since this region is syntenic to barley chromosome 3HS, attempt was made to map wheat STS markers too.

The RFLP marker ABG70 was located on the top of chromosome 3HS, based on A. Kleinhofs' Bins at http://barleygenomics.wsu.edu/arnis/linkage_maps/maps-svg.html. Using sequence information provided at NCBI for ABG70 (Accession # L44083), an STS marker was developed.

Linkage analysis

Disease and marker data were used to determine the chromosomal location of *Rph5*. For genetic mapping and linkage analysis, the computer program MAPMAKER version 3.0b was used (Lander et al. 1987). Linkage maps were constructed based on an LOD threshold of 3.0 and maximum Haldane distance of 50 cM.

Results

Strategies for identification of target region in rice chromosome 1S syntenic to *Rph5* region in barley

Barley leaf rust resistance gene *Rph5* was genetically mapped between the two RFLP markers VT1 and C970 in an F_2 population of 93 individuals. The genetic distance between these two markers is 0.7 cM. This small region, flanking the *Rph5* locus, was selected as a target for saturation mapping with barley gene-based markers syntenic to rice 1S chromosome. Importantly, rice clone C970 was also mapped to the tip of rice chromosome 1S (http://www.gramene.org/japonica/contigview?chr=1&vc_start=645764&vc_end=646132&bottom=%7Cbump_Markers%3Aon). Thus, the *Rph5* locus of barley chromosome 3HS is syntenous to the distal region of chromosome 1S of rice.

In order to delimit the syntenic region in rice, nucleotide sequences of C970 (GenBank accession #D15622) and VT1 (Mammadov et al. 2003) were used as queries to screen the GenBank database using blastn program (Altschul et al. 1997). The blastn search was limited to *Oryza sativa* non-redundant (NR) database. C970 detected 2 rice PAC clones AP003219 and AP002882. VT1 did not reveal any significant similarities with rice sequences. In order to determine the location of the C970 clone with respect to the retrieved PAC clones, the entire sequences of the two PAC clones and C970 were used to construct TC. Results indicated that C970 is located at the intersection of the two rice PAC clones (Fig. 3.1). In addition to C970 and VT1, the sequence of RFLP clone CDO549, which was previously mapped 0.2 cM distal from VT1 in the Bowman × Magnif 102 population (Mammadov et al 2003), was used as a query to blastn the rice NR database. CDO549 retrieved the rice PAC clones AP002845 and AP002863 with a significant E value (9e-33) (Table 3.1). Thus, the *Rph5* locus spanning from C970 to CDO549 markers in barley was syntenic to the segment of rice chromosome 1S bordered by the proximal and distal rice PAC clones AP003219 and AP002863, respectively. Apart from these two PAC clones, this region of the rice genome is represented by AP002882, AP002845, and AP002818, which are contiguous to AP003219 and AP002863 (http://www.gramene.org/japonica/SeqTable?chr=1). The genomic sequences

of these five rice PAC clones were blastned against the barley dbEST. AP003219 detected 102 barley ESTs with E-values varying from 7e-98 to 2e-12. A majority of the barley ESTs (80%) were syntenic to the distal region of rice PAC clone AP003219 (Fig. 3.2). In contrast to AP003219, the other three PAC clones detected large numbers of barley ESTs. For instance, 862 barley ESTs were syntenic to AP002863 with E-values varying from e-122 to 6e-10. The five rice PAC clones in this study detected a total of 3,962 barley ESTs. As can be seen from Fig. 3.2, the level of redundancy among the blastn hits is quite high. In order to remove the redundancy, we assembled the EST sequences into TCs. This approach reduced the final number of candidate gene fragments to 94, which were either represented by EST singletons or EST TCs.

Saturation of the *Rph5* region with gene-based markers

In total, 94 non-redundant barley EST sequences were used to design primers for PCR amplification. Primers were designed to flank the source of potential polymorphisms, including microsatellites and indels. The expected size of the PCR product was desirable to be not larger than 300 bp to enable polyacrylamide gel electrophoresis (PAGE) assays. When the sources of polymorphism were scattered within one TC or singleton of 1 kb + size, we designed several primer combinations in order to cover the whole span of the target sequence. The IDs of the developed EST-derived barley STS markers contain useful information about the origin of the marker (Fig. 3.3). As a result, we designed 174 primer combinations for amplifying the 94 non-redundant EST sequences syntenic to the five rice PAC clones. Out of the 94 EST sequences, 10 were polymorphic between the two parental lines and were subsequently mapped to a region of 8.8 cM between markers VT1 and MWG691 (Fig. 3.1A). EST sequences syntenic to portion of rice PAC clone AP003219, non-overlapping with AP002882, as well as to the entire AP002818 clone (Fig. 3.3B, shown in black color) were monomorphic between the two parents. Two markers TC2882-BF25 and TC2882-BM37.3 were mapped 1.5 cM and 3.6 cM proximal to *Rph5*, respectively (Fig. 3.1A). The former is syntenic to the rice PAC clone AP002882, while the latter is syntenic to the region of the rice genome located at the intersection of AP002882 and AP003219 (Table 3.1 and Fig. 3.1B). Markers TC2845-BJ55.2 and TC2845-BQ46.1 were syntenic to both

rice PAC clones AP002845 and AP002863 (Table 3.1 and Fig. 3.1B) and were located 0.1 cM and 0.2 cM, respectively, proximal to *Rph5* (Fig. 3.1A). Remarkably, five EST markers, syntenic to both AP002863 and AP002845, co-segregate with *Rph5* (Fig. 3.1A). Marker TC2863-16.1, exhibiting synteny with AP002863, was mapped 0.6 cM distal to the gene, hence, delimiting the distal border of *Rph5*. In addition to EST markers, we mapped the barley STS marker ABG70, which also co-segregates with *Rph5* (Fig. 3.3A). With respect to the wheat STS markers, only one out of 28 (STS3B-66) is polymorphic in the Bowman × Magnif 102 population and was mapped 12.8 cM proximal to *Rph5* (Fig. 3.1A). In total, we saturated the *Rph5* region with 11 new molecular markers, including six co-segregating sequences (Fig. 3.1A and Table 3.2).

Discussion

In this study synteny-based maker saturation of the barley *Rph5* leaf rust resistance gene region was achieved utilizing the abundant rice genomic information available and the rapidly growing barley EST sequence resources. Rice RFLP clone C970 was previously mapped 0.5 cM proximal to *Rph5* in barley (Mammadov et al. 2003). Since this marker was also mapped onto the distal region of rice chromosome 1S, it was used as an anchor marker to identify the syntenic region in the rice genome. As a result, a 585 kb region of rice chromosome 1S was identified as being syntenic to the *Rph5* region spanning from CDO549 to C970. This segment of rice chromosome 1S is comprised of 5 PAC clones (Fig. 3.1B), which were used as queries to blastn barley EST database. An important aspect of this analysis was the reduction in redundancy of the retrieved EST sequences. These ESTs (3,962 sequences) were assembled into TCs that led to a reduction in the number of redundant sequences by 97.6%. The remaining 2.4% were represented by non-redundant EST sequences, which served as a template for the development of STS markers. By a similar approach, Perovic et al. (2004) were successful in the saturation of a 20 cM region around another barley leaf rust resistance gene, *Rph16,* with barley EST sequences. Using a "two-step *in silico* selection of candidate orthologous genes", they reduced EST redundancy by 96%. In the present study, 10 EST-based STS markers from the distal TC2863-16.1 to the proximal TC2882-BM37.3 were mapped between VT1 and MWG691 markers, covering a distance of 4.2

cM (Fig. 3.1A). Based on location of EST markers, the region of barley genome, flanked by markers CDO549 and MWG691, is colinear with the corresponding region in rice, bracketed by PAC clones AP002863 and AP003219 (Fig. 3.1B). However, small translocations were observed in the order of genes in barley related to syntenic rice clones AP002863 and AP002845 (Fig. 3.1A, B). Brunner et al. (2003) and Perovic et al. (2004) noted similar observations while saturating regions of the barley leaf rust resistance genes *Rph7* and *rph16*, respectively, with ESTs originating from rice chromosome 1S. In general, rearrangements in syntenic regions appear to be a common attribute at the DNA level between species (Tarchini et al. 2000; Dubcovsky et al. 2001; Song et al. 2002).

Eight different types of genes were identified at the *Rph5* region (Table 3.1). Apart from genes encoding putative acetoacyl-CoA-thiolase (TC2882-BF25), protein kinase homolog (TC2863-37.1 and TC2863-37.2), endoplasmatic reticulum retrieval protein Rer1A (TC2863-12.4), limonene cyclase-like protein (TC2863-32.1 and TC2845-BQ46.1), no function based on similarity with known proteins could be assigned to the other genes. Importantly, linkage analysis indicates that five genes (TC2863-37.1, TC2863-37.2, TC2863-32.1, TC2863-12.4 and TC2863-19.2) are putative candidates for *Rph5*. However, none of the mapped sequences belong to the NBS-LRR class of disease resistance genes. Barley leaf rust resistance genes may belong to a different or novel class of R genes. For instance, the wheat leaf rust resistance gene, *Lrk10* (GenBank accession # U51330), encodes a receptor-like kinase (Feuillet et al. 1997). From this point of view, TC2863-37.1 is of great interest, as it is similar to a protein kinase homolog in *Arabidopsis thaliana*. Meanwhile, barley STS marker ABG70, which also co-segregates with *Rph5,* does not exhibit any similarity to any GenBank sequence, including rice. Thus, microcolinearity between rice and barley in the ABG70 locus is broken. Even though ABG70 is not an expressed sequence, this marker might represent the portion of the candidate gene for *Rph5*. The fact that ABG70 does not have an ortholog in rice can be explained by the phenomenon that leaf rust does not affect rice and, consequently, as a result of high selection pressure on this category of genes, rice must have lost the *Rph5* ortholog. In fact, disruption in synteny among cereals at loci of disease resistance gene homologs is not a rare phenomenon (Leister et al. 1998). For instance, synteny-based cloning of barley stem rust resistance gene, *Rpg1*, was unsuccessful, because an ortholog

of this gene was absent in the rice genome (Han et al. 1999). Later, *Rpg1* was isolated by a positional cloning approach and it was confirmed that rice genome does not possess the *Rpg1* orthologous gene (Brueggemann et al. 2002). Perovic et al. (2004) could not confirm colinearity among rice and barley at the barley leaf rust resistance gene *Rph16* locus "since no obvious resistance gene candidate could be determined in rice that would cosegregate in barley with the *Rph16*". However, Brunner et al. (2003) claimed that barley leaf rust resistance gene *Rph7,* which is located on the same chromosome as *Rph5,* could belong to a new class of disease resistance genes, particularly HGA genes, which are very well conserved in rice. HGA genes are unknown protein family in rice. HGA stands for amino acids histidine (H), glycine (G) and alanine, "which are the core of the most conserved domain located in the C terminus of the protein" (Feuillet, personal communication).

Our next step towards map-based cloning of *Rph5* is to continue high-resolution mapping of *Rph5* with the remaining 600 F_2 individuals. Genotyping of these individuals will be done using molecular markers co-segregating or closely linked to the gene. For instance, barley co-dominant STS markers ABG70, TC2863-16.1, TC2863-37.1, TC2863-12.4, TC2845-BJ55.2 and TC2845-BQ46.1 are great candidates for screening the mapping population to identify additional recombinants, if they exist. Simultaneously, these markers will be used as probes to screen the available cv. Morex BAC library to construct a BAC contig spanning the *Rph5* region. This will determine physical to genetic ratio at the *Rph5* locus, which is very valuable information for positional cloning of the gene. BAC clones comprising the *Rph5* region also will be sequenced in order to isolate the susceptible allele of *Rph5*. However, BAC clones from Morex library may not contain susceptible allele of *Rph5*, because of deletion of the entire gene from the Morex genome.

Table 3.1. Rice PAC [phage (P1)-derived artificial chromosome] clones detected from the public database by BLASTing with molecular markers mapped within the *Rph5* region in barley.

***Rph5* markers**	**Rice PAC clones**	**Sequences within rice PAC clones syntenic to *Rph5* region**		**Score**	**E-value**
		Beginning	**End**		
TC2863-12.4	AP002863	70888	70947	224	4e-55
TC2863-16.1	AP002863	83022	82966	93	3e-17
TC2863-37.1 and	AP002863	143423	143483	121	1e-25
TC2863-37.2	AP002845	31786	31846	121	1e-25
TC2863-32.1	AP002863	165652	165712	80	5e-13
	AP002845	54075	54122	80	5e-13
TC2845-BQ46.1	AP002863	165652	165759	80	5e-13
	AP002845	54015	54122	80	5e-13
TC2845-BJ55.2	AP002863	169412	169662	246	5e-62
	AP002845	57775	58025	246	5e-62
TC2863-19.2	AP002863	169412	170059	355	1e-95
	AP002845	57775	58422	355	1e-95
CDO549	AP002863	177705	177828	143	9e-33
	AP002845	66068	66191	143	9e-33
TC2882-BF25	AP002882	24711	24807	129	7e-28
TC2882-BM37.3	AP002882	105798	105907	74	8e-12
	AP003219	14937	15046	74	8e-12
C970	AP002882	121902	122935	2008	0.0
	AP003219	31041	32061	2008	0.0

Table 3.2. Primer sequences of the barley STS markers mapped to the *Rph5* region.

Marker	Forward primer (5' to 3')	Reverse primer (5' to 3')	PCR product size, bp
ABG70	AAACAGGAGACAACGGGACCAA	GCAATGCAACGCCACGAG	244
TC2863-37.1	CGGCTTCAACGGCATCATCAT	TGCTTCCCTCGGGTCAAATCC	233
TC2863-37.2	GCACCGCCCGCATACATCA	TGGTCCTCGAGGGCTACAAAGAAG	177
TC2863-19.2	GCTGTTGATGGCACGGACGACGAC	GGATATGCCAAGGACACCGATGAC	236
TC2863-12.4	TACAAATACGTGCCCTTCAACATC	AGCCTCGTCGACTCTATCTTCC	154
TC2863-32.1	CCATGACTGGGGACCTTACACGACTA	TTGAGAAGGACAGGCCGAACACG	249
TC2863-16.1	AGCAGCCCCCTTTTCTTTGTCCGTCTCTC	CCCTTGGCCTTGAGCAGCGAGCAC	128
TC2845-BJ55.2	AACCAGGCTGCCATCTTTCTAT	CGCCTTACGCTTCCCGAGTGA	227
TC2845-BQ46.1	TACGAGGAACATACATAAGTCTGC	GTTCACCTCATACCCCATTG	259
TC2882-BM37.3	GGTGGAGGAGGTGGCGGGAACTAC	AAGAATTGATGGACTACGAACTGA	184
TC2882-BF25	GGACAGCGGCGCGTTTGATT	GGCTTTCTCCCCACTGACTA	300

Table 3.3 Putative function of the EST-based markers mapped to *Rph5* region in barley.

STS marker	Similar proteins	Score/E-value
TC2882-BM37.3	No similarity	
TC2845-BQ46.1	Limonene cyclase-like protein	270/2e-95
TC2882-BF25	Putative acetoacyl-CoA-thiolase [*Oryza sativa*]	427/e-130
TC2845-BJ55.2	Unknown protein in rice	291/e-124
STS3B-66	ATP-dependent metalloprotease FtsH1 [*Arabidopsis thaliana*]	518/e-145
TC2863-37.1	Protein kinase homolog [*Arabidopsis thaliana*]	72/9e-12
TC2863-37.2	Protein kinase homolog [*Arabidopsis thaliana*]	72/9e-12
TC2863-19.2	Unknown, expressed protein [*Arabidopsis thaliana*]	178/1e-43
TC2863-12.4	Endoplasmatic reticulum retrieval protein Rer1A protein (AtRer1A) [*Oryza sativa*]	239/9e-62
TC2863-32.1	Limonene cyclase-like protein [*Oryza sativa*]	256/1e-67
TC2863-16.1	Hypothetical protein	54/2e-06

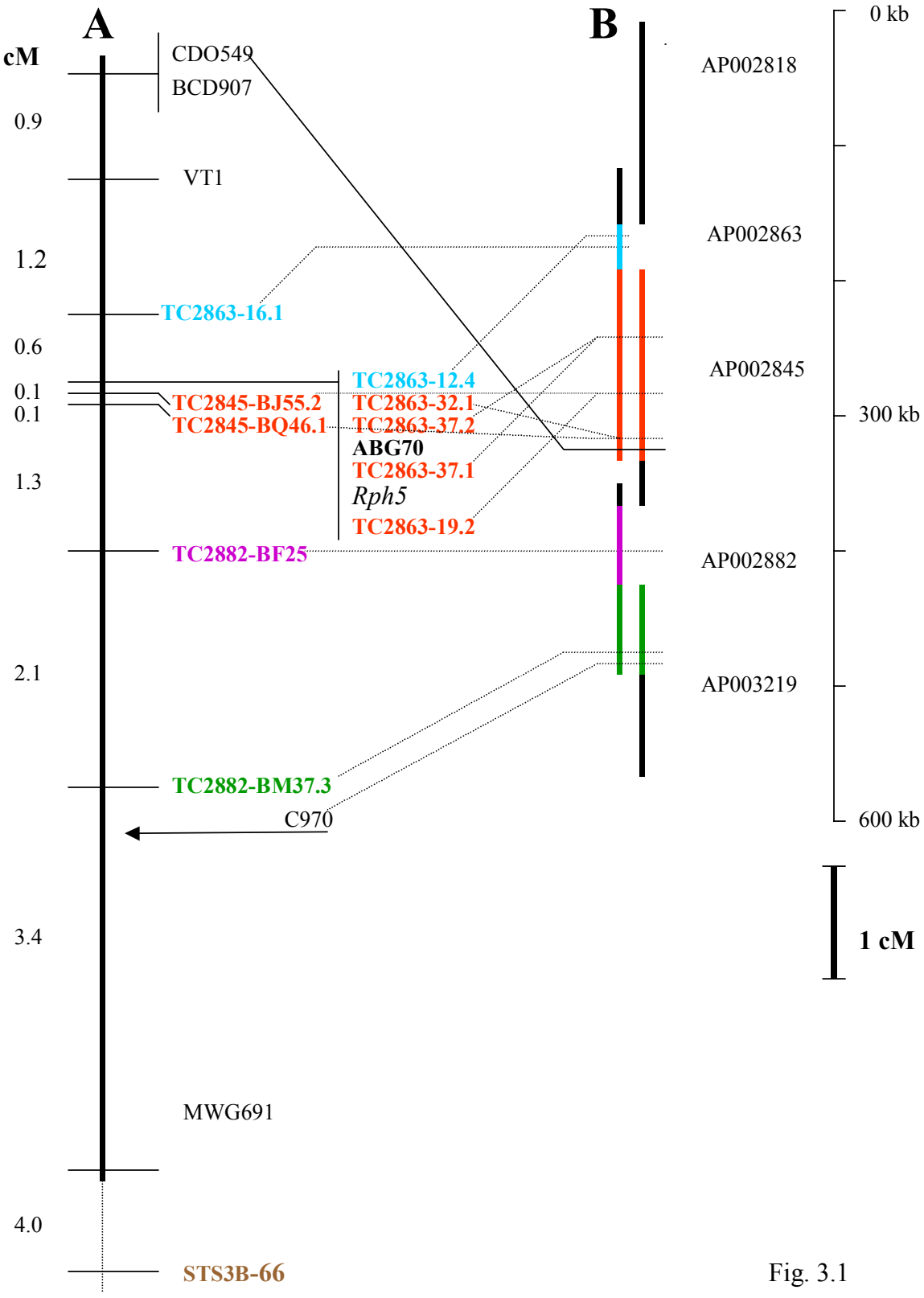

A
B
cM
CDO549
BCD907
0.9
VT1
1.2
TC2863-16.1
0.6
0.1
0.1
TC2863-12.4
TC2845-BJ55.2
TC2863-32.1
TC2845-BQ46.1
TC2863-37.2
ABG70
1.3
TC2863-37.1
Rph5
TC2863-19.2
TC2882-BF25
2.1
TC2882-BM37.3
C970
3.4
MWG691
4.0
STS3B-66
AP002818
AP002863
AP002845
AP002882
AP003219
0 kb
300 kb
600 kb
1 cM

Fig. 3.1

Figure 3.1. A, B. Comparative mapping of the *Rph5* region in barley and rice.

A. High-resolution genetic map of the *Rph5* region resulting from the 'Bowman' × 'Magnif 102' population. Molecular markers shown in color are barley EST-based STS sequences. STS markers shown in blue were syntenic to the distal region of the rice PAC clones AP002863, in red – to the overlapping region of AP002863 and AP002845; in purple – to AP002882 and in green – to the overlapping region of AP002882 and AP003219. Wheat EST is shown in brown. The non-EST-based STS marker ABG70 is shown in black and bold. Markers, previously mapped in Mammadov et al. (2003), are shown in regular font. The approximate location of the rice RFLP clone C970 is depicted by an arrow. This clone was previously tested in 93 F_2 individuals (Mammadov et al. 2003) only, and was not used in this study in linkage analysis. Since C970 is 12 kb proximal from the segment of the rice clone AP003219, which is syntenic to TC2882-BM-37.3 (http://www.gramene.org/japonica/contigview?chr=1&vc_start=645764&vc_end=646132&bottom=%7Cbump_Markers%3Aon), we assumed that genetically this clone is also located proximal to TC2882-BM-37.3. **B**. Physical map of the 585 kb length distal portion of the rice chromosome 1S syntenic to the *Rph5* region of barley chromosome 3HS, represented by overlapping rice PAC clones. The length of the rice contig was calculated using PAC coordinates on the rice chromosome 1S (http://www.gramene.org/japonica/SeqTable?chr=1). The color code of the rice PAC clones corresponds to the color code of the syntenic polymorphic EST-based STS markers. Portions of the PAC clones shown in black are syntenic to the barley ESTs, which were monomorphic in our mapping population. The distal part of each PAC clone is the its 5' end and proximal – 3' end. Dotted lines connecting A and B indicate colinear markers between barley and rice, while solid lines show deviation from macro-colineraity. Several EST-derived STS markers are syntenic to the same region within the rice PAC clone. That is why, lines from these markers (A) are projected to the single point on the rice PAC clone (B).

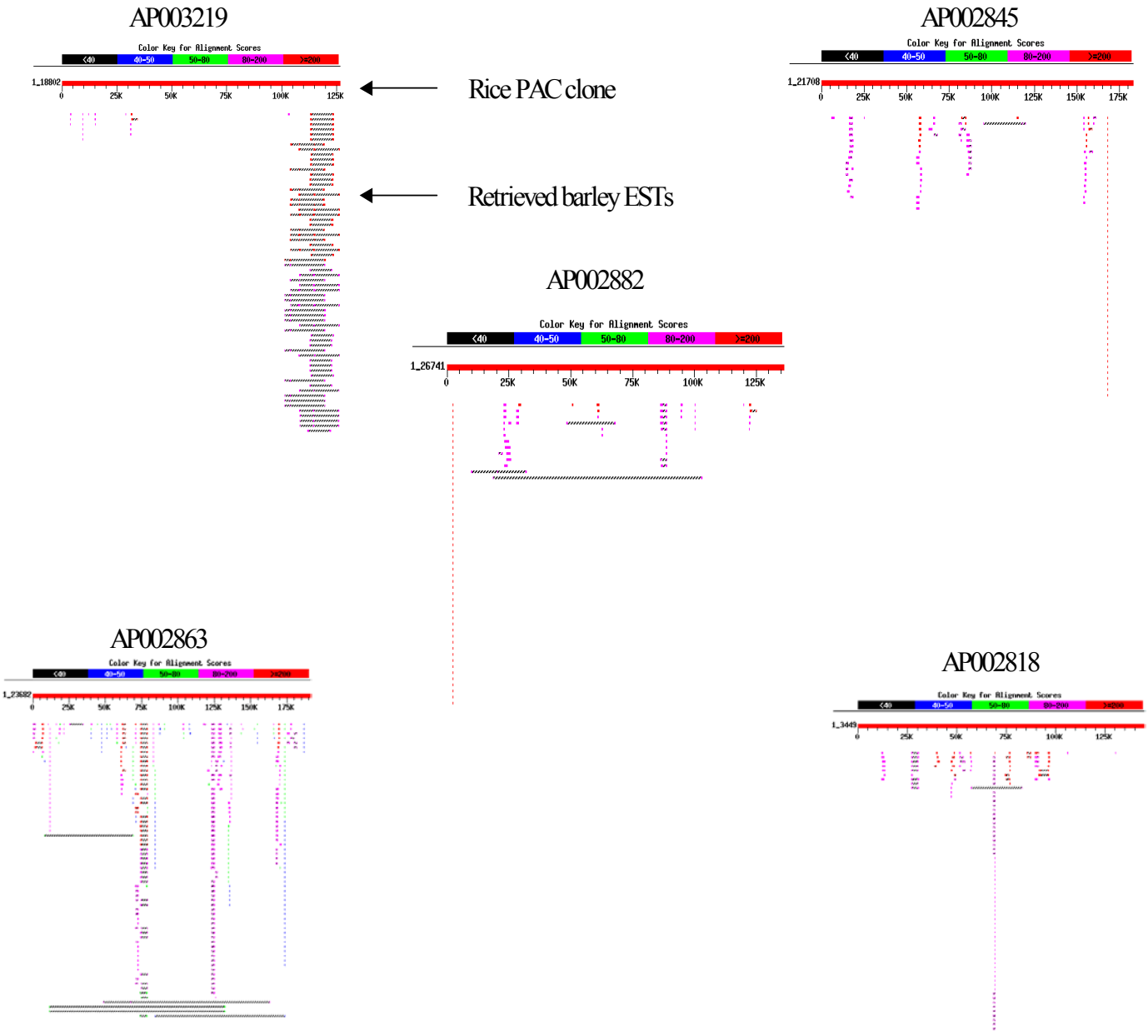


Figure 3.2. Distribution of barley expressed sequence tag (EST) hits on the query represented by rice phage (P1)-derived artificial chromosome (PAC) clones.

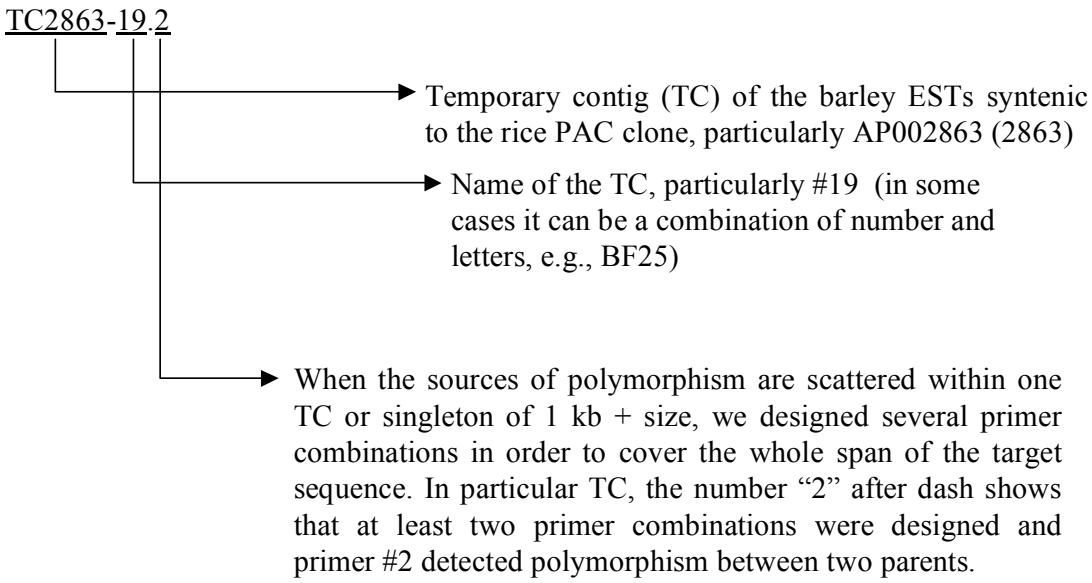


Figure 3.3. How to read the IDs of EST-derived barley STS markers

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., and Liman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**: 3389-3402.

Avramova, Z., Tikhonov, A., SanMiguel P., Jin, Y.-K., Liu, C., Woo, S.-S., Wing, R.A., and Bennetzen, J.L. 1996. Gene identification in a complex chromosomal continuum by local genomic cross-referencing. Plant J. **10**: 1163-1168.

Bennetzen, J.L., and Freeling, M. 1993. Grasses as a single genetic system: genome composition, collinearity and compatibility. Trends Genet. **9**: 259-261.

Brodny, U., and Rivadeneira, M. 1996. Physiological specialization of *Puccinia hordei* in Israel and Ecuador: 1992 to 1994. Can. J. Plant Pathol. **18**: 375-378.

Brueggeman, R., Rostoks, N., Kudrna, D., Kilian, A., Han, F., Chen, J., Druka, A., Steffenson, B., and Kleinhofs A. 2002. The barley stem rust-resistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinases. Proc. Natl. Acad. Sci. U.S.A. **99**: 9328-9333.

Brunner, S., Keller, B., and Feuillet, C. 2003 A large rearrangement involving genes and low copy DNA interrupts the micro-colinearity between rice and barley at the *Rph7* locus. Genetics, **164**: 673-683.

Büschges, R., Hollrichter, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., van Daelen, R., van der Lee, T., Diergaarde, P., Groenendijk, J., Töpsch, S., Vos, P., Salamini, F., Schulze Lefert, P. 1997. The barley *Mlo* gene: a novel control element of plant pathogen resistance. Cell, **88**: 695-705.

Collins, N., Park, R., Spielmeyer W., Ellis, J., and Pryor, A.J. 2001. Resistance gene analogs in barley and their relationships to rust resistance genes. Genome, **44**: 375-381.

Druka, A., Kudrna D., Han F., Kilian, A., Steffenson, B., Frisch, D., Tomkins, J., Wing, R., and Kleinhofs, A. 2000. Physical mapping of the barley stem rust resistance gene *rpg4*. Mol. Gen. Genet. **264**: 283-290.

Dubcovsky, J., Ramakrishna, W., San Miguel, P.J., Busso, C.S., Yan, L., Shiloff, B.A., Benetzen, J.L. 2001. Comparative sequence analysis of colinear barley and rice bacterial artificial chromosomes. Plant Physiol. **125**: 1342-1353.

Dunford, R.P., Yano, M., Kurata, N., Sasaki, T., Huestis, G., Rocheford, T., Laurie, D.A. 2002. Comparative mapping of the barley *Ppd-H11* photoperiod response gene region, which lies close to a junction between two rice linkage segments. Genetics, **161**: 825-834.

Feng, Q., Zhang, Y., Hao, P., Wang, S., et al. 2002. Sequence and analysis of rice chromosome 4. Nature, **420**: 316-320.

Fetch, T.G., Steffenson, B.J., and Jin, Y. 1998. Worldwide virulence of *Puccinia hord*ei on barley. Phytopathology, **88**: 28.

Feuillet, C., Schachermayr, G., and keller, B. 1997. Molecular cloning of a new receptor-like kinase gene encoded at the *Lr10* disease resistance locus of wheat. Plant J. **11**: 45-52.

Franckowiak, J.D., Jin, Y., and Steffenson, B.J. 1997. Recommended allele symbols for leaf rust resistance genes in barley. Barley Genet. Newsl. **27**: 36-44.

Goff, S.A., Ricke, D., Lan, T.H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions A et al. 2002. A draft sequence of the rice genome (*Oryza sativa L. ssp. japonica*). Science, **296**: 92-100.

Han, F., Kilian, A, Chen, J.P., Kundrna, D., Steffenson, B., Yamamoto, K., Matsumoto, T., Sasaki, T., and Kleinhofs, A. 1999. Sequence analysis of a rice BAC covering the syntenous barley *Rpg1* region. Genome, **42**: 1071-1076.

Jin, Y., Cui, G.H., Steffenson, B.J., and Franckowiak, J.D. 1996. New leaf rust resistance genes in barley and their allelic and linkage relationships with other *Rph* genes. Phytopathology, **86**: 887-890.

Lahaye, T., Hartmann, S., Töpsch, S., Freialdenhoven, A., Yano, M., Schulze-Lefert, P. 1998. High-resolution genetic and physical mapping of the *Rar1* locus in barley. Theor. Appl. Genet. **97**: 526-534.

Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Daly, J.M., Lincoln, S.E., and Newberg, L. 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics, **1**: 174-181.

Leister, D., Kurth, J., Laurie, D.A., Yano, M., Sasaki, T., Devos, K., Graner, A., and Schulce-Lefert, P. 1998. Rapid reorganization of resistance gene homologues in cereals genomes. Proc. Natl. Acad. Sci. U.S.A. **95**: 370-375.

Levine, M.N., and Cherewick, W.J. 1952. Studies on dwarf leaf rust of barley. U.S. Department of Agriculture Technical Bulletin. No. 1056, Washington D.C.

Liu, S., and Anderson, J.A. 2003. Targeted molecular mapping of a major wheat QTL for Fusarium head blight resistance using wheat ESTs and synteny with rice. Genome, **46**: 817-823.

Liu, Z.-W., Biyashev, R.M., and Saghai Maroof, M.A.. 1996. Development of simple sequence repeat DNA and their integration into a barley linkage map. Theor. Appl. Genet. **93**: 869-876.

Mammadov, J.A., Zwonitzer, J.C., Biyashev, R.M., Griffey, C.A., Jin, Y., Steffenson, B.J., and Saghai Maroof, M.A. 2003. Molecular mapping of leaf rust resistance gene *Rph5* in barley. Crop Sci. **43**: 388-393.

Moore, G. 1995. Cereal genome evolution: pastoral pursuits with 'Lego' genomes. Curr. Opin. Genet. Dev. **5**: 717–724.

Parlevliet, J.E. 1976. The genetics of seedling resistance to leaf rust, *Puccinia hordei* Otth in some spring barley cultivars. Euphytica, **25**: 249-254.

Perovic, D., Stein, N., Zhang, H., Drescher, A., Prasad, M., Kota, R., Kopahnke, D., Graner, A. 2004. An integrated approach for comparative mapping in rice and barley with special reference to the *Rph16* resistance locus. Funct. Integr. Genomics, **4**: 74-83.

Ramsay, L., Macaulay, M., degli Ivanissevich, S., MacLean, K., Cardle, L., Fuller, J., Edwards, K.J., Tuvesson, S., Morgante, M., Massarie, A., Maestri, E., Marmiroli, N., Sjakste, T., Ganalg, M., Powell, W., and Waugh, R. 2000. A simple sequence repeat-based linkage map of barley. Genetics, **156**: 1997-2005.

Saghai Maroof, M.A., Biyashev, R.B., Yang G.P., Zhang Q., Allard R.W. 1994**.** Extraordinarily polymorphic microsatellite DNA in barley: species diversity, chromosomal locations and population dynamics. Proc. Natl. Acad. Sci. U.S.A. **91**: 5466–5470.

Saghai Maroof, M.A., Yang, G.P., Biyashev, M.P., Maughan, P.J., and Zhang, Q. 1996. Analysis of the barley and rice genomes by comparative RFLP linkage mapping. Theor. Appl. Genet. **92**: 541–551.

Sasaki, T., Matsumoto, T., Yamamoto, K., et al. 2002. The genome sequence and structure of rice chromosome 1. Nature, **420**: 312-316.

Schmierer, D., Drader, T., Horsley, R., and Kleinhofs, A. 2003. Saturation mapping of a major Fusarium head blight QTL on barley chromosome 2H. Proceedings of 2003 National Fusarium head blight forum, pp 36-39.

Smilde, W.D., Haluskova, J., Sasaki, T., and Graner, A. 2001. New evidence for the synteny of rice chromosome 1 and barley chromosome 3H from rice expressed sequence tags. Genome, **44**: 361-367.

Song, R., Llaca V., and Messing, J. 2002. Mosaic organization of orthologous sequences in grass genomes. Genome Res. **12**: 1549-1555.

Tarchini, R., Biddle, P., Wineland, R., Tingey, S., and Rafalski, A. 2000. The complete sequence of the 340 kb of DNA around *Adh1-Adh2* region reveals interrupted colinearity with maize chromosome 4. Plant Cell, **12**: 381-391.

Tikhonov, A.P., SanMiguel, P.J., Nakajima, Y., Gorenstein, N.M., Bennetzen, J.L., and Avramova, Z. 1999. Colinearity and its exceptions in orthologous *adh* regions of maize and sorghum. Proc. Natl. Acad. Sci. U.S.A. **96**: 7409-7414.

Wei, F., Gobelman-Werner, K., Morroll, S.M., Kurth, J., Mao, L., Wing, R., Leister, D., Schultze-Lefert, P., and Wise, R.P. 1999. The *Mla* (powdery mildew) resistance cluster is associated with three NBS-LRR gene families and suppressed recombination within a 240-kb DNA interval on chromosomal 5S (1HS) of barley. Genetics, **153**: 1929-1948.

Yu, Y., Tomkins, J.P., Waugh, R., Frisch, D.A., Kudrna, D., Kleinhofs, A., Brueggeman, R.S., Muehlbauer, G.J., Wise, R.P., and Wing, R.A. 2000. A bacterial artificial chromosome library for barley (*Hordeum vulgare* L.) and the identification of clones containing putative resistance genes. Theor. Appl. Genet. **101**: 1093-1099.

CHAPTER 4

PHYSICAL MAPPING AND SEQUENCE ANALYSIS OF BARLEY RESISTANCE GENE ANALOGS

Abstract

Using degenerate primers, designed from the conserved motifs of the nucleotide-binding site (NBS) region in tobacco *N* and *Arabidopsis RPS*2 genes, 190 resistance gene analog (RGA) clones were previously isolated from barley (*Hordeum vulgare*)genomic DNA. A total of 15 single- and low- copy RGAs were genetically mapped onto chromosomes 1H to 7H (except 5H) using three barley double haploid (DH) mapping populations: Steptoe × Morex, Harrington × TR306, and LUGC × Bowman. Sequence analysis of RGAs indicated that they are members of a diverse group. As a result of BLAST search, eight RGAs proved unique since they did not detect any significant hit. Five RGAs are putatively functional, because they detected several expressed sequence tag (EST) matches with highly significant E-values. To physically map the RGAs, 26 sequences were used to screen a 6.3X cv. 'Morex' BAC library. After fingerprinting analysis, eight contigs were constructed, incorporating 62 BAC clones. These BAC contigs are of great importance for positional cloning of disease resistance genes, because they span the regions where various barley R genes including those for Fusarium head blight and kernel discoloration, net blotch, leaf rust, barley yellow dwarf virus, cereal cyst nematode, stripe rust and powdery mildew, have been genetically mapped.

Keywords: barley, RGA, genetic mapping, physical mapping, BAC contig, TIR-NBS-LRR

Introduction

Evolution of R genes

Classical and modern molecular genetics have increasingly demonstrated that resistance genes have a tendency to be clustered in the genome. However, some R loci are single genes with multiple alleles. For example, the *L* gene, conferring resistance to flax rust, and *Rpm*1 in Arabidopsis, have 13 and 2 alleles at one locus, respectively. In contrast, *Cf*4/9 and *Xa*21 genes in tomato and rice, respectively, are organized into clusters (reviewed by Michelmore and Meyers 1998). DNA recombination plays very important role in the evolution of scattered R genes as well as gene clusters. In general, there are several types of DNA recombination involved in the evolution of the above-mentioned genes such as interallelic recombination leading to gene conversion, intragenic unequal crossing-over, and intergenic unequal crossing-over and gene conversion.

DNA recombination and evolution of gene clusters

The best model describing the role of DNA recombination in the evolution of gene clusters is "Birth-and-Death Model" (Mitchelmore and Meyers 1998). Originally this model was designed to explain the organization and evolution of the vertebrate major histocompatibility complex (MHC). However, Mitchelmore and Meyers (1998) thought that the evolution of gene clusters in plants and vertebrates might be similar. Interestingly, this model proposes that hot spots of recombination are located within LRR region, mainly because of repeated nature of that motif. Birth-and-death model includes several steps, at which different types of DNA recombination are involved in the development of R gene clusters:

- Interallelic recombination and gene conversion alter the combinations and orientation of the arrays of solvent-exposed residues in LRR region. At this step there were no qualitative changes in the content of the solvent-exposed residues.
- Interallelic unequal crossing-over, resulting from mispairing within the LRR region, as well as mutations change the amino-acid components of the LRR region. This phenomenon creates new recognition specificity as a response to

actively evolving pathogens. This process will continue until the selection of a new variant, encoding increasingly effective resistance genes.

- Rare unequal crossing-over will cause duplications (birth) and deletions (death) of either single genes or blocks of genes. These processes will lead to the formation of paralogs, genes, which occurr as a result of duplication of the ancestral gene. This stage is a beginning in the formation of a gene cluster, where unequal crossing-over plays crucial role.
- Newly formed duplicated sequences share a high degree of similarity and are unstable, because they are prone to undergo additional rounds of intergenic unequal crossing-over that leads to further duplications and deletions. Also, intergenic unequal crossing-over can lead to occurrence of chimeras between the paralogs. Eventually, rapid divergence of intergenic regions decreases the frequency of unequal crossing-over. Variants and derivatives become fixed in the haplotype. Duplicated genes undergo diversifying selection. New gene cluster consists of genes with altered recognition specificities as well as pseudogenes or non-functional genes. In summary, DNA recombination plays crucial role in the formation of gene clusters. However, the rate of unequal crossing-over is not high at all stages of their development. According to Michelmore and Meyers (1998), a high rate of crossing-over and gene conversion tend to homogenize sequences. Meanhwile, there are some opposite opinions about that (Jelesko, personal communications). However, sequence analysis of genes within the *Dm* (Meyers et al. 1998) and *Cf* (Wulff et al. 2001) clusters shows that sequences within clusters are different. This indicates that unequal crossing-over between and within coding regions has occurred but has been infrequent (Michelmore and Meyers 1998). In contrast, Jelesko (personal communication) argues that unequal crossing-over and gene conversion is more active.

DNA recombination and evolution of scattered genes

Similar to the development of gene clusters, small multigene families, probably, also undergo interallelic recombination and gene conversion at the very beginning of their evolution. However, the rate of interallelic recombination is much higher in small

multigene families than between members of a gene cluster, because a higher rate of DNA recombination will sequence-wise homogenize the alleles (Michelmore and Meyers 1998). Indeed, sequence analysis of *L* alleles indicated that 8 out of 11 were 90% identical. Additionally, comparison of DNA sequences of *L* alleles revealed a mosaic nature of sequence similarities. Both facts can be explained by extensive and multiple intragenic sequence shuffling (Ellis et al. 1999). In contrast to members of gene clusters, alleles at the same locus rarely undergo unequal crossing-over. And the major sources of allelic diversity are point mutations and transposable elements rather than DNA recombination.

In conclusion, gene clusters most likely evolved based on "birth-and-death" model. Interallelic recombination is a principal mechanism in producing the variation in recognition specificities. Although intergenic unequal crossing-over and gene conversion are also important, they are infrequent and do not play a key role in the evolution of new specificities. In evolution of a single gene or small multigene families, interallelic recombination occurs at a very high rate and results in homogenizing of the alleles. However, mutations and transposable elements are the main mechanisms of development of allelic variation rather than interallelic recombination.

The structure of R genes

The majority of cloned plant disease resistance genes encode a putative nucleotide binding site (NBS) domain and a leucine-rich repeat (LRR) domain. In general, NBS is a common protein domain essential for the catalytic activity of various prokaryotic and eukaryotic proteins. Particularly, NBS is required for the ATP- or GTP-binding that is thought to modify interaction between R gene products and other members of the defense signal transduction (Bent 1996). The primary sequence of NBS is so distinct that protein sequences can be assigned to separate subgroups based on conserved motifs found within the domain (Traut 1994). The most common conserved motif is the phosphate-binding loop or "P-loop" (GxGGxGKTT), which was found in both ATP- and GTP-binding proteins (Saraste et al. 1990). Also, NBS domain contains additional conserved sites such as kinase-2 (xxLDDVW/D), kinase-3a (GxxxxxTTR) and GLPLAL, which presumably participate in activation of the resistance pathway (Traut 1994).

The LRR domain is a serial repeat of approximately 24 amino acids with leucine and other hydrophobic residues at regular intervals. The putative function of this domain is the mediation of protein × protein interactions (Ellis et al. 1997; Kobe and Kajava 2001) and is known to be involved in ligand interactions in porcine ribonuclease inhibitor (PRI) (Kobe and Deisenhofer 1994). In PRI, residues corresponding to hypervariable residues in R gene products are part of a β-strand/ β-turn structure of LRR with a consensus sequence of xxLxLxx. The conserved leucine residues (L) project into the hydrophobic core, whereas the other residues (x) form a solvent-exposed surface that is involved in ligand binding (Kobe and Deisenhofer 1995). In R genes, conserved posititions in the consensus sequence contain a variety of aliphatic residues. R genes are unlikely to have as regular a structure as PRI, because the amino acids in the backbone are more variable and there is less evidence that they form regular α-helices (Hammond-Kossack and Jones 1997). A structure for LRR regions with arrays of potential ligand-binding surfaces has several implications for R gene function. The most important implications are the extremely large number of binding specificities that could be encoded by groups of genes with such arrays and the ease in which new binding specificities could be generated by recombination and gene conversion. In addition to different combinations of LRRs providing different binding characteristics, variation in amino acids in the backbone between the hypervariable regions might change the relative orientations of the β -strands providing another level of variation for binding specificity. Comparative analyses of R genes from different species have revealed that solvent-exposed positions in LRRs are hypervariable and subject to positive selection. They can vary from 14 to > 40 amino acids (Jones and Jones 1997; Bergelson et al. 2001; Mondragon-Palomino et al. 2002), which reflects the role of LRR regions in the recognition of rapidly evolving pathogen ligands (Ellis et al. 2000; Dodds et al. 2001).

NBS-LRR proteins can be subdivided further into TIR and non-TIR proteins based on the presence or absence of amino-terminal Toll/Interleukin-1 receptor homology region (TIR) (Parker et al. 1997; Rock et al 1998). The second subgroup of NBS-LRR genes does not contain TIR domain and have been recently reported to possess a coiled-coil, or leucine zipper domain (Pan et al. 2000). Interestingly, TIR proteins are widely distributed in dicot species but have not been detected among cereals (Meyers et al. 1999;

Goff et al. 2002; Cannon et al. 2002). In contrast, non-TIR proteins are present throughout angiosperms (Pan et al. 2000; Jeong et al. 2001).

PCR-cloning of Resistance Gene Analogs

PCR-cloning of resistance gene analogs (RGA) has been an active area of research during the last decade. Using degenerate primers designed on the basis of conserved motifs of the nucleotide binding site (NBS) region of previously cloned disease resistance genes, researchers have PCR-amplified from a particular organism genes orthologous to one already isolated from another organism (Collins et al. 2001; Rostoks et al. 2002; Mohler et al. 2002; Madsen et al. 2003). With this technique, other investigators were successful in cloning the paralogous genes – member of the multigene family, for which at least one cloned member was available (Kanazin et al. 1996; Yu et al. 1996; Leister et al. 1998; Mago et al. 1999; Penuela et al. 2002). RGAs have been isolated from many plant species such as soybean (Yu et al. 1996; Penuela et al. 2002), potato (Leister et al. 1996), tomato (Ohmori et al. 1998), common bean (Rivkin et al. 1999), coffee (Noir et al. 2001), Arabidopsis (Aarts et al. 1998), sunflower (Gentzbittel et al. 1998), lettuce (Shen et al. 1998), grapevine (Donald et al. 2002), chickpea (Huettel et al. 2002), apple (Lee et al. 2003), chicory (Plocik et al. 2004) and grain crops including rice, wheat, barley and maize (Collins et al. 2001; Leister et al. 1998; Seah et al. 1998).
Genetic mapping of RGAs demonstrated their clustering in the vicinity of and even co-segregating with previously mapped R genes (Graham et al. 2000; Collins et al. 2001; Kalavacharia et al. 2002; Flandez-Galvez et al. 2003). This phenomenon makes RGAs the indispensable marker for discovery of R genes, as well as being useful for studies of the evolution of gene families and plant genomes (Shi et al. 2001; Pan et al. 2000; Jeong et al. 2001). However, information provided by genetic maps is not always sufficient to propose a putative function for an RGA. For example, Ilag et al. (2000) transformed rice cultivars susceptible to bacterial leaf blight with RGAs isolated using homology with the *A. thaliana RPS2* gene. However, transformed rice plants did not exhibit resistance to the pathogen indicating that these RGAs were not candidate disease resistance genes. Mainly, this fact can be explained by the complex structure of RGA clusters, which consist of active and/or inactive variants conditioning multiple resistance specificities for

a single pathogenic organism; also some RGAs may represent defeated disease resistance genes or genes that perform functions unrelated to disease resistance (Dangle and Jones 2001; Backes et al. 2003). Additionally, RGA clustering may complicate map-based cloning approaches since it increases the number of R gene candidates in the region of interest (Leister et al. 1999). Physical mapping of RGAs can provide an insight to understanding their rearrangement in the plant genome, determination of the actual span of the RGA clusters, and may be very useful as a prerequisite for map-based cloning of R genes. The objectives of this study were (1) to develop physical maps for barley RGA, (2) analyse their physical organization in barley genome, (3) carry out their sequence analysis and (4) demonstrate their association with previously mapped R genes.

Materials and methods

Barley RGAs

Barley RGAs used in this study were developed and genetically mapped by Liu et al. (2004). Liu et al. (2004) used two pairs of degenerate primers, designed from the conserved motifs of the NBS in tobacco *N* and *Arabidopsis RPS*2 genes. For the first pair of the primers, the forward primer (5'-GGAATGGGNGGNGTNGGNAARAC-3') was designed based on kinase 1a region, and the reverse primer (5'-YCTAGTTGTRAYDATDAYYYTRC –3') was designed near the kinase 3a region (Yu et al. 1996). This primer pair is hereafter referred to as BN primers and clones derived from the sequences amplified with this primer pair are referred to as BN class RGAs. For the second pair of primers, the forward primer (5'-GGIGGIGTIGGIAAIACIAC-3') was slightly different from the forward primer of BN primers, and the reverse primer of the second pair (5'-ARIGCIARIGGIARICC-3') was designed from the downstream region (GLPLAL). The second primer pair is hereafter referred to as GL primers and clones as GL class RGAs.

GL and BN primers were used to ampify genomic DNA of barley cultivars 'Steptoe', 'Morex', 'Harrington', 'TR306', and 'LUGC'. PCR-amplification of barley genomic DNA using BN- and GL-class primers resulted in major products of expected sizes of 340 bp and 540 bp, respectively. These PCR products were gel-purified and cloned. A total of 109 BN-class and 81 GL-class RGAs were analyzed by RFLP using a

set of diagnostic blots containing six barley parental DNA digested with six restriction enzymes (Liu et al. 2004). Clones were grouped based on the specific RFLP patterns they detected on the set of barley diagnostic lines. High copy number clones were discarded. Remaining single- or low-copy BN and GL RGAs were grouped into six and ten classes, respectively. According to RFLP patterns, majority of the RGAs, 70% of the BN and 53% of GL clones, belonged to one class, Class III and Class XII, respectively (Table 4.1). At least one representative clone was sequenced from each class (Table 4.1). DNA sequences from three BN and five GL clones contained open reading frames (ORF). These clones, representing eight different classes, are BN1-C7, BN1-C8, BN1-D5, GL2-B1, GL2-B4, GL2-B11, GL2-F5 and GL3-H11 (Table 4.1). Also, sequence analysis showed that three BN classes represented by BN1-A10, BN1-D10 and BN2-D3 and five GL classes represented by GL1-A2, GL2-D5, GL2-E4, and GL4-A2 and GL4-A4 were interrupted by stop codons (Table 4.1).

Three barley double haploid (DH) populations Steptoe × Morex (hereafter, referred to as SM) (150 DH lines) (Kleinhofs et al. 1993), Harrington × TR306 (hereafter, referred to as HT) (150 DH lines) (Kasha and Kleinhofs 1994), and LUGC × Bowman BC (hereafter, referred to as LB) (106 DH lines) (P. Hayes, personal communication) were used for genetic mapping of RGA sequences identified in this study. A total of 15 sequences were placed on chromosomes 1H to 7H (except 5H). One hundred sixty four RFLP markers were used to map seven RGAs, including BN1-A10, GL2-B11, GL3-H11, GL2-E4, GL2-F5, GL2-B4, GL2-B1, in the SM population. Employing 76 molecular markers, five RGAs, including BN1-D5, BN2-E8, BN1-C7 and GL1-A2 and BN2-D3, were mapped in the HT population. Finally, three RGAs, GL2-D5, GL4-E1 and GL2-B11, were mapped in the LB population, using 15 RFLP markers. Utilizing common markers in all three mapping populations, using RFLPs as anchor markers, consensus maps were created for all chromosomes except 5H.

BAC library screening

Barley RGAs were used to screen the 6.3X cv. Morex Bacterial Artificial Chromosome library produced at Clemson University Genomics Institute (Yu et al. 2000). This library contains 313,344 clones with average insert size of 106 Kb. High-

density filters were gridded robotically using a Genrtix Q-BOT in a 4×4 double-spotted array on 22.5-cm^2 filters. This gridding pattern allows 18,432 clones to be presented per filter. Library screening was performed using 17 filters (labeled A-Q), which cover the whole barley genome. The arrayed BAC colony filters were hybridized with ^{32}P-labeled GL and BN barley RGAs. In order to avoid cross-hybridization of the RGA clone vector to BAC clone vector, we used a pure insert as a probe. The insert was isolated from the RGA clone by digesting 10 μg of RGA clones with *Xba*I. Digest was analyzed by electrophoresis in 0.8% agarose gel, cut out from the gel and purified using Qigene Kit (Qiagen Inc., Valencia, CA). BAC colony filters were processed and hybridized with the ^{32}P-labeled RGAs using standard techniques (Sambrook et al. 1989). Filters were exposed to the Kodak film (New Haven, Conn) for 48-72 hours.

Fingerprinting of BAC clones and BAC contig development

BAC clones were isolated from the 6.3X cv. Morex BAC library (Yu et al. 2000) by hybridizing the arrayed BAC colony filters with ^{32}P-labeled GL and BN class of RGAs. BAC DNA was extracted using the standard alkaline lyses procedure (Sambrook et al. 1989). Fingerprinting of BAC clones was performed as described in Marra et al. (1997), with some modifications. Briefly, for each BAC DNA sample, individual restriction digests consisted of 35 μL of H_2O, 5 μL of 10X buffer II (Gibco BRL, Rockville, Md.), 5 μL of *Hin*dIII (10 U/μL), and 5 μL of miniprep BAC DNA. Digestion was achieved by incubation at 37°C for 3 h. Digested DNA was separated in a 0.8% agarose gel. Samples were electrophoresed at 50 V overnight (18-20 h) at room temperature in 1X TAE buffer. After electrophoresis, gels were stained with ethidium bromide and photographed using Kodak 1D Image Analysis Software (Rocheser, NY). BAC contigs were constructed manually based on the overlapping bands shared by the individual BACs as detected by single RGA sequences. After imaging, the gel was Southern-blotted onto membranes (Hybond N+; Amersham, Pscataway, NJ) and hybridized with the corresponding ^{32}P-labeled GL or BN class of RGAs to characterize the positives and determine the location and copy number of the RGA clones. To determine the length of the BAC contigs, 3-6 μl DNA of the individual BAC clones, comprising a contig, was digested with 10 U of *Not*I for 4 h. Digests were analyzed by

pulsed-field electrophoresis in 1.0% agarose CHEF gel at 6.0 V/cm, with a 90 second pulse, for 20 hours, at 14°C in 0.5x TBE buffer.

Bioinformatic methods

Database search

The GenBank non-redundant database (NR) of nucleotide sequences as well as expressed sequence tag (EST) database (dbEST) at the National Center for Biotechnology Information (NCBI) were screened with RGAs using blastn program (Altschul et al. 1997). Deduced protein sequences of experimental RGAs were compared to protein sequences in the GenBank NR database using blastn program (Altschul et al. 1997).

Analysis of conserved motif structures

Deduced amino acid sequences of the RGAs were subjected to domain and motif analyses. The NBS domain was defined as the region extending from the P-loop to the GLPLAL motif. PANAL (http://www.mgd.ahc.umn.edu/panal) and InterProScan (http://www.ebi.ac.uk/InterProScan/) were used to analyze the protein sequences of the experimental RGAs.

Multiple sequence alignment

Multiple sequence alignment of nucleotide and amino acid sequences were implemented using public ClustalX software (Thompson et al. 1997). Then, complete sequence alignment was opened at BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and manually edited. Since P-loop and Kinase-3 regions of the sequences were primer-derived, they were excluded from alignment. BioEdit software package was used to generate neighbor-joining phylogenetic trees. First unrooted phylogenetic tree compared nucleotide sequences of RGAs as well as their matches from NR database and dbEST. The second phylogenetic tree compared the amino acid sequences of RGAs and known plant disease resistance genes, including *RPP5, N, M, L6, Pi-b, Xa1, Cre3, I2c-1, Mi, Prf, RPS5*. Human *Apaf-1* gene was used as an outgroup to root the second tree.

Results

Physical mapping of RGAs

Twenty six barley RGAs were used as probes to screen a 6.3X genome equivalent Morex barley BAC library (Table 4.1). Nine barley RGAs including 3 BN and 6 GL clones, detected a low number of positive BACs, which had strong hybridization signals, while 11 BN clones including BN1-D10, BN1-C3, BN1-C8, BN1-C6, BN1-A2, BN1-C7, BN1-H3, BN1-D4, BN2-F2, BN1-D7 and BN1-C11 did not reveal any positives. This is strong evidence that the above-mentioned 20 RGAs are not widespread in barley genome. Six other RGAs including BN2-D3, GL2-E4, GL4-A4, GL2-D5, GL4-A2 and GL4-E1 hybridized to a large number of BAC clones. BAC contigs were developed only for nine low-copy RGAs. DNA of forty-five BAC positives for GL clones and 17 BAC positives for BN clones were subject to fingerprint analysis (Table 4.2).

After fingerprint analysis, 7 contigs were developed (Fig. 4.1-4.7) incorporating 62 BAC clones (Table 4.2). Two BAC contigs were constructed for three RGAs, genetically mapped to the 2H chromosome. BN1-D5 and BN2-E8 detected the same 7 BAC clones (Table 4.2, Fig. 4.1) that confirmed their close genetic location on the 2H chromosome (1.1 cM on the consensus map) (Fig. 4.8B). A contig of 250 Kb length was constructed for these RGAs. BN1-D5 and BN2-E8 each have 2 RGA copies (Table 4.2). Another RGA, GL2-F5, was genetically mapped on the long arm of the 2H chromosome (Fig. 4.8B). This RGA detected 15 positive BACs. A BAC contig of 280 Kb length was identified to harbor three copies of GL2-F5 (Fig. 4.2 and Table 4.2).

Some of the contigs are of great interest in terms of prospects for the positional cloning of R genes. In our consensus map, two RGAs BN1-A10 and GL2-B11 were located about 30 cM away from each other in the centromeric region of the chromosome 1H (Fig 4.8B.). Despite the large genetic distance between the two RGAs, they detected 4 common BAC clones along with unique ones. Overall physical span of the region between the two RGAs was about 213 Kb (Table 4.2 and Fig. 4.3). Taking into account that these RGAs flank *Mla12* gene, the above-mentioned BACs could be very useful in map-based cloning of this powdery mildew resistance gene. Another powdery mildew resistance gene *Mlg*, co-segregated with GL1-A2 RGA on the chromosome 4H based on 134 DH lines of the Harrington × TR306 and could be a potential target for cloning (Fig.

4.8B). A BAC contig of 190 Kb (Table 4.2 and Fig. 4.4), developed for GL1-A2, may harbor this gene. Two RGAs, GL2-B1 and GL2-B4, were mapped on the chromosome 3H (Fig 4.8B). Based on the anchor markers, GL2-B1 was mapped on the centromeric region and GL2-B4 - on the long arm of 3H chromosome. A contig of 205 Kb length for GL2-B1 was comprised of 7 BAC clones (Table 4.2, Fig. 4.5). Three BAC clones contained 2 copies and 4 BAC clones – four copies of GL2-B1 RGA. GL2-B4 was a single copy RGA, and detected 4 BAC clones, which were assembled into a contig of 250 Kb length (Table 4.2 and Fig. 4.6). Finally, another single copy RGA, GL3-H11, mapped onto the 6HL chromosome and detected 4 BAC clones. The overall length of the BAC contig for this RGA was 180 Kb (Table 4.2 and Fig. 4.7).

In summary, the number of RGAs per contig ranged from 1 to 3 with an average of 1.8 RGAs. BAC contig size ranged from 180 (GL3-H11) to 280 Kb (GL2-F5) with an average of 202 Kb. A minimum of one RGA per 190 Kb and a maximum of one RGA per 53.3 kb were observed among the contigs (Table 4.2).

Association of RGAs with disease resistance genes

RGAs are potentially derived from disease resistance genes, and, therefore, are expected to be located in the vicinity of other disease resistance genes. Comparison of locations of RGAs with positions of previously mapped R genes was done based on the position of common markers linked to RGAs in our maps as well as R genes in other maps. Three RGAs, GL2-E4, GL2-D5 and GL4-E1 were mapped onto chromosome 7H (Fig. 4.8B). GL2-E4 was mapped onto the centromeric region in the SM population. This region harbors several QTLs conferring resistance to Fusarium head blight (FHB) and kernel discoloration (KD) (De la Pena et al. 1999) as well as one single gene, *Rpt4* conferring resistance to the spot form of net blotch (NB) (Williams et al. 1999). Two other RGAs, GL2-D5 and GL4-E1, were clustered in the long arm of chromosome 7H. This region contains *Rph19* (a leaf rust resistance gene) (Park and Karakousis 2002), QTL for barley yellow dwarf virus (BYDV) resistance (Toojinda et al. 2000) and *Rphq9 (*QTL for partial resistance to leaf rust) (Qi et al. 1998, 2000) (Fig. 4.8C).

Four RGAs, BN1-D5, BN2-E8, BN1-C7 and GL2-F5 were positioned onto the long arm of the chromosome 2H covering a genetic distance of about 30 cM (Fig. 4.8B).

R genes located in this chromosomal region include *Ha2* – cereal cyst nematode resistance gene (Kretschmer et al., 1997) as well as QTLs for resistance to FHB (De la Pena et al., 1999) and BYDV (Scheurer et al., 2001) (Fig. 4.8C). GL2-B1 and GL2-B4 were assigned to the short and long arms of chromosome 3H, respectively in SM population (Fig. 4.8B). Position of GL2-B1 clone is of great interest, since several QTLs such as those for FHB (De la Pena et al., 1999), BYDV (Scheurer et al., 2001) and Net Blotch (NB) (Richter et al., 1998) as well as the single BYDV resistance gene *Yd2* (Collins et al. 1996; Paltridge et al. 1998) were previously mapped onto that region. GL2-B4 mapped to the vicinity of the QTLs for resistance to stripe rust (SR) (Toojinda et al., 2000) and Kernel discoloration (KD) (De la Pena et al., 1999) (Fig. 4.8B). Remarkably, the GL1-A2 clone co-segregated with a powdery mildew resistance gene *Mlg* on the chromosome 4H based on HT population (Fig. 4.8B). The close association between GL1-A2 and *Mlg* was confirmed by NIL analysis (Liu et al 2004). GL1-A2 clone was used to probe a blot containing the *Mlg* donor parent 'Goldfoil', the susceptible parent Manchuria and the corresponding *Mlg* NIL. GL1-A2 detected DNA fragment of the same size in NIL and Goldfoil, while a different size fragment was observed in Manchuria. GL4-A2 and BN1-A10 were mapped near a powdery mildew resistance gene *Mla12* on chromosome 1H (Fig. 4.8B). BN2-D3 was identified near the region of *Mlh* locus, the other powdery mildew resistance gene on chromosome 6H (Fig. 4.8B).

Sequence analysis of the barley RGAs

Nucleotide sequence comparison, implemented by the ClustalX multiple sequence analysis program, indicated that the identified sequences were members of diverse groups of RGAs. The most divergent RGAs (GL4-E4 and BN1-D10) shared only 20% nucleotide identity and in fact is less if one excludes the highly conserved P-loop and Kinase-3 sequences, which served as templates for designing the original primer sets. The most related RGAs were 90% identical. Diverse groups of RGAs were identified in this study. In order to determine whether these RGAs were unique, they were used as queries to screen the GenBank database of non-redundant nucleotide sequences by means of blastn program. Table 4.3 summarizes the results of the BLAST search. Only one RGA, GL4-A4, was found to be unique, as it did not detect any significant hits. In addition,

matches to the NBS region of seven other RGAs including GL2-D5, GL4-A2, GL4-E1, GL2-E4, BN1-D4, BN2-F2 and BN1-A10 had poor E-values varying from $2e^{-04}$ to $1e^{-11}$ (Table 4.3). Twelve RGAs, including 11 BNs and one GL, detected matches with E-values of $1e^{-87}$ and higher; while 4 GL sequences were 100% identical to previously reported barley RGAs: GL3-H11 vs. AF032680; GL2-F5 vs. AJ495837; GL2-B11 vs. AF032682 and GL2-B4 vs AJ507098 (Table 4.3).

All RGAs were also used as queries to screen dbEST, in order to identify whether or not they are expressed. BN class RGAs detected several EST matches with high E-values. For example, BN1-C6 detected a rice EST with an accession number CB635373 (1e-57); BN1-C3 and BN1-H3 were similar to soybean EST BE805840 with E-values of $1e^{-87}$ and $2e^{-87}$, respectively. Some of the GL class RGAs also exhibited high similarity to the expressed sequences from dbEST. GL2-B4 was similar to barley EST BQ465185 (E-value = $7e^{-85}$) and GL2-B11 - to the noble cane EST CA214958 (E-value = $2e^{-36}$). Three GL (GL2-B1, G4-A2 and GL4-A4) and five BN sequences (BN1-D4, BN1-A10, BN2-F2, BN1-D10 and BN1-D5) did not match any ESTs. The rest of the RGA sequences had relatively low similarity to EST sequences with E-values varying from $1e^{-05}$ to $8e^{-13}$ (Table 4.3).

Twenty five barley RGAs from this study, except BN2-E8 (no sequence information is available), as well as the matching RGAs and ESTs from the public database having E-values lower than e^{-80} (except AF427791, which represents the entire BAC sequence) were used to construct a phylogenetic unrooted tree. All RGAs and ESTs were divided into five well distinguishable groups (Fig. 4.9). Although our RGAs were developed from barley, not all matches from NR and dbEST were barley sequences. The matches included not only monocots such as wheat, rice, oat, Aegilops, Elymus and cane, but also dicots, involving mostly soybean, *Medicago trancatula* and kidney beans. From this point of view, group 1 was of great importance, because it contained both monocot and dicot sequences. For example, BN1-C3 and BN1-H3 were highly similar to soybean RGAs AY182243 and AF541963 (e-value = e^{-113}) as well as RGA from wheat/Elymus alien addition line AY242389 (e-value = e^{-93}). Also, this group included other RGAs from wheat and Elymus. In light of the existing debate about whether or not TIR-NBS-LRR type of disease resistance genes exists among monocots, these findings have

important implications. We aligned deduced amino-acid sequences of the RGAs from this study representing only open reading frames (ORF) and retrieved Elymus and wheat RGAs from the group 1 (Fig. 4.9) with previously cloned disease resistance genes, including well-known TIR-NBS-LRR and non-TIR-NBS-LRR genes and constructed a neighbor-joining rooted tree, where human *Apaf1* gene was used as an outgroup to anchor the tree (Fig. 4.10A). RGAs such as GL2-F5, GL3-H11, GL2-B11 and GL2-B1 were grouped together with non-TIR genes *Cre3, Xa1, I2C-1, RPS5, Prf and Mi* genes, which confer resistance to wheat nematode (Lagudah et al. 1997), bacterial blight in rice (Yoshimura et al. 1998), soil-borne fungus *Fusarium oxysporum f sp lycopersici* in tomato (Ori et al. 1997), bacterial pathogen *Pseudomonas syringae* (Simonich et al. 1995; Salmeron et al. 1996) and root-knot nematode (Rossi et al. 1998), respectively. BN1-D5 and GL2-B4 were grouped with *RPM1* and *Pi-b* genes, respectively, which condition resistance to bacterial pathogen *Pseudomonas syringae* (Boyes et al. 1998) and rice blast disease. *RPM1* and *Pi-b* genes also belong to non-TIR-NBS-LRR. However, a majority of BN class RGAs were clustered with known TIR-NBS-LRR genes such as tobacco *N* gene, conferring resistance to tobacco mosaic virus (Whitham et al. 1993), and the rust resistance genes *L6* and *M* of flax (Lawrence et al. 1995; Anderson et al. 1997) (Fig. 4.10A). Based on the literature, TIR-NBS-LRR genes have been widely distributed among dicots (Pan et al., 2000, Penuela et al., 2002) yet have not been detected in cereal database (Madsen et al., 2003). Remarkably, our studies revealed a group of barley RGAs, which potentially could belong to the above-mentioned class of disease resistance genes. According to Meyers et al. (1999), Jeong et al. (2001) and Penuela et al. (2002), the final amino acid in the kinase-2 domain of the NBS region with 95% accuracy distinguishes the two classes of R genes: tryptophan (W) residue in non-TIR and an aspartic acid (D) or its uncharged derivative aspartate (N) in TIR class of R genes (Fig. 4.10B). Based on this fact, we concluded that some of our RGAs might represent TIR-NBS-LRR class of R genes. Through January 2002, BLAST search with our putative TIR-NBS-LRR-like barley RGAs did not detect any sequences among monocots, all retrieved matches were soybean or other dicot sequences. However, recently several sequences from Elymus, wheat as well as some wheat × Elymus alien addition lines were detected by BN1-C3, BN1-H3, BN1-A2 and BN1-C7 barley RGAs during BLAST

search. These sequences were included in the phylogenetic tree and, remarkably, they were also grouped with TIR-NBS-LRR class of R genes (Fig. 4.10A). Multiple sequence alignment demonstrated that not all wheat and elymus sequences contained conserved aspartic acid (D) within kinase-2 domain. In fact, three sequences AAP20702, AAP03075 (wheat/Elymus alien addition lines) and AAP03073 (Elymus) did not contain kinase-2 domain at all, while the rest of the sequences AAP03077 (wheat), AAP03076 and AAP13546 (wheat/Elymus alien addition lines) and AAP03074 (Elymus) had D residue within kinase-2 domain. Consequently, the presence of W or D residue within kinase-2 is not always an indicator of assignment of a gene to certain classes of R genes. Unfortunately, the length of the experimental sequences did not allow us to determine whether or not they contain TIR domains. We blastned our putative TIR-NBS-LRR RGAs against the high-throughput genomic sequences database (dbHTGS) hoping to detect full-length gene matches. As a result BN1-C3 retrieved a whole soybean BAC sequence with a GenBank accession number AF541963 (e^{-113}). Previously published sequence analysis of this BAC clone (91F11 cv. 'Williams 82') identified 16 different disease resistance-like genes with homology to the TIR-NBS-LRR class (Graham et al. 2002). Deduced amino acid sequences of BN1-C3 as well as closely related experimental RGAs BN1-H3, BN1-A2 and BN1-C7 were also used to search NR database using blastp program. As we expected, these RGAs retrieved several genes from the soybean BAC 91F11. Based on this evidence, we can support a hypothesis that TIR-NBS-LRR genes may exist in monocots.

Discussion

The discovery of conserved sequence motifs among plant disease resistance genes has resulted in their use to design candidate gene approaches for the identification of resistance genes and for the analysis of their distribution in plant genomes. In our laboratory, 16 different classes of RGAs were previously isolated from barley using two pairs of degenerate primers (Liu et al. 2004). Twenty six barley RGAs, including at least one from each of the sixteen classes, were used to screen a 6.3X genome equivalent cv. Morex barley BAC library. Only nine barley RGAs detected low number of positive BACs, while the rest of the RGAs either detected none or too many BAC clones (one

RGA detected more than 300 positives). BAC contigs were developed only for low-copy RGAs. As a result, we were able to construct seven BAC contigs on six chromosomes except 5H. These contigs incorporated 62 BAC clones. Two RGAs, BN2-E8 and BN1-D5, are located on the same BAC contig. Characterization of BAC clones showed that four RGAs were subject to duplication events and had 2-3 copies per contig. The average copy number of an RGA per contig was about 1.8. R genes are frequently reported to occur in clusters (Michelmore and Meyers 1998). In the Arabidopsis genome, 33% of the R genes are organized in pairs and 36% in clusters of three to nine members (The Arabidopsis Genome Initiative, 2000). In barley, these numbers are more or less consistent with those of Arabidopsis genome. For instance, 10% of RGAs, mapped by Madsen et al. (2003), were organized in pairs, 30% in clusters and 60% were represented as single genes. Leister et al. (1999) reported that 30% of barley RGAs was organized in clusters and the rest 70% as single genes. In our study, out of 15 (53% eight RGAs) were grouped in pairs and the remaining seven (47%) mapped as single genes.

The results of genome-wide mapping of RGAs identified a number of new barley RGA loci: on chromosome 1H (BN1-A10 and GL4-A2); on chromosome 2H (BN1-C7); on chromosome 4H (GL1-A2); on chromosome 6H (BN2-D3) and on chromosome 7H (GL2-D5, GL4-E1 and GL2-E4) (Liu et al. 2004). BLAST search did not indicate any significant sequence matches for these RGAs and therefore they can be considered as unique RGA loci. However, some RGAs such as GL2-B11, GL2-B1 and BN1-D5 exhibited high similarity with previously reported sequences. These RGAs were very similar to b4 (Leister et al. 1998), DR2 (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=30171228) and rga S-120 (Madsen et al. 2003), respectively. Moreover, several RGAs were even identical to the previously published sequences. GL3-H11 was the same as the RGAs from different species, including b2 (its b2.3 allele) (Leister et al., 1998) and S-129 (Madsen et al., 2003) in barley, RGA1 in wheat (Goodwin and Hu, 1998) and rae3 gene in *Aegilops ventricosa* (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=10638388). RGAs reported in this study represent partial sequences stretching from P-loop/Kinase-1a to GLPLAL motifs. The absence of full-length sequences makes it difficult to predict the

structure of the genes and their correspondence to certain classes of R genes. However, several RGAs were identical to previously published full-length barley RGAs identified by Madsen et al. (2003). This fact indicated something about their nature. For instance, GL2-B4 and GL2-F5 were identical to rga-S-L8 and rga-S-217, which belong to CC-NBS-LRR class of R genes. Another interesting finding was that several barley RGAs, including BN1-D7, BN1-C8, BN1-C11, BN1-C3, BN1-H3, BN1-A2 and BN1-C7, exhibited high similarity with TIR-NBS-LRR genes. The existing hypothesis is that TIR-NBS-LRR genes are absent from monocot genomes. Using our TIR-like barley RGAs as queries, a GenBank was searched and several sequences were retrieved from monocots Elymus, wheat as well as some wheat × Elymus alien addition lines. Those TIR-like barley RGAs and the retrieved monocot RGAs were grouped together with well-known TIR-NBS-LRR genes in a phylogenetic tree. This finding is a good suggests that TIR-NBS-LRR type sequences may be underrepresented in but are not completely absent from monocot genomes. Recent observations by Bai et al (2002) and Zhou et al. (2004) support the afore-mentioned findings. Bai et al. (2002) identified 2 genes with TIR domain in rice. However, these two genes did not encode any obvious LRR domain and according to the authors were otherwise divergent from the NBS-LRR type R genes. Three TIR genes in rice were discovered by Zhou et al. (2004). They point out that the rice genome harbors 535 NBS-coding sequences, meaning that only 1% of the genes can potentially be TIR type.

Several authors have reported close associations between RGAs and disease resistance loci and QTLs (Wang et al. 2001; Graham et al. 2000). In some cases genetic linkage of RGAs with previously mapped R genes manifest their physical proximity (~100 Kb) (Leister et al. 1999; Wei et al. 2002). This fact makes them extremely attractive for map-based cloning of disease resistance genes. In addition, such RGAs can be useful markers in marker-assisted selection of resistant genotypes. In this study, many experimental RGAs were mapped in the vicinity of known R genes. One of the most interesting associations, between GL1-A2 and the powdery mildew resistance gene *Mlg*, was tested by co-segregation analysis. In the Harrington × TR306 mapping population (134 DH lines), this RGA co-segregated with *Mlg* gene. The close linkage between GL1-A2 and *Mlg* was also confirmed by RFLP analysis of NILs. BAC contig of GL1-A2

developed in this study can be crucial in cloning of *Mlg* gene. Other correlations between our RGAs and known disease resistance genes were established by comparison of anchor (common) markers among different mapping populations.

Although the RGA approach to the discovery of R genes is very important and was successful in some studies as mentioned above, a majority of RGAs are pseudogenes and do not have any functional specificity (Michelmore and Meyers 1998). From this point of view, EST-RGA resources can be extremely useful in the identification of expressed RGA sequences thus eliminating many pseudogenes. In this study, experimental RGAs were BLASTed against dbEST. As a result, 20% of RGAs (5 out of 25) showed similarity to ESTs with E values varying from $2e^{-36}$ to $1e^{-87}$.

Table 4.1. Classification and chromosomal location of PCR derived barley RGA clones.

RGA*	Class	Copy No†	Sequence‡	Chromosome
BN clones				
BN1-C7	I	2	ORF	2H
BN1-D5		2	ORF	2H
BN2-E8	II		ORF	2H
BN1-C6			ORF	Mono§
BN1-C8		2	ORF	Mono
BN1-D7			ORF	Mono
BN1-A2			ORF	Mono
BN1-C11			ORF	Mono
BN1-D4	III		ORF	Mono
BN1-C3			ORF	Mono
BN1-H3			ORF	Mono
BN2-F2			ORF	Mono
BN1-A10	IV	3	Stop	1H
BN2-D3	V	4	Stop	6H
BN1-D10	VI	3	Stop	Mono
GL clones				
GL2-B1	VII	2	ORF	3H
GL2-B4	VIII	2	ORF	3H
GL2-B11	IX	2	ORF	1H
GL2-F5	X	1	ORF	2H
GL3-H11	XI	2	ORF	6H
GL1-A2	XII	1	Stop	4H
GL2-D5	XIII	2	Stop	7H
GL4-E1			Stop	7H
GL2-E4	XIV	5	Stop	7H
GL4-A2	XV	6	Stop	1H
GL4-A4	XVI	5	Stop	Mono

* BN and GL RGAs represent PCR products of size 340 or 530 bp, respectively.
† Approximate copy number as determined by the number of RFLPs for a given RGA.
‡ Sequences with open reading frame (ORF) or containing stop codon.
§ Monomorphic among parental lines of three mapping populations

Table 4.2. Main characteristics of the barley RGA BAC contigs.

RGA	BAC clones detected by RGAs (size in kb)	RGA copy number per contig	Contig length, kb	Number of RGAs per kb
BN1-D5 BN2-E8	32M12* (80), 49B23 (225), 127O10 (115), 359E3 (140), 548P20 (120), 639F13 (150), 683C1 (110)	2	250†	125
GL2-B1	180H8 (115), 197C17 (79), 359K17 (110), 439P23 (109), 492H15 (90), 597H24 (110), 581H15 (70)	2	205	102.5
GL2-B4	110M5 (90), 592K3 (212), 663N15 (120), 790A16 (110)	1	250	125
GL3-H11	318D17 (110), 376P1 (110), 513L17 (50), 799M2 (135)	1	180	90
GL1-A2	187E10 (70), 226G24 (180), 322D2 (80), 431M9 (130), 408L3 (125), 588P16 (110), 652D15 (105), 223C5 (125), 245D12(130), 431O9 (120), 608N16 (100), 717H21 (110), 684B15 (125)	1	190	190
BN1-A10	338H21 (100), 338G21 (90), 690M20 (100), 722E13 (100), 737H17 (100), **364A17 (97), 367J7 (97), 447O14 (102), 614O13 (90), 472L21 (120)**	1	213‡	53.3
GL2-B11	17OI7 (100), 553L7 (109)	3		
GL2-F5	72F12 (130), 264K4 (130), 251A24 (109), 365L24 (80), 358A8 (125), 413J19 (115), 460D13 (117), 460N22 (105), 454L18 (110), 513M20 (130), 595C19 (109), 650E1 (110), 661F8 (105), 707L5 (110), 731O2 (110)	3	280	93

* BAC IDs refer to 6.3X cv. Morex Bacterial Artificial Chromosome library produced at Clemson University Genomics Institute (Yu et al. 2000). clone.

† BN1-D5 and BN2-E8 detected the same BAC clones and were physically located at the same position within the BAC

‡ BN1-A10 and GL2-B11 detected 5 common BAC clones (they are shown in bold) and we constructed one joint contig for both RGAs.

Table 4.3. Expressed Sequence Tags (ESTs) and resistance gene analogs (RGAs) detected from the public database by BLASTing with barley RGAs from this study.

RGA	Non-redundant database			EST database		
	Accession #	E-value*	Organism	Accession #	E-value	Organism
BN1-A10	AF456243	1e-11	Rice			
BN1-C6	AK067669	e-161	Rice	CB635373	1e-57	Rice
BN1-D4	AF363799	3e-09	Kidney bean			
BN2-F2	AF363799	4e-08	Kidney bean			
BN1-H3	AY182243	e-113	Soybean	BE805840	2e-87	Soybean
	AY242389	2e-93	Wheat/Elymus AAL†			
	AY249524	5e-91	Elymus			
	AY249526	7e-84	Wheat/Elymus AAL			
BN1-C3	AF541963	e-113	Soybean	BE805840	1e-87	Soybean
	AY182243	e-104	Soybean			
	AY242389	2e-75	Wheat/Elymus AAL			
	AY249526	4e-73	Wheat/Elymus AAL			
	AY249528	4e-72	Elymus			
BN1-A2	AF222876	e-124	Soybean	CF922652	8e-13	Soybean
	AY249525	8e-13	Elymus			
	AY249527	8e-13	Wheat/elymus AAL			
	AY238935	8e-13	Wheat/elymus AAL			
	AY249528	8e-13	Wheat			
BN1-D7	AF222875	e-165	Soybean	BU926353	4e-08	Soybean
BN1-C8	AF222875	e-159	Soybean	BU926353	7e-04	Soybean
BN1-C11	AF222875	e-161	Soybean	BU926353	2e-07	Soybean
BN1-D10	AB022170	e-143	Rice			
BN1-D5	AJ507100	e-154	Barley			
	AJ506122	e-153	Barley			
	AL731613	2e-31	Rice			
BN1-C7	AF222876	2-129	Soybean	CF922652	8e-13	Soybean
	AF541963	3e-40	Soybean	BQ741424	8e-12	Soybean
	AY249525	8e-13	Elymus			
	AY249527	8e-13	Wheat/Elymus AAL			
	AY238935	8e-13	Wheat/Elymus AAL			
	AY249528	8e-13	Wheat			
BN2-D3	AY182245	e-162	Soybean	BI265907	4e-07	Barrel Medic‡
GL2-B1	AY242388	e-151	Elymus			
	AF146274	e-146	Barley			
GL2-B4	AJ507098	0	Barley	BQ465185	7e-85	Barley
	AJ506140	0	Barley			

Table 4.3. Continued

GL3-H11	AF032680	0	Barley	AU030779	3e-13	Rice
	AJ495836	0	Barley			
	AF087518	0	Wheat			
	AJ249945	0	*Aegilops ventricosa*			
	AJ249944	0	Aegilops ventricosa			
GL2-F5	AJ495837	0	Barley	AU030779	3e-13	Rice
	AJ507090	0	Barley			
	AF032683	0	Barley			
	AF087520	0	Wheat			
GL2-B11	AF032682	0	Barley	CA214958	2e-36	Sugarcane
	AF087519	0	Wheat			
	AST296001	0	Black oat			
GL1-A2				BQ487414	5e-06	Wheat
GL4-A2	AY146587	1e-09	Durum Wheat			
GL4-E1	AF427791	2e-04	Barley	CB865449	1e-05	Barley
GL4-A4						
GL2-E4				CA697262	8e-08	Wheat
GL2-D5	AF427791	2e-04	Barley	AJ463531	9e-06	Barley
				CB865449	1e-05	Barley

*E-value (Expected value) provide information about the likelihood that a given sequence alignment is significant. An alignment's E-value indicates the number of alignments one expects to find with a score greater than or equal to the observed alignment's score in a search against a random database. Thus, a large E-value (5 or 10) indicates that the alignment probably has occurred by chance, and that the target sequence has been aligned to an unrelated sequence in the database. E-values of 0.1 amd 0.05 are typically used as cutoffs in sequence databse searches (Gibas and Jambeck 2001). The BLAST programs report E-value rather than P-values because it is easier to understand the difference between, for example, E-value of 5 and 10 than P-values of 0.993 and 0.99995. However, when $E < 0.01$, P-values and E-value are nearly identical (http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html#head2).

†Wheat/Elymus AAL –Wheat/Elymus Alien Addition Lines

‡Barrel medic –*Medicago trancatula*

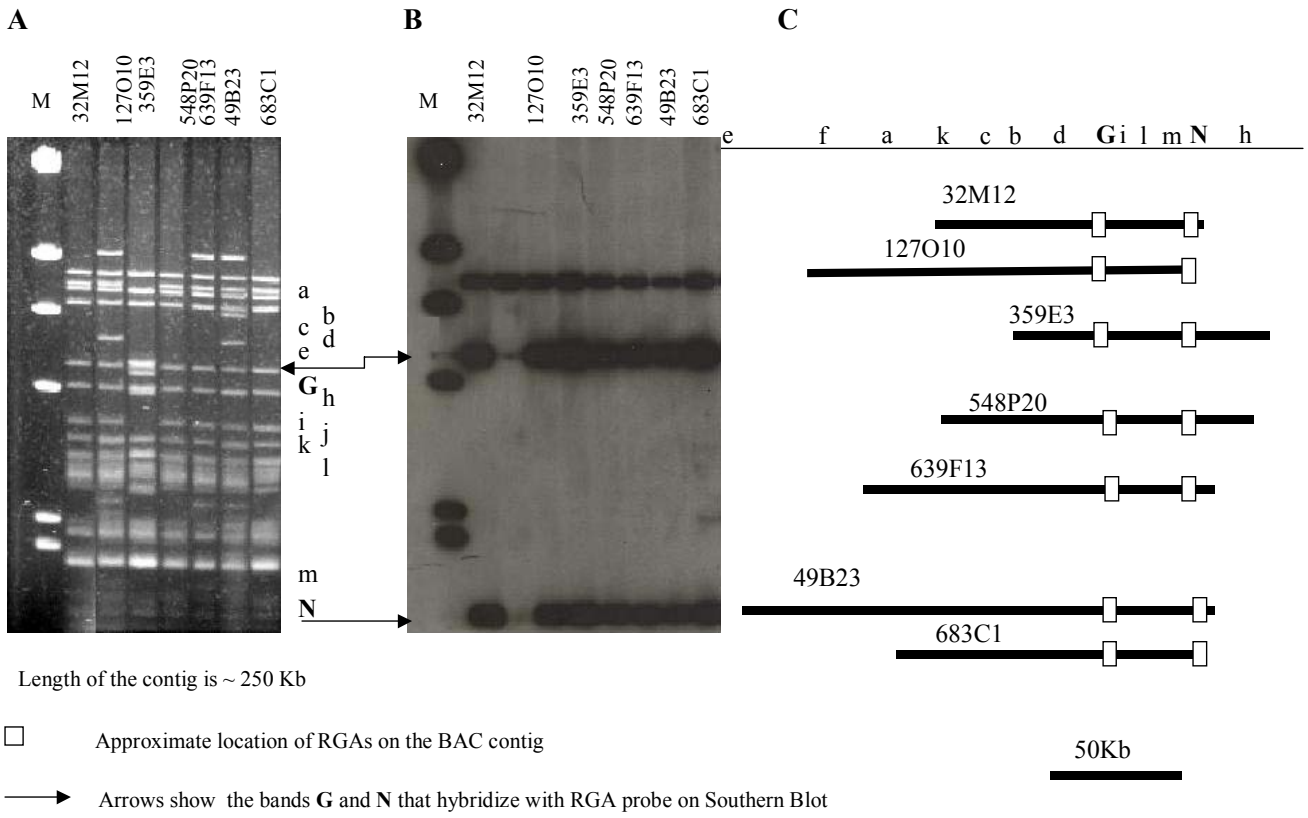


Figure 4.1. BAC contig for BN1-D5 and BN2-E8 barley RGAs. These two RGAs detected the same BAC clones.

A. Agarose gel image of the *Hin*dIII fingerprinted BAC clones. **B.** Autoradiography of southern blot containing *Hin*dIII fingerprinted BAC clones. **C.** BAC contig. BAC IDs corresponds to addresses of BAC clones from Morex library (Clemson University Genomics Institute).

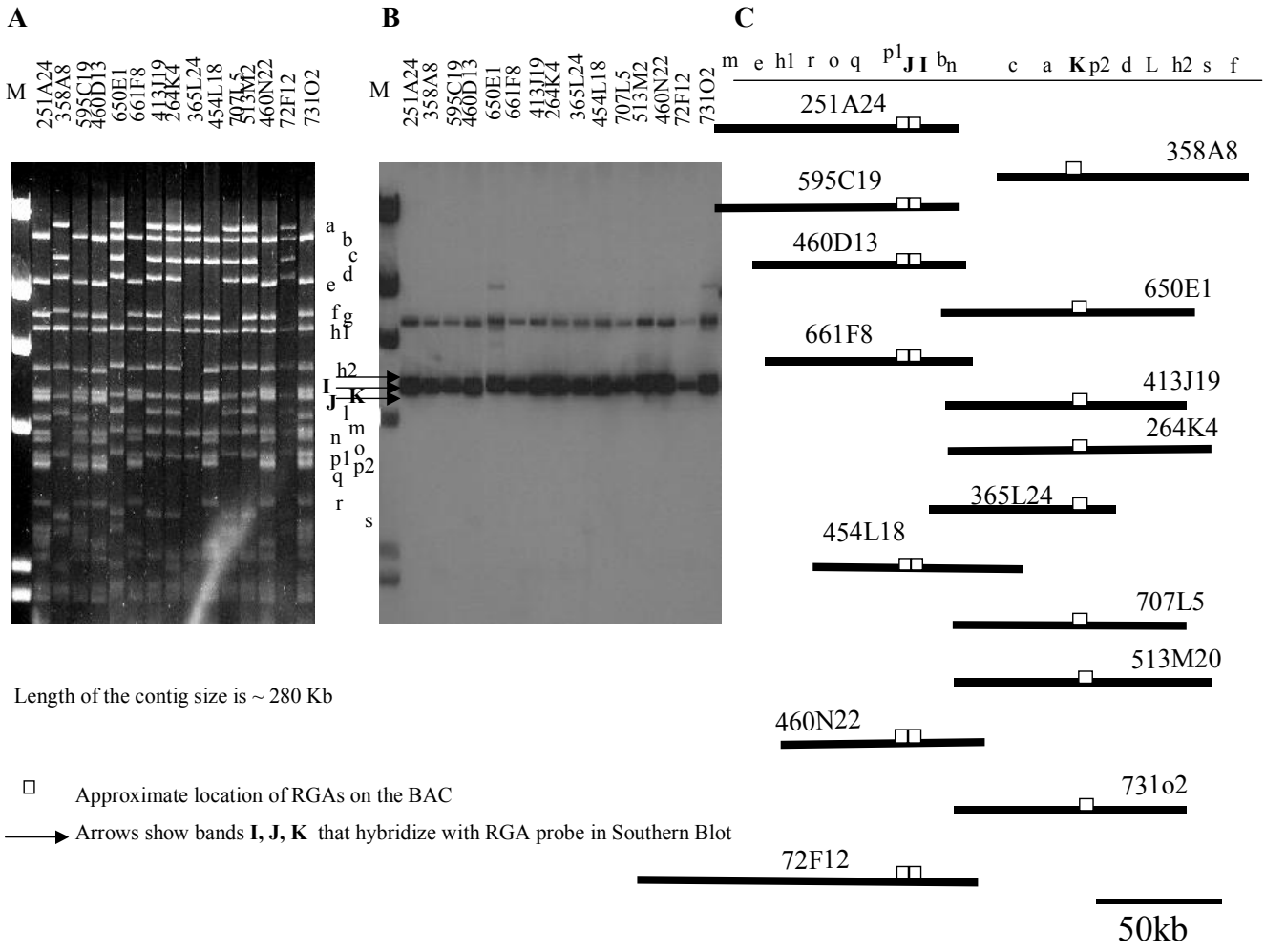


Figure 4.2. BAC contig for GL2-F5 barley RGA.

A. Agarose gel image of the *Hin*dIII fingerprinted BAC clones. **B.** Autoradiography of southern blot containing *Hin*dIII fingerprinted BAC clones. **C.** BAC contig. BAC IDs corresponds to addresses of BAC clones from Morex library (Clemson University Genomics Institute).

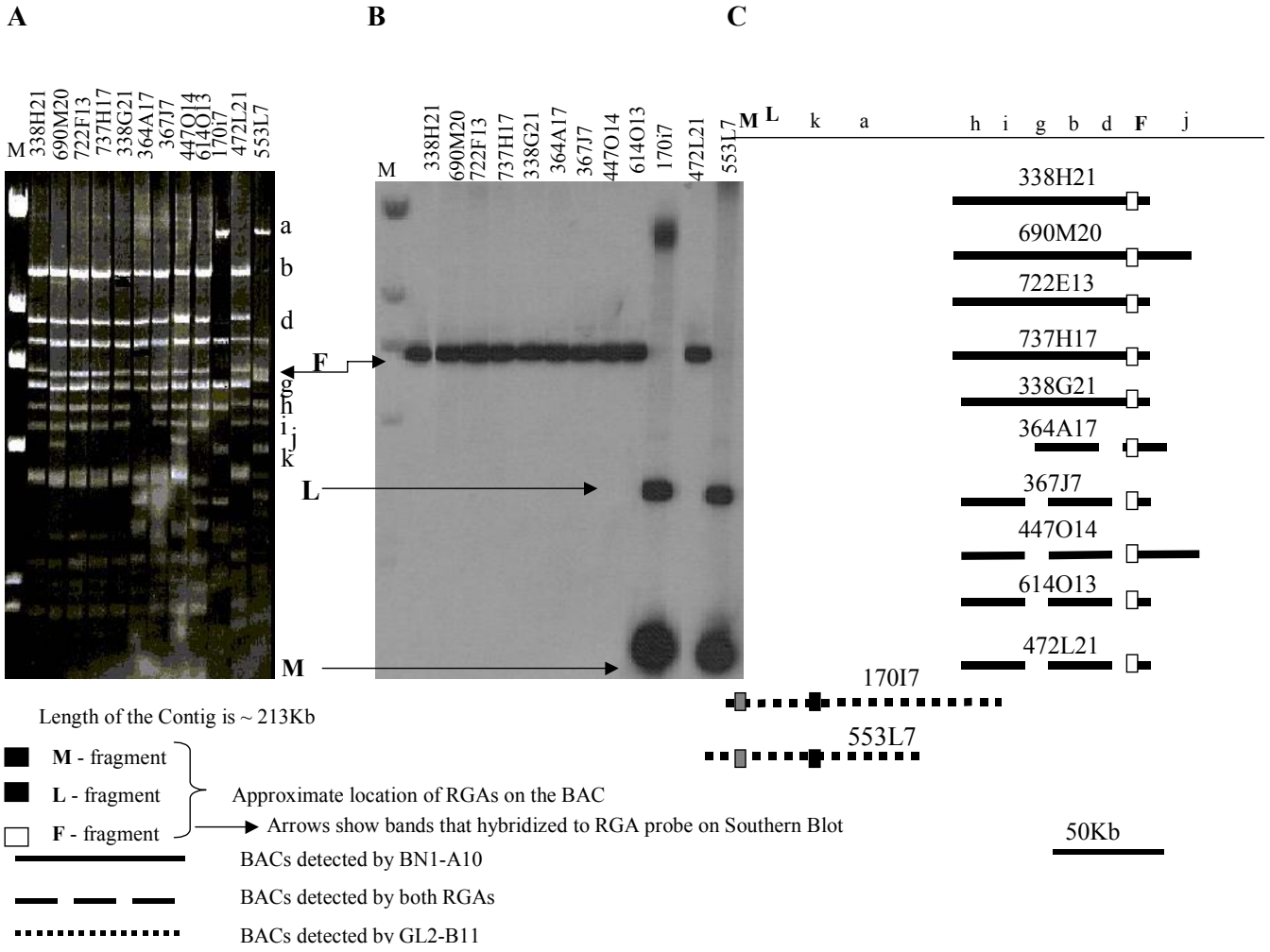


Figure 4.3. BAC contig for BN1-A10 and GL2-B11 barley RGAs.

A. Agarose gel image of the *Hin*dIII fingerprinted BAC clones. **B.** Autoradiography of southern blot containing *Hin*dIII fingerprinted BAC clones. **C.** BAC contig. BAC IDs corresponds to addresses of BAC clones from Morex library (Clemson University Genomics Institute).

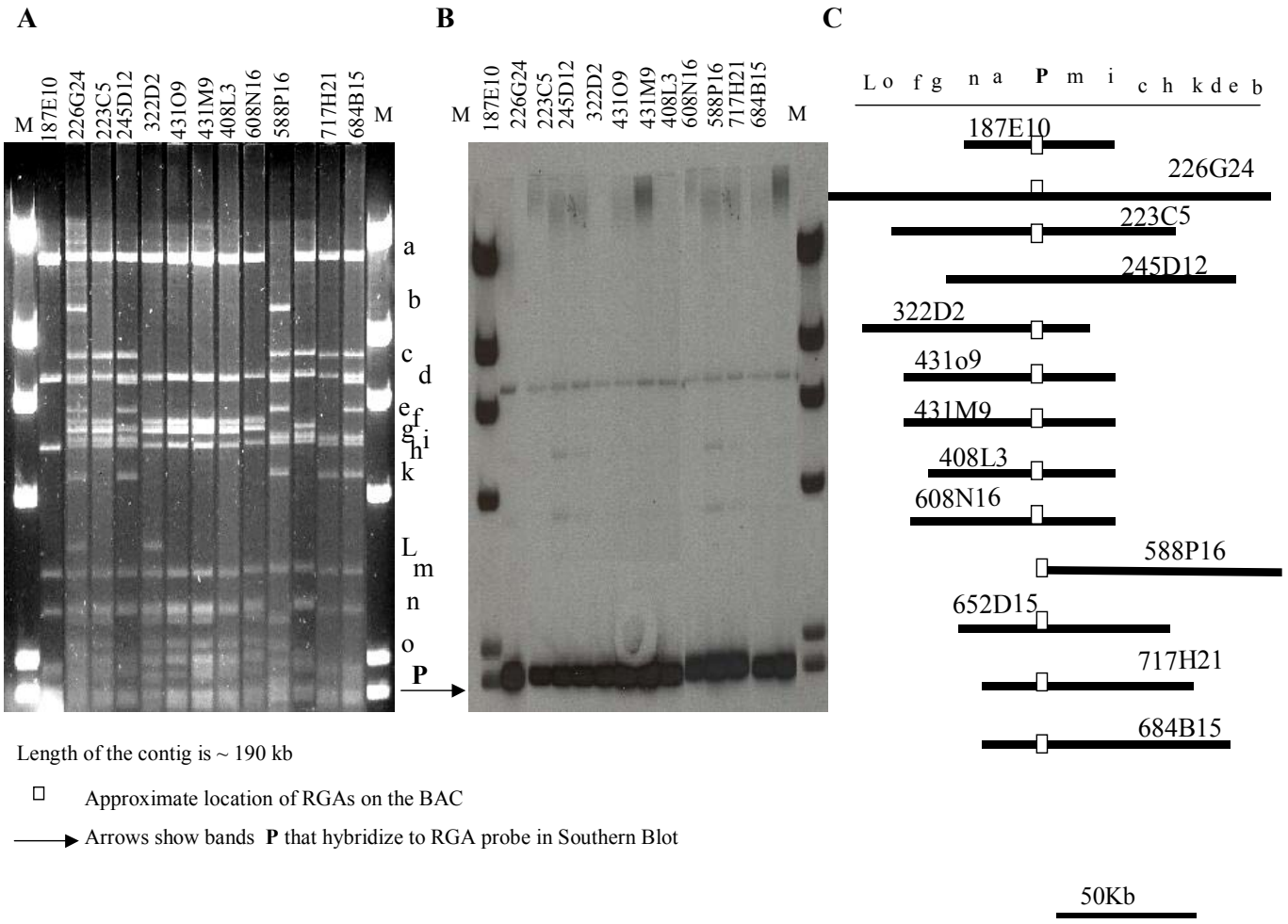


Figure 4.4. BAC contig for GL1-A2 barley RGA.

A. Agarose gel image of the *Hin*dIII fingerprinted BAC clones. **B.** Autoradiography of southern blot containing *Hin*dIII fingerprinted BAC clones. **C.** BAC contig. BAC IDs corresponds to addresses of BAC clones from Morex library (Clemson University Genomics Institute).

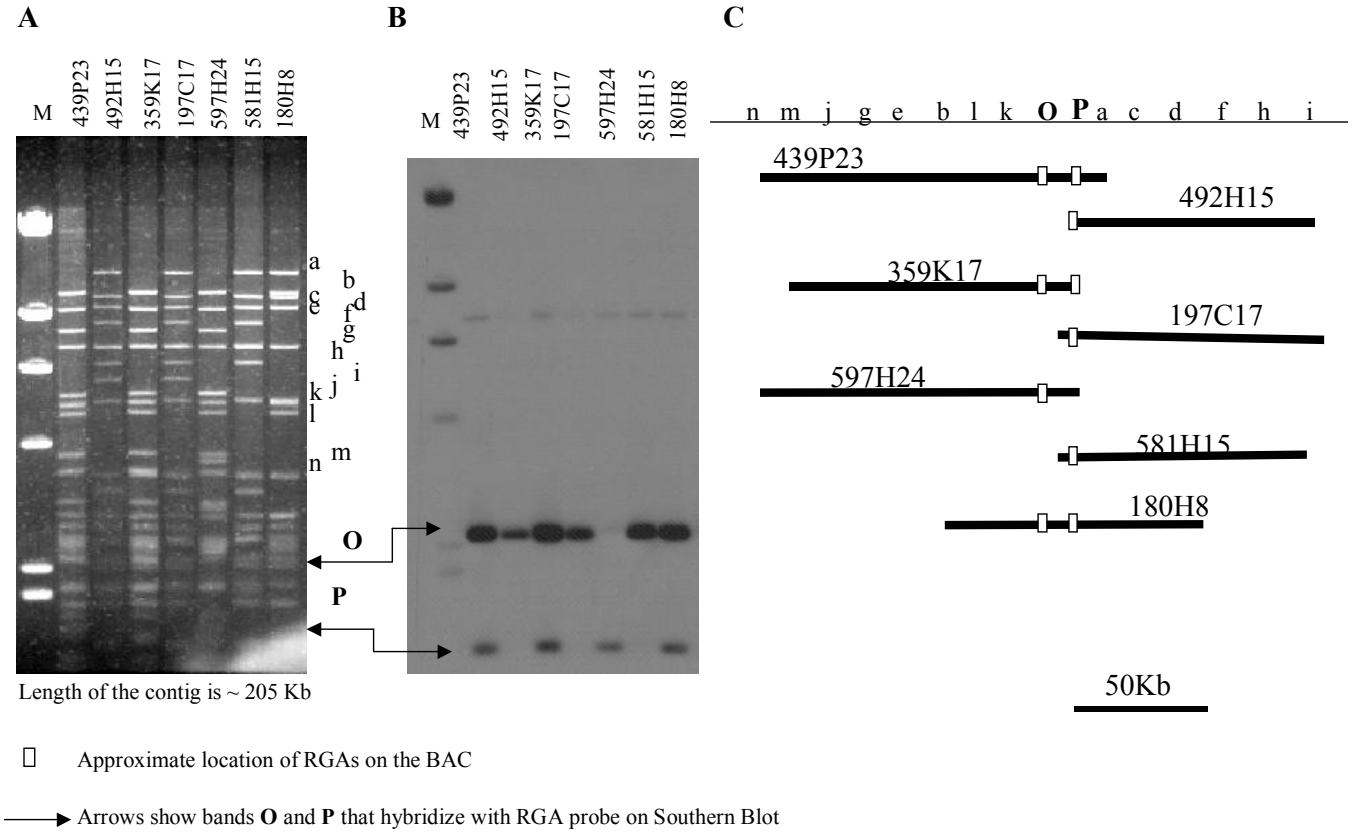


Figure 4.5. BAC contig for GL2-B1 barley RGA.

A. Agarose gel image of the *Hin*dIII fingerprinted BAC clones. **B.** Autoradiography of southern blot containing *Hin*dIII fingerprinted BAC clones. **C.** BAC contig. BAC IDs corresponds to addresses of BAC clones from Morex library (Clemson University Genomics Institute).

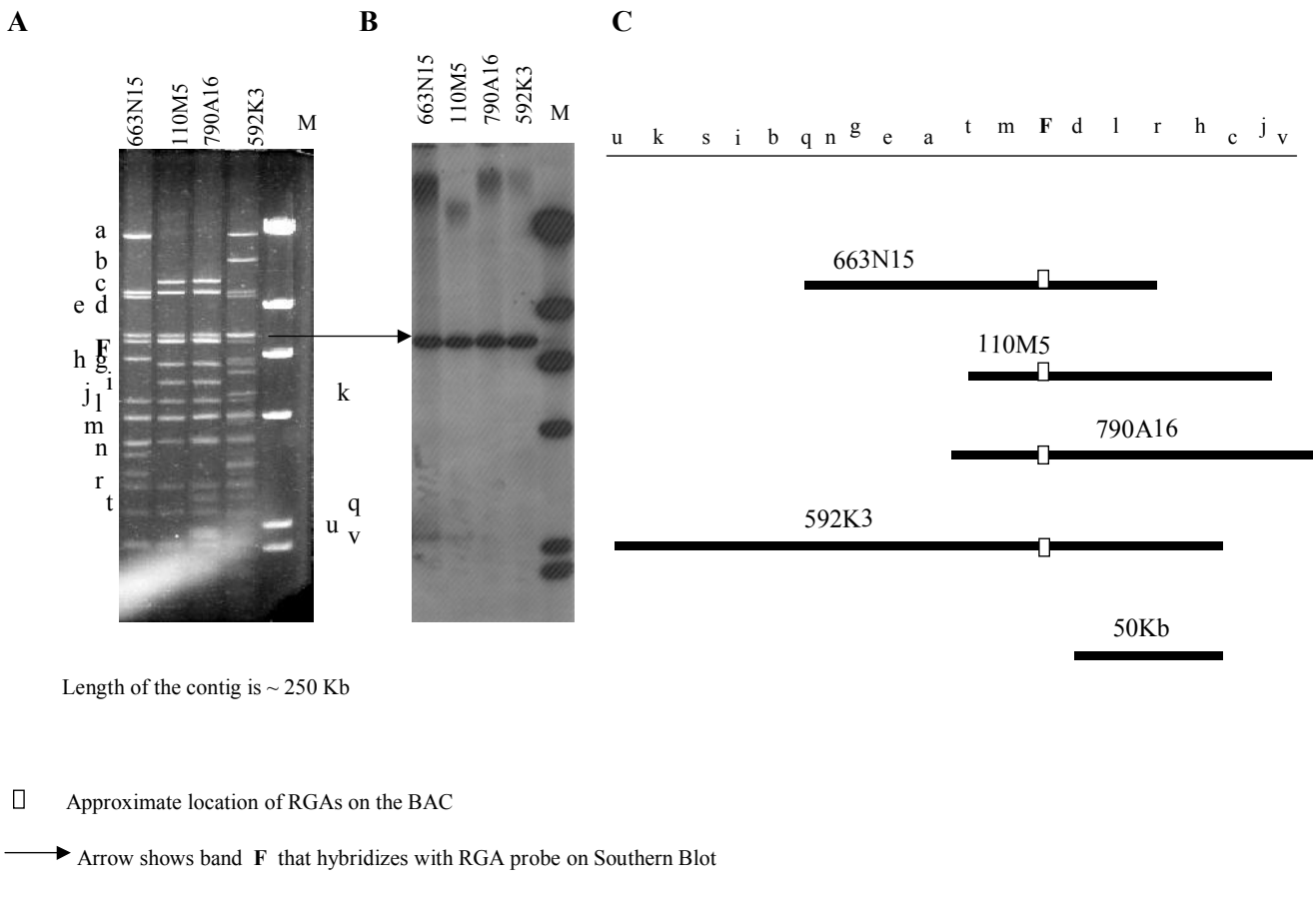


Figure 4.6. BAC contig for GL2-B4 barley RGA.

A. Agarose gel image of the *Hin*dIII fingerprinted BAC clones. **B.** Autoradiography of southern blot containing *Hin*dIII fingerprinted BAC clones. **C.** BAC contig. BAC IDs corresponds to addresses of BAC clones from Morex library (Clemson University Genomics Institute).

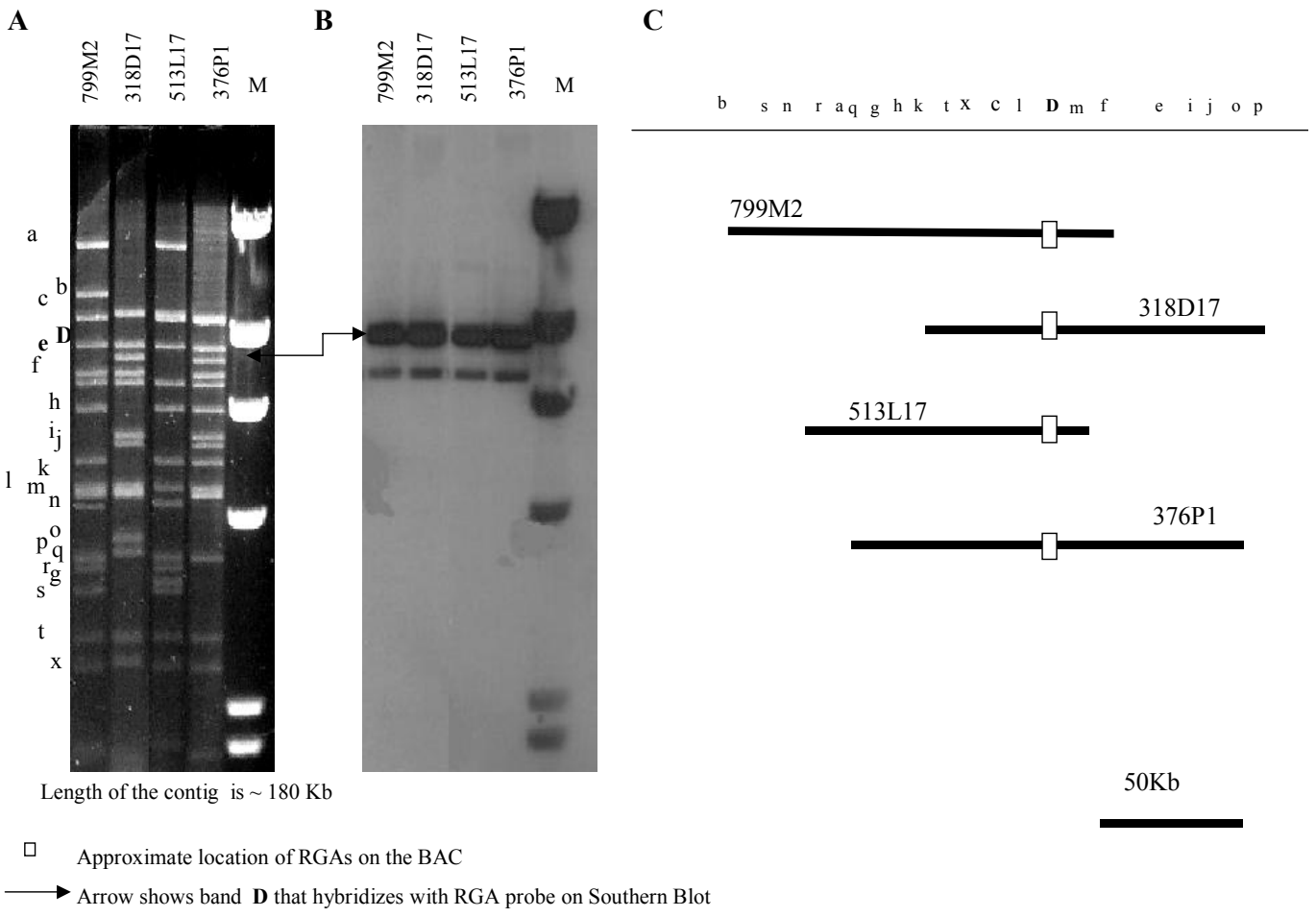


Figure 4.7. BAC contig for GL3-H11 barley RGA.

A. Agarose gel image of the *Hin*dIII fingerprinted BAC clones. **B.** Autoradiography of southern blot containing *Hin*dIII fingerprinted BAC clones. **C.** BAC contig. BAC IDs corresponds to addresses of BAC clones from Morex library (Clemson University Genomics Institute).

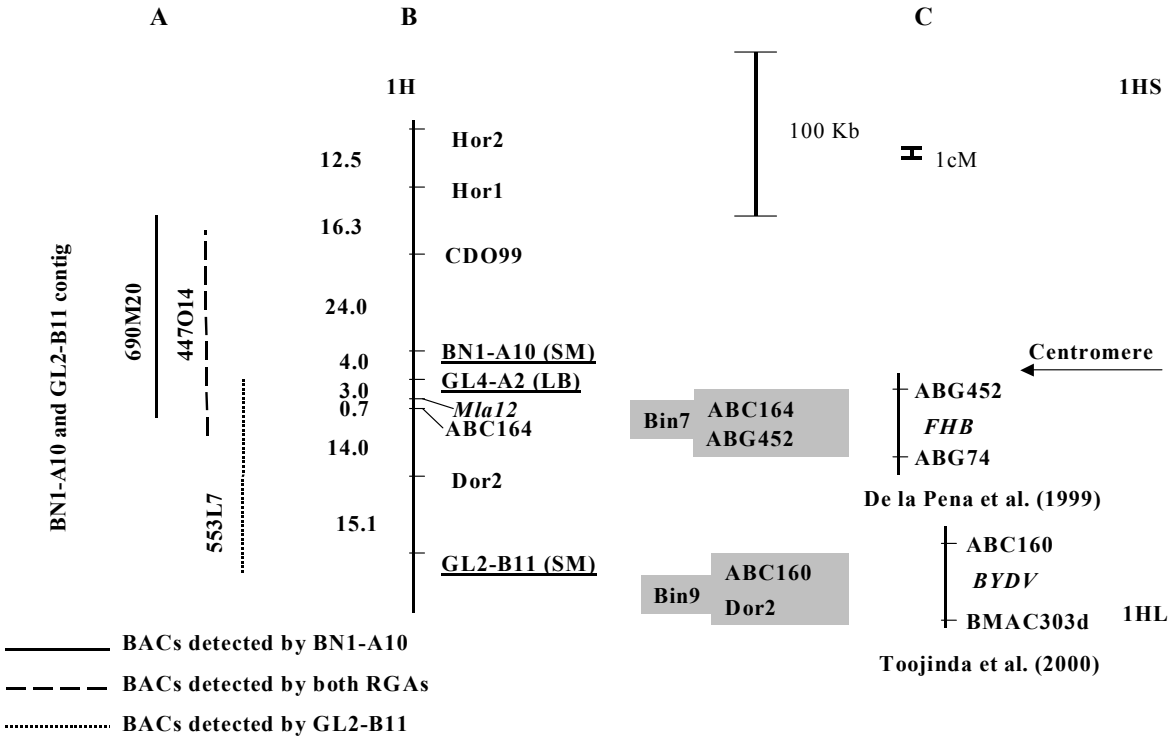


Figure 4.8. Physical (A) and genetic (B) map of barley showing RGAs and previously mapped R genes (C).

Barley RGAs (underlined) were genetically mapped to chromosomes 1H to 7H (except 5H) in three double haploid mapping populations, including Steptoe × Morex (SM), Harrington × TR306 (HT) and LUGC × Bowman (LB). Based on the positions of the anchor (common) markers in our genetic maps and the fragments of the published genetic maps of R genes, it was established that barley RGAs are located in the vicinity of known disease resistance genes. The shaded boxes are portions of the A. Kleinhofs' Bins at http://barleygenomics.wsu.edu/arnis/linkage_maps/maps-svg.html. Eight BAC contigs were developed. Only those BACs, which comprise minimum tiling pass, are depicted on the contig. Overlapping BACs are not shown.

Figure 4.8. Continued

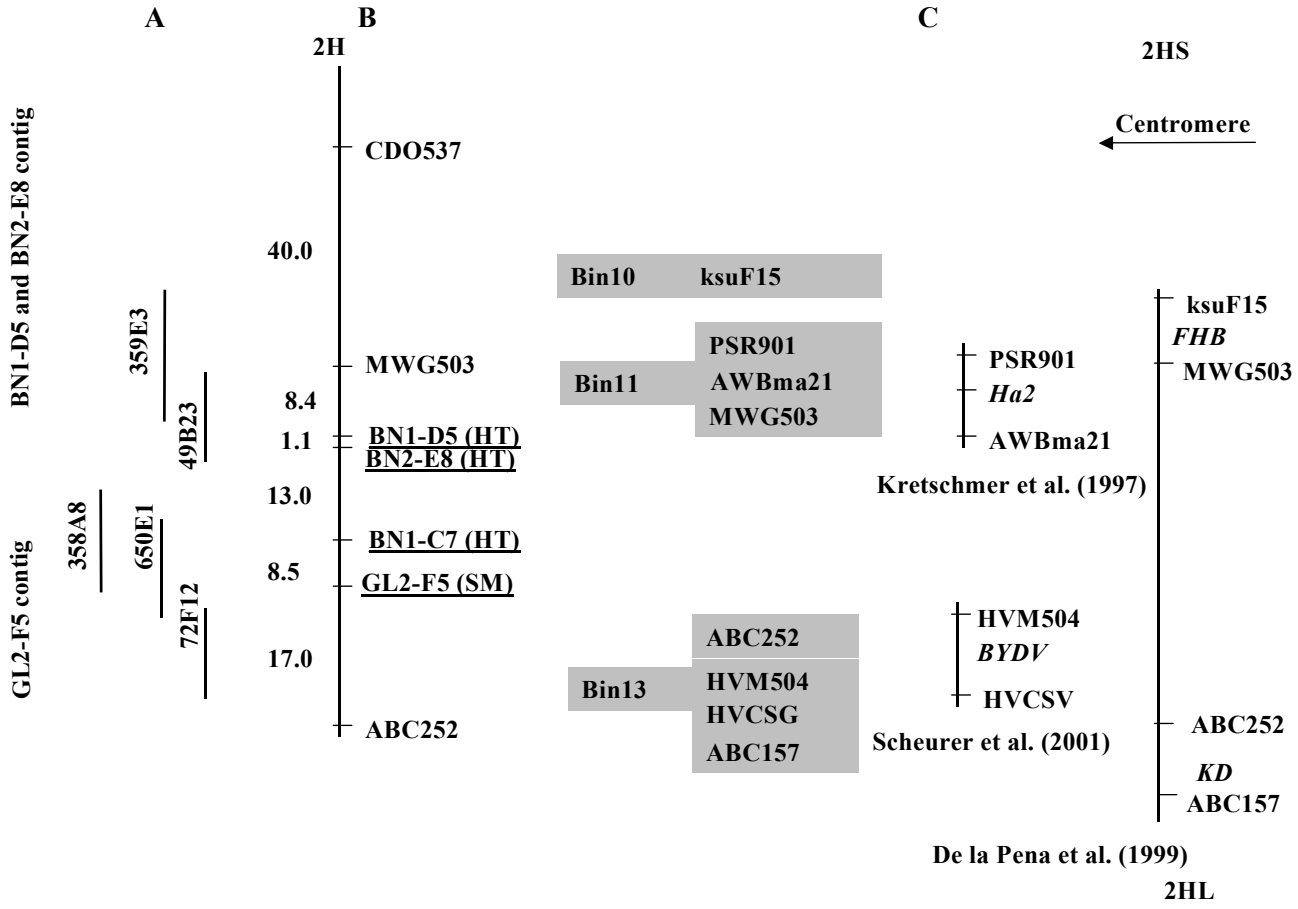


Figure 4.8. Continued

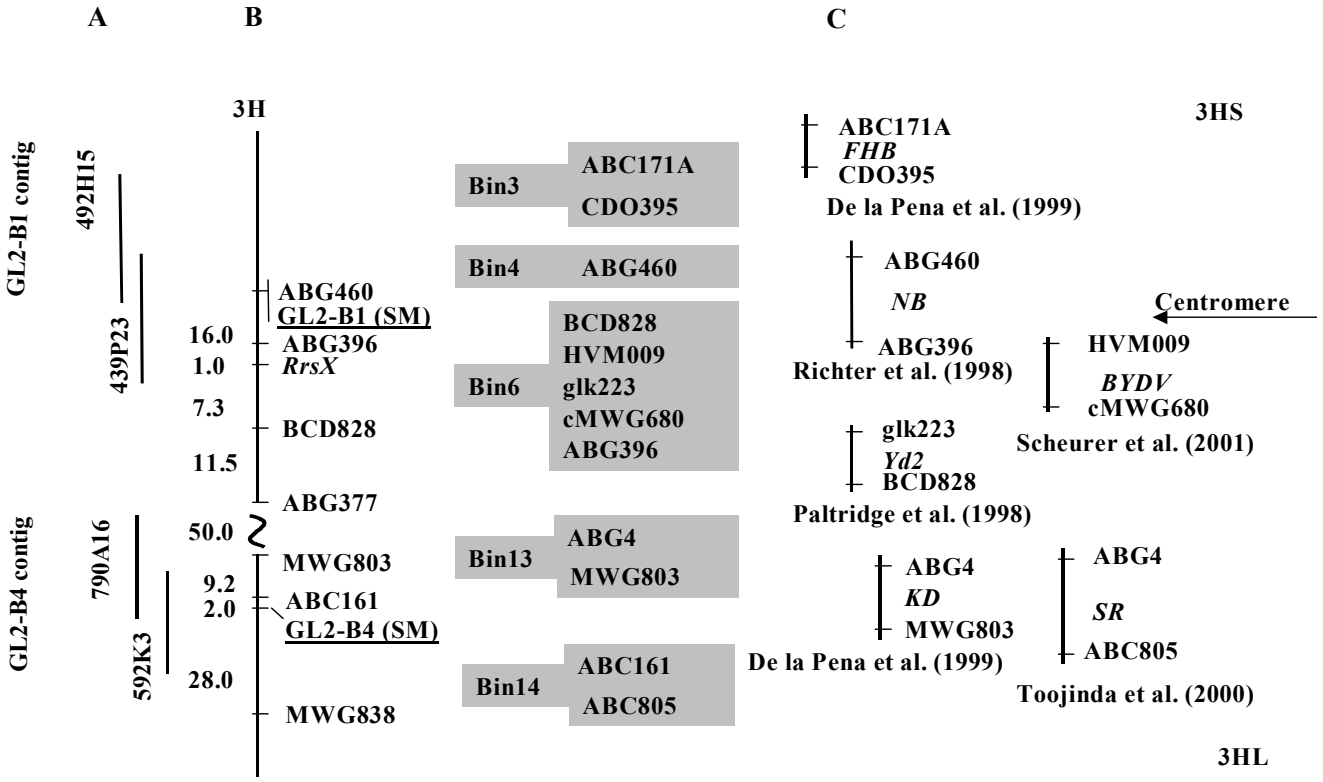


Figure 4.8. Continued

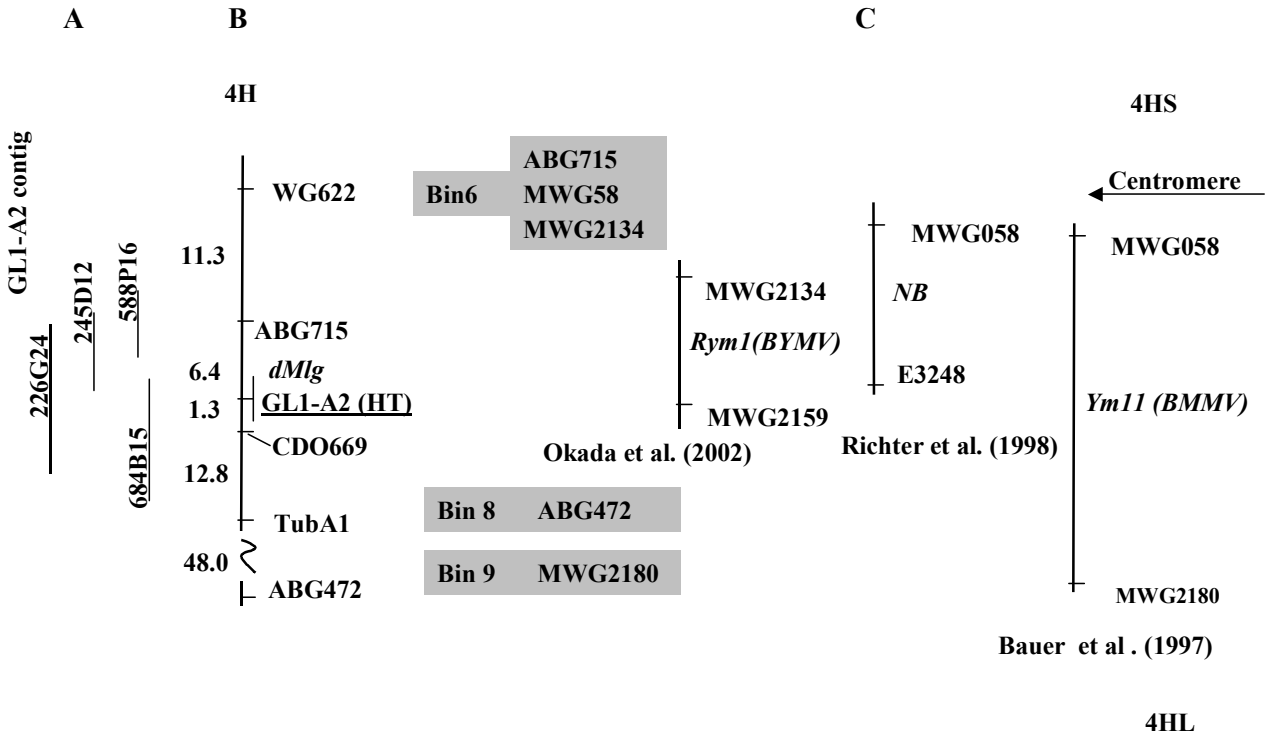


Figure 4.8. Continued

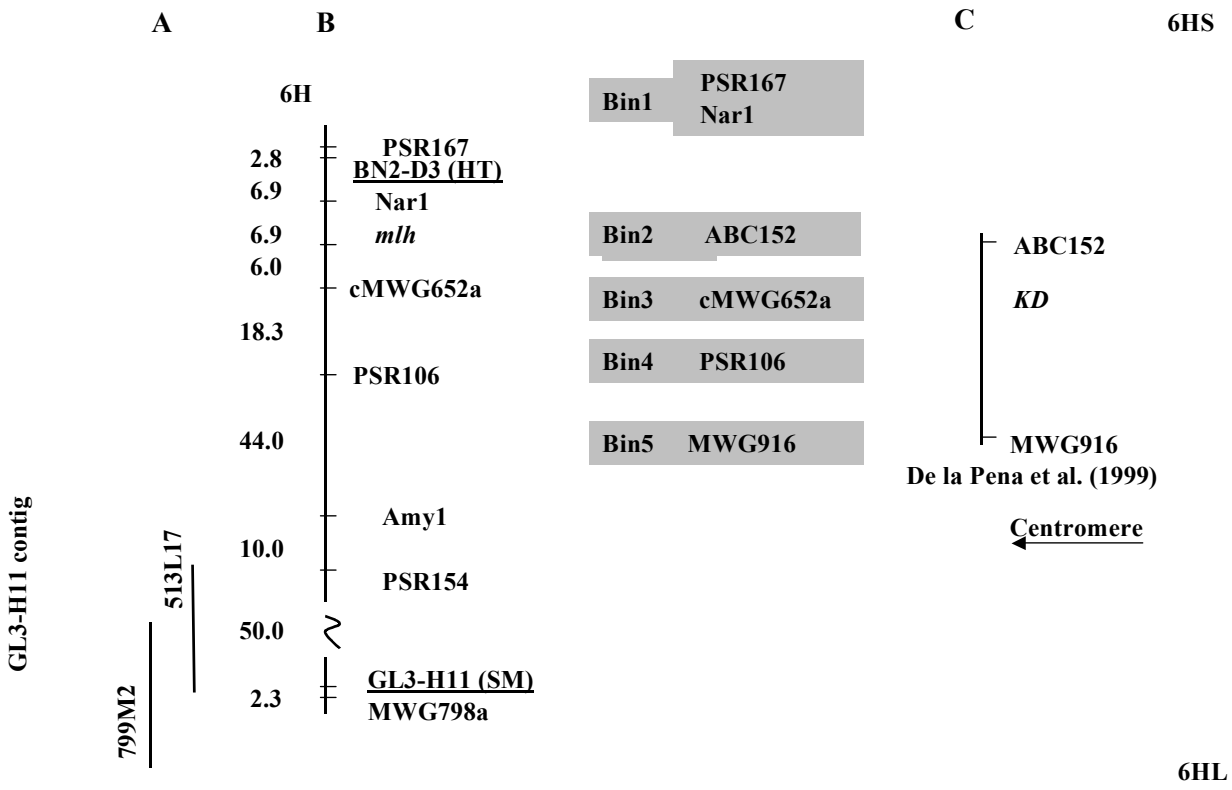
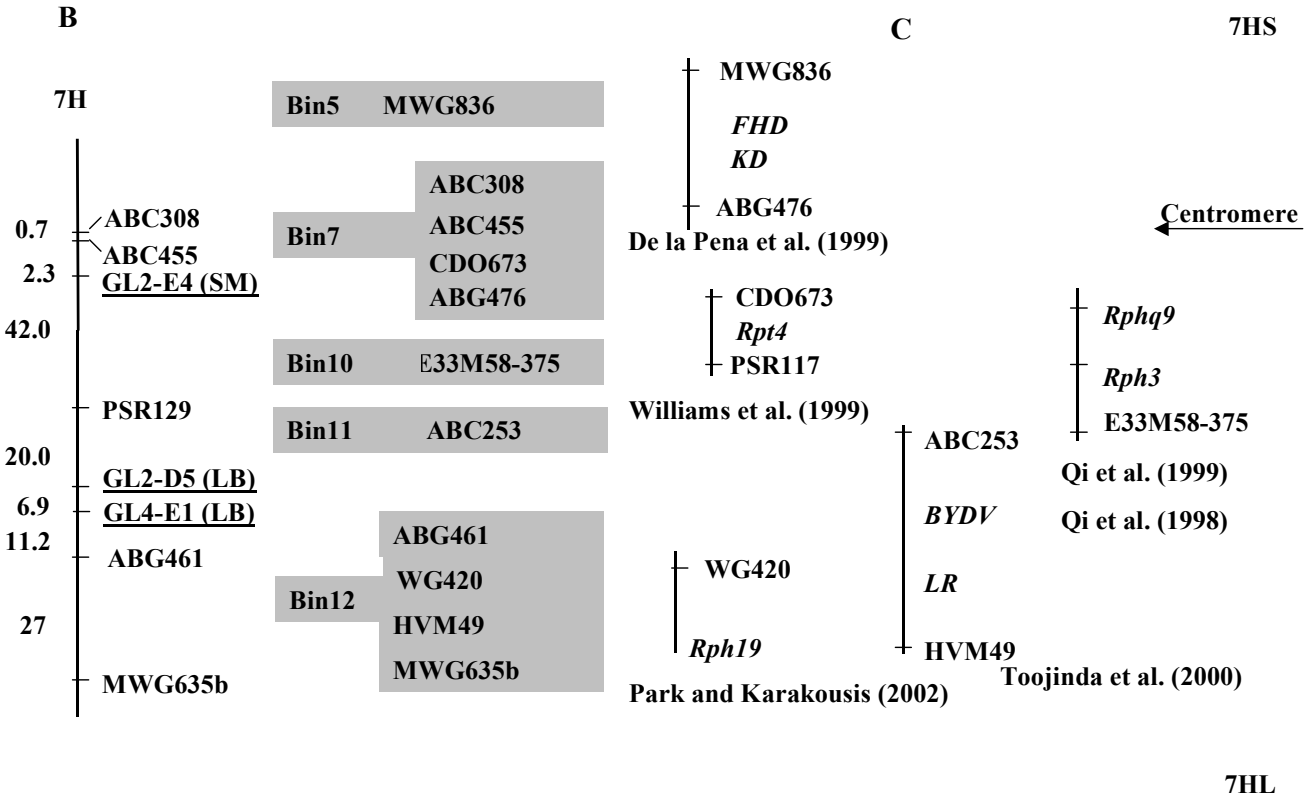


Figure 4.8. Continued



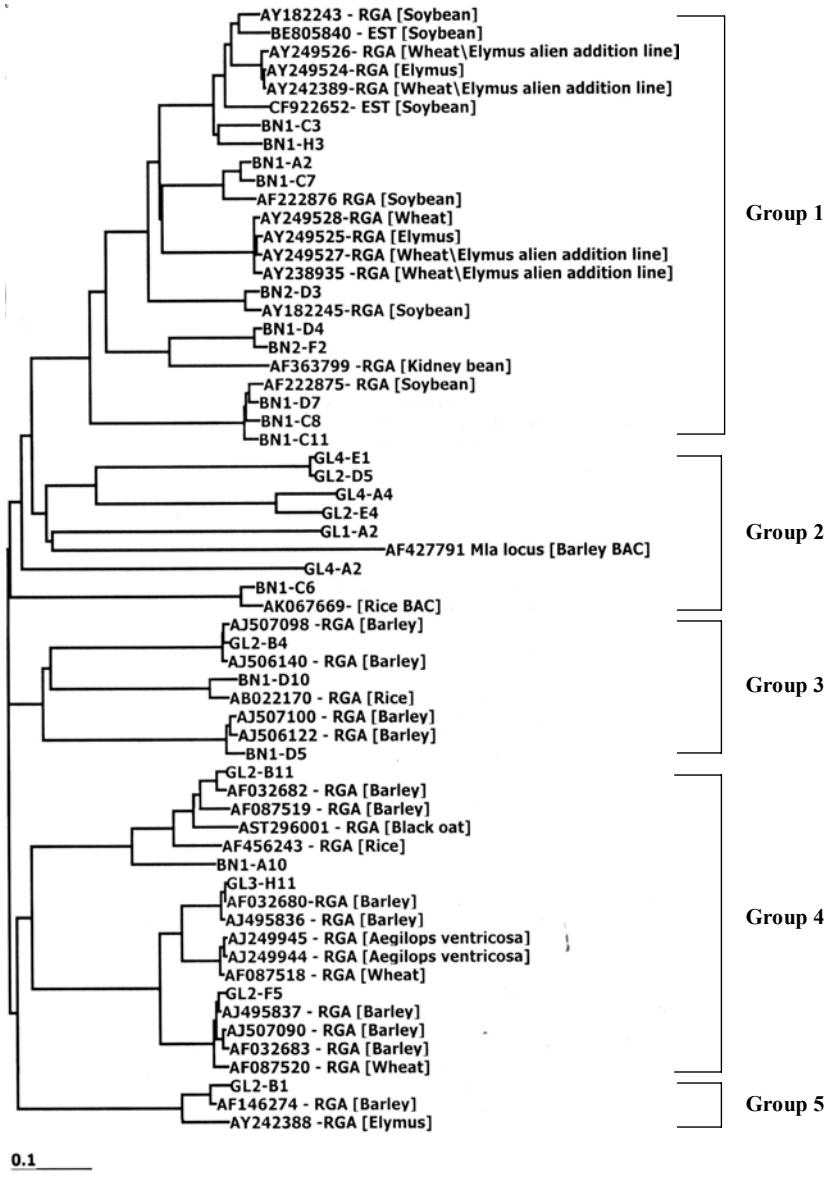


Figure 4.9. Phylogenetic tree of GL- (540 bp sequences) and BN- (340 bp sequences) class barley RGAs with previously published RGAs and expressed sequence tags (ESTs).

GL- and BN-class barley RGAs were BLASTed against non-redundant (NR) and expressed sequence tag (dbEST) databases. Based on nucleotide sequence comparisons of barley RGAs and their retrieved homologues from NR and dbESTs databases, a phylogenetic tree was constructed by neighbor-joining method (Saitou and Nei 1978) implemented in BioEdit software package after the ClustalX alignment of the above-mentioned sequences. As a result, all RGAs and ESTs were divided into five distinct groups.

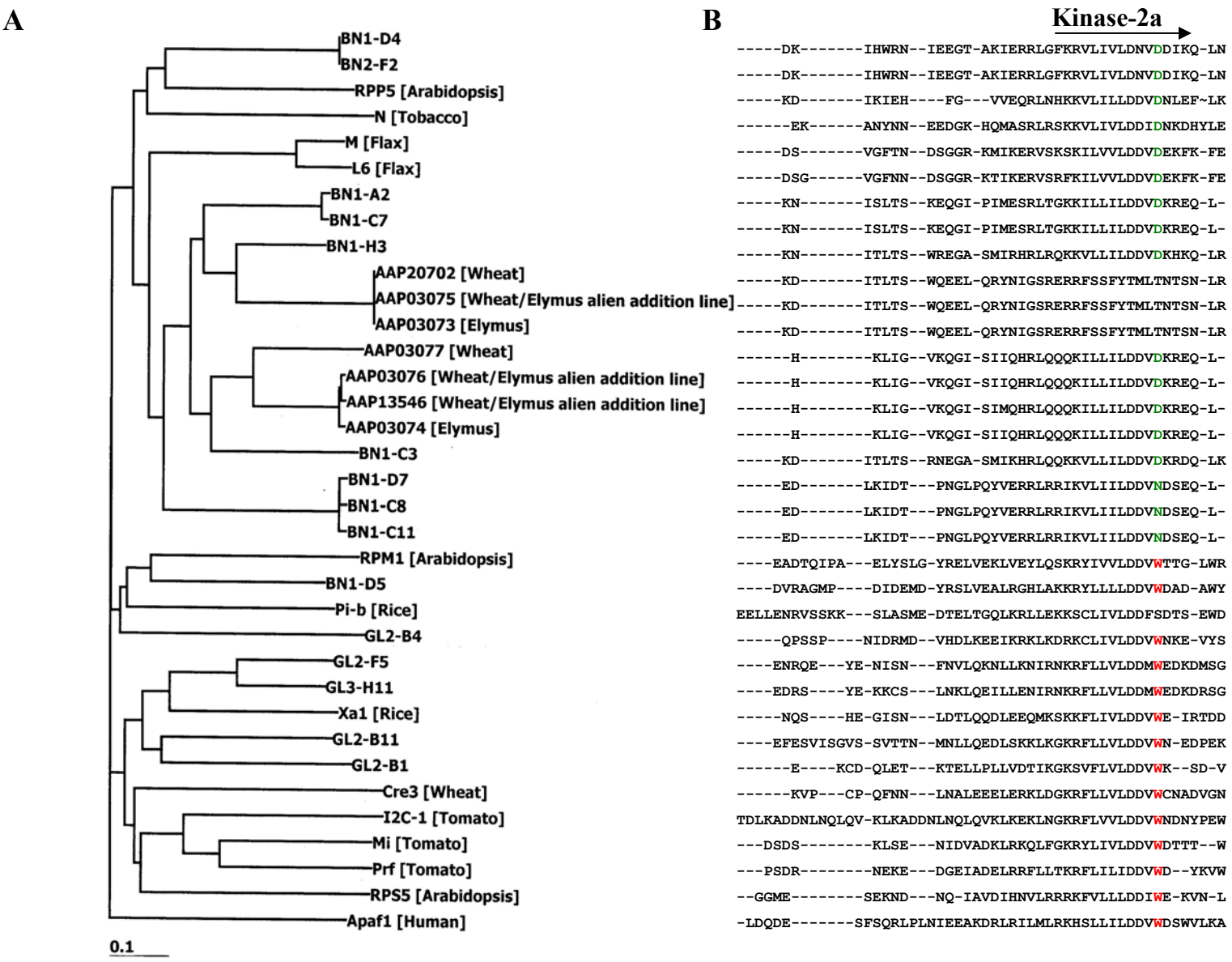


Figure 4.10. A, B. Comparison of the deduced amino acid sequences of 16 barley RGAs (selected from this study) and representing open reading frames (ORF), several TIR-NBS-LRR type monocot RGAs (retrieved from the public sequence database), and previously cloned R genes.

Sequence comparisons were done from p-loop-encoded region to the putative kinase 3a-encoded region. The rooted phylogenetic tree **(A)** was constructed after alignment of deduced amino acid sequences **(B)** with ClustalX followed by manual editing at BioEdit. The tree was constructed by neihbor-joing method (Saitou and Nei 1987) implemented at ClustalX. The branch lengths are proportional to the average substitutions per site as indicated by the scale. Elymus and wheat RGAs are identified by their GenBank accession numbers. The W and D/N demarcate conserved tryptophans (in green) and aspartic acid/aspartate (in red), which have been proposed to distinguish non-TIR-NBS-LRR from TIR-NBS-LRR resistance genes, respectively (Meyers et al. 1999). Human *Apaf-1,* gene was used to anchor the tree.

References

Aarts, M.G.M., Hekkert, B. L., Holub, E.B., Beynon, J.L., Stiekema, W.J., and Pereira, A. 1998. Identification of R gene homologous DNA fragments genetically linked to disease resistance loci in *Arabidopsis thaliana*. Mol. Plant-Microbe Interact. **11**: 251-258.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., and Liman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**: 3389-3402.

Anderson, P.A., Lawrence, G.J., Morrish, B.C., Ayliffe, M.A., Finnegan, E.J., and Ellis, J.G. 1997. Inactivation of the flax rust resistance gene *M* associated with loss of a repeated unit within the leucine-rich repeat coding region. Plant Cell, **9**: 641 651.

Backes, G., Madsen, L.H., Jaiser, H., Stougaard, J., Herz, M., Mohler, V., and Jahoor A. 2003. Localization of genes for resistance against *Blumeria graminis f.sp. hordei* and *Puccinia graminis* in a cross between a barley cultivar and a wild barley (*Hordeum vulgare ssp. spontaneum*) line. Theor. Appl. Genet. **106**: 353-362.

Bai J., Pennil, L.A., Ning, J., Lee, S.W., Ramalingam, J., Webb, C.R., Zhao, B., Sun, Q., Nelson, J.C., Leach, J.E., and Hulbert, S.H. 2002. Diversity in nucleotide binding site-leucine-rich repeat genes in cereals. Genome Res. **12**: 1871-1884.

Bauer, E., Weyen, J., Schiemann, A., Graner, A., and Ordon, F. 1997. Molecular mapping of novel resistance genes against Barley Mild Mosaic Virus (BMMV). Theor. Appl. Genet. **95**: 1263-1269.

Bent, A.F. 1996. Plant disease resistance genes: Function meets structure. Plant Cell, **8**: 1757-1771.

Bergelson, J., Kreitman, M., Stahl, E. and Tian, D. Evolutionary dynamics of R genes. 2001. Science. **292**: 2281-2285.

Boyes, D.C., Nam, J., and Dangl, J.L. 1998. The *Arabidopsis thaliana RPM1* disease resistance gene product is a peripheral plasma membrane protein that is degraded coincident with the hypersensitive response. Proc. Natl. Acad. Sci. U.S.A. **95**: 15849-15854.

Cannon, S.B., Zhu, H., Baumgarten, A.M., Spangler, R., May, G., Cook, D.R., Young, N.D. 2002. Diversity, distribution, and ancient taxonomic relationships within the TIR and non-TIR NBS-LRR resistance gene subfamilies. J. Mol. Evol. **54**: 548-562.

Collins, N.C., Paltrige, N.G., Ford, C.M., and Symons, R.H. 1996: The *Yd2* gene for barley yellow dwarf virus resistance maps close to the centromere on the long arm of barley chromosome 3. Theor. Appl. Genet. **92**: 858-864.

Collins, N., Park, R., Spielmeyer W., Ellis, J., and Pryor, A.J. 2001. Resistance gene analogs in barley and their relationships to rust resistance genes. Genome, **44**: 375-381.

Dangle, J.L., and Jones, J.D.G. 2001. Plant pathogens and integrated defence responses to infection. Nature, **411**: 826-833.

De la Pena, R.C., Smith, K.P., Capettini, F., Muehlbauer, G.J., Gallo-Meagher, M., Dill-Macky, R., Somers, D.A., and Rasmusson, D.C. 1999. Quantitative trait loci associated with resistance to Fusarium head blight and kernel discoloration in barley. Theor. Appl. Genet. **99**: 561-569.

Dodds, P.N., Lawrence, G.L., and Ellis, J.G. 2001. Contrasting modes of evolution acting on the complex *N* locus for rust resistance in flax. Plant J. **27**: 439-453.

Donald, T. M., Pellerone, F., Adam-Blondon, A-F., Bouquet, A., Thomas, M. R., and Dry, I. B. 2002. Identification of resistance gene analogs linked to a powdery mildew resistance locus in grapevine. Theor. Appl. Genet. **104**: 610 – 618.

Ellis, J., Dodds, P., and Pryor, T. 2000. Structure, function and evolution of plant disease resistance genes. Curr. Opin. Plant Biol. **3**: 278-284.

Ellis, J., Lawrence, G., Ayliffe, M., Anderson, P., Collins, N., Finnegan, J., Frost, D., Luck, J., and Pryor, T. 1997. Advances in the molecular genetic analysis of the flax-flax rust interaction. Annu. Rev. Phytopathol. **35**: 271-291.

Ellis, J.G., Lawrence, G.J., Luck, J.E., and Dodds, P.N. 1999. Identification of regions in alleles of the flax rust resistance gene *L* that determine differences in gene-foR gene specificity. Plant Cell, **11**: 495-506.

Flandez-Galvez, H., Ades P.K., Ford, R, Pang, E.C.K., and Taylor, P.W.J. 2003. QTL analysis for ascochyta blight resistance in an intraspecific population of chickpea (*Cicer arietinum* L.). Theor. Appl. Genet. **107**: 1257-1265.

Gentzbittel, L., Mouzeyar, S., Badaoui, S., Mestries, E., Vear, F., Labrouhe, D.T.D., and Nicolas, P. 1998. Cloning of molecular markers for disease resistance in sunflower, *Helianthus annuus,* L. Theor. Appl. Genet. **96**: 519-525.

Gibas, C. and Jambeck, P. 2001. Sequence analysis, pairwise alignment, and database searching. *In* Developing bioinformatics computer skills. *Edited by* L. LeJeune O'Reilly& Associates, Sebastopol, CA, p.187.

Goff, S.A., Ricke, D., Lan, T.H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions A et al. 2002. A draft sequence of the rice genome (*Oryza sativa L. ssp. japonica*). Science, **296**: 92-100.

Goodwin, S.B., and Hu, X. 1998. Cloning and analysis of four resistance gene analogs from wheat using conserved primers. Proceedings of the 9th International Wheat Genetics Symposium, Saskatoon, Sk Vol. 3, pp. 11-13.

Graham, M.A., Marek, L.F., Lohnes, D., Cregan, P., and Shoemaker, R.C. 2000. Expression and genome organization of resistance gene analogs in soybean. Genome, **43**: 86-93.

Graham, M.A., Marek, L.F., and Shoemaker, R. 2002. Organization, expression and evolution of a disease resistance gene cluster in soybean. Genetics, **162**: 1961-1977.

Hammond-Kosack, K.E. and J.D.G. Jones. 1997. Plant disease resistance genes. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48**: 575-607.

Huettel, B., Santra, D., Muehlbauer, F., and Kahl, G. 2002. Resistance gene analogues of chickpea (*Cicer arietinum* L.): isolation, genetic mapping and association with a Fusarium resistance gene cluster. Theor. Appl. Genet. **105**: 479-490.

Ilag, L.L., Yadav, R.C., Huang, N., Ronald, P.C., and Ausubel, F.M. 2000. Isolation and characterization of disease resistance gene homologues from rice cultivar IR64. Gene, **255**: 245–255.

Jeong, S.C., Hayes, A.J., Biyashev, R.M., and Saghai Maroof, M.A. 2001. Diversity and evolution of a non-TIR-NBS sequence family that clusters to a chromosomal "hotspot" for disease resistance genes in soybean. Theor. Appl. Genet. **103**: 406-414.

Jones, D., and Jones, J.D.G. 1997. The role of leucine-rich repeat proteins in plant defenses. Adv. Bot. Res. Adv. Plant. Pathol. **24**: 89-167.

Kalavacharia, V., Stavely, J.R., Myers, J.R., and McClean, P.E. 2002. *Crg*, a gene required for Ur-3-mediated rust resistance in common bean, maps to a resistance gene analog cluster. Mol. Plant Microbe Interact. **13**: 1237-42.

Kanazin, V., Marek, L.F. and Shoemaker, R.C. 1996. Resistance gene analogs are conserved and clustered in soybean. Proc. Natl. Acad. Sci. U.S.A. **93**: 11746-11750.

Kasha, K.J. and Kleinhofs, A. 1994. Mapping of the barley cross Harrington × TR306. Barley Genet. Newsl. **23**: 65-69.

Kleinhofs, A., Kilian, A., Saghai Maroof, M.A., Biyashev, R.M., Hayes, P., Chen F.Q., Lapitan, N., Fenwick, A., Blake, T.K., Kanazin, V., Ananiev, E., Dahleen, L., Kudrna, D., Bollinger, J., Knapp, S.J., Liu, B., Sorrels, M., Heun, M., Franckowiak,

J.D., Hoffman, D., Skadsen, R., and Steffenson, B.J. 1993. A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. Theor. Appl. Genet. **86**: 705-712.

Kobe, B. and J. Deisenhofer. 1994. The leucine-rich repeat: A versatile binding motif. Trends Biochem Sci. **19**: 415-421.

Kobe, B. and J. Deisenhofer 1995. A structural basis of the interactions between leucine-rich repeats and protein ligands. Nature **374**: 183-186.

Kobe, B, and Kajava, A.V. 2001. The leucine-rich repeat as a protein recognition motif. Curr. Opin. Struct. Biol. **11:** 725–732.

Kretscher, J.M., Chalmers, K.J., Manning, S., Karakousis, A., Barr, A.R., Islam, A.K.M.R., Logue, S.J., Choe, Y.W., Barker, S.J., Lance, R.C.M, and Langridge, P. 1997. RFLP mapping of the *Ha2* cereal cyst nematode resistance gene in barley. Theor. Appl. Genet. **94**:1060-1064.

Lagudah, E.S, Moullet, O., and Appels, R.. 1997. Map-based cloning of a gene sequence encoding a nucleotide-binding domain and a leucine-rich region at the *Cre3* nematode resistance locus of wheat. Genome, **40**: 659-65.

Lawrence, G.J., Finnegan, E.J., Ayliffe, M.A., and Ellis, J.G. 1995. The *L6* gene for flax rust resistance is related to Arabidopsis bacterial resistance gene *RPS2* and the tobacco viral resistance gene *N*. Plant Cell, **7**: 1195-1206.

Lee, S-Y, Seo, J.-S., Rodriguez-Lanetty, M., and Lee, D.-H. 2003. Comparative analysis of superfamilies of NBS-encoding disease resistance gene analogs in cultivated and wild apple species. Mol. Gen. Genomics, **269**: 101-108.

Leister, D., Ballvora, A., Salamini, F. and Gebhardt, C. 1996. A PCR based apparoach for isolating pathogen resistance genes from potato with potential for wide application in plants. Nature Genetics, **14**: 421-429.

Leister, D., Kurth, J., Laurie, D.A., Yano, M., Sasaki, T., Devos, K., Graner, A. and Schulze-Lefert, P. 1998. Rapid reorganization of resistance gene homologues in cereal genomes. Proc. Natl. Acad. Sci. U.S.A. **95**: 370-375.

Leister D., Kurth, J., Laurie, D.M., Yano, M., Sasaki, T., Graner, A., and Schulze-Lefert, P. 1999. RFLP and physical mapping of resistance gene homologues in rice (*O. sativa*) and barley (*H. vulgare*). Theor. Appl. Genet. **98**: 509-520.

Liu, Z., Biyashev, R.M., Mammadov, J.A., and Saghai Maroof, M.A. 2004. Identification and mapping of disease resistance-related DNA sequences in barley. Czech Journal of Genetics and Plant Breeding, **40**: 46.

Madsen, L.H., Collins, N.C., Rakwalska, M., Backes, G., Sandal, N., Krusell, L., Jensen, J., Waterman, E. H., Jahoor, A., Ayliffe, M., Pryor, A. J., Langridge, P., Schulze-Lefert, P., and Stougaard, J. 2003. Barley disease resistance gene analogs of the NBS-LRR class: identification and mapping. Mol. Gen. Genomics, **269**: 150-161.

Mago, R., Nair, S., and Mohan, M. 1999. Resistance gene analogues from rice: cloning, sequencing and mapping. Theor. Appl. Genet. **99**: 50-57.

Manninen, O., Kalendar, R., Robinson, J., and Schulman, A.H. 2000. Application of BARE-1 retrotransposon markers to the mapping of a major resistance gene for net blotch in barley. Mol. Gen. Genet. **264**: 325-34.

Marra, M.A., Kucaba, T.A., Dietrich, N.L., Green, E.D., Brownstein, B., Wilson, R.K., McDonald, K.M., Hillier, L.W., McPherson, J.D., and Waterston, R.H. 1997. High throughput fingerprint analysis of large-insert clones. Genome Res. **7**: 1072-84.

Meyers, B.C., Chin, D.B., Shen, K.A, Sivaramakrishnan, S., Lavelle, D.O., Zhang, Z., and Michelmore, R.W. 1998. The major resistance gene cluster in lettuce is highly duplicated and spans several megabases. Plant Cell, **10**:1817-1832.

Meyers, B.C., Dickerman, A.W., Michelmore, R.W., Sivaramakrishnan, S., Sobral, B.W., and Young, N.D. 1999. Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant J. **20**: 317-332.

Michelmore, R.W., and Meyers, B.C. 1998. Clusters of resistance genes in plants evolve by divergent selection and birth-and-death process. Genome Res. **8**:1113-1130.

Mohler, V., Klahr, A., Wenzel, G., and Schwarz, G. 2002. A resistance gene analog useful for targeting disease resistance genes against different pathogens on group 1S chromosomes of barley, wheat and rye. Theor. Appl. Genet. **105**: 364-368.

Mondragon-Palomino, M., Meyers, B.C., Michelmore, R.W., and Gaut, B.S. 2002. Patterns of positive selection in the complete NBS-LRR gene family of *Arabidopsis thaliana*. Genome Res. **12:** 1305–1315

Noir, S., Combes, M.-C., Anthony, F., and Lashermes, P. 2001. Origin, diversity and evolution of NBS-type disease-resistance gene homologues in coffee trees (*Coffea L.*). Mol. Gen. Genomics. **265**: 654-662.

Ohmori, T., Murata, M., and Motoyoshi, F. 1998. Characterization of disease resistance gene-like sequences in near-isogenic lines of tomato. Theor. Appl. Genet. **96**: 331-338.

Ori, N., Eshed, Y., Paran, I., Presting, G., Aviv, D., Tanksley, S., Zamir, D., and Fluhr, R. 1997. The *I2C* family from the wilt disease resistance locus *I2* belongs to the

nucleotide binding, leucine-rich repeat superfamily of plant resistance genes. Plant Cell, **9**: 521-532.

Paltridge, N.G., Collins, N.C., Bendahmane, A., and Symons, R.H. 1998. Development of YLM, a codominant PCR marker closely linked to the *Yd2* gene for resistance to barley yellow dwarf disease. Theor. Appl. Genet. **96**: 1170-1177.

Pan, Q., Wendel, J., and Fluhr, R. 2000. Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. J. Mol. Evol. **50**: 203-213.

Park, R.F., and Karakousis, A. 2002. Characterization and mapping of the gene *Rph19* conferring resistance to Puccinia hordei in the cultivar 'Reka I' and several Australian barleys. Plant Breeding, **121**: 232-236.

Parker, J.E., Coleman, M.J., Szabo, V., Frost, L.N., Schimdt, R., van der Biezen, E.A., Moores, T., Dean, C., Daniles, M.J., and Jones, J.D.G. 1997. The *Arabidopsis* downy mildew resistance gene *RPP5* shares similarity to the toll and interleukin-1 receptors with *N* and *L6*. Plant Cell, **9**: 879-894.

Penuela, S., Danesh, D., and Young N.D. 2002. Targeted isolation, sequence analysis, and physical mapping of non-TIR NBS-LRR genes in soybean. Theor. Appl. Genet. **104**: 261–272.

Plocik, A., Layden, J., and Kesseli, R. 2004. Comparative analysis of NBS domain sequences of NBS-LRR disease resistance genes from sunflower, lettuce, and chicory. Mol. Phylogenet. Evol. **31**: 53 163.

Qi, X., Fekadu, F., Sijtsma, D., Niks, R.E., Lindhout, P., and Stam, P. 2000. The evidence for abundance of QTLs for partial resistance to *Puccinia hordei* on the barley genome. Mol. Breed. **6**: 1-9.

Qi, X., Stam, P., and Lindhout, P. 1998. Use of locus-specific AFLP markers to construct a high-density molecular map in barley. Theor. Appl. Genet. **96**: 376-384.

Richter, K., Schondelmaier, J., and Jung, C. 1998. Mapping of quantitative trait loci affecting *Drechslera teres* resistance in barley with molecular markers. Theor. Appl. Genet. **97**: 1225-1234

Rivkin, M.I., Vallejos, C.E., and McClean, P.E. 1999. Disease-resistance related sequences in common bean. Genome, **42**: 41-47.

Rock, F.L., Hardiman, G., Timans, J.C., Kastelein, R.A., and Bazan, J.F. 1998. A family of human receptors structurally related to Drosophila Toll. Proc. Natl. Acad. Sci. U.S.A. **95**: 588-593.

Rossi, M., Goggin, L.F.L., Milligan, B.S.B., Kaloshian, I., Ullman, D.E., and Wiiliamson, V.M. 1998. The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. Proc. Natl. Acad. Sci. U.S.A. **95**: 9750-9754.

Rostoks, N., Zale, J., Soule, J., Brueggeman, R., Druka, A., Kudrna, D., Steffenson, B., and Kleinhofs, A. 2002. A barley gene family homologous to the maize rust resistance gene *Rp1-D*. Theor. Appl. Genet. **104**: 1298–1306.

Salmeron, J.M., Oldroyd, G.E.D., Rommens, C.M.T., Scofield, S.R., Kim, H.-S., Lavelle, D.T., Dahlbeck, D., and Staskawicz, B.J. 1996. Tomato *Prf* is a member of the leucine-rich-repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. Cell, **86**: 123–133.

Sambrook, J., Frisch E., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Saraste, M., Sibbald, P.R., and Wittinghofer, A. 1990. The P-loop – common motif in ATP- and GTP-binding proteins. Tr. Biochem. Sci. **15**: 430-434.

Scheurer, K.S., Friedt, W., Huth, W., Waugh, R., and Ordon, F. 2001. QTL analysis of tolerance to a German strain of BYDV-PAV in barley (*Hordeum vulgare* L.). Theor. Appl. Genet. **103**: 1074-1083.

Seah, S., Sivasithamparam, K., Karakousis, A., and Lagudah, E. 1998. Cloning and characterization of a family of disease resistance gene analogs from wheat and barley. Teor. Appl. Genet. **97**: 937-945.

Shen, K.A., Meyers, B.C., Faridi, M.N.I., Chin, D.B., Stelly, D.M., and Michelmore, R.W. 1998. Resistance gene candidates identified by PCR with degenerate oligonucleotide primers map to clusters of resistance genes in lettuce. Mol. Plant-Microbe Interact. **11**: 815-823.

Shi, Z.X., Chen, X.M., Line, R.F., Leung, H., and Wellings, C.R. 2001. Development of resistance gene analog polymorphism markers for the *Yr9* gene resistance to wheat stripe rust. Genome, **44**: 509-516.

Simonich, M.T., and Innes, R.W. 1995. A disease resistance gene in *Arabidopsis* with specificity for the *avrPph3* gene of *Pseudomonas syringae* pv. *phaseolicola*. Mol. Plant-Microbe Interact. **8**: 637-640.

The Arabidopsis Genome Initiative. 2000. Nature, **408**: 796-815.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Hihhins, D.G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **24**: 4876-4882.

Toojinda, T., Broers, L.H., Chen, X.M., Hayes, P.M., Kleinhofs, A., Korte, J., Kudrna, D., Leung, H., Line, R.F., Powell, W., Ramsay, L., Vivar, H., and Waugh, R. 2000. Mapping quantitative and qualitative disease resistance genes in a double haploid population of barley (*Hordeum vulgare*). Theor. Appl. Genet. **101**: 580-589.

Traut, T.W. 1994. The functions and consensus motifs of nine types of peptides segments that form different types of nucleotide-binding sites. Eur. J. Biochem. **222**: 9-19.

Wang, Z., Taramino, D., Yang, D., Liu, G., Tingey, S.V., Miao, G., and Wang, G. 2001. Rice EST with disease resistance gene - or defense-response gene-like sequences mapped to regions containing major resistance genes or QTLs. Mol. Genet. Genomics, **265**: 302-310.

Wei, F., Wing, R.A., and Wise, R.P. 2002. Genome dynamics and evolution of the *Mla* (powdery mildew) resistance locus in barley. Plant Cell, **14**:1903-1917.

Williams, K.J., Lichon, A., Gianquitto, P., Kretschmer, J.M., Karakousis, A., Manning, S., Langridge P., and Wallwork H. 1999. Identification and mapping of a gene conferring resistance to the spot form of net blotch (*Pyrenophora teres f. maculata*) in barley. Theor. Appl. Genet. **99**: 323-327.

Whitham, S., McCormick, S., and Baker, B. 1996. The *N* gene of tobacco confers resistance to tobacco mosaic virus in transgenic mosaic virus in transgenic tomato. Proc. Natl. Acad. Sci. U.S.A. **93**: 8776-8781.

Wulff, B.B.H., Thomas, C.M., Smoker, M., Grant, M., and Jones, J.D.G. 2001. Domain swapping and gene shuffling identify sequences required for induction of an Avr-dependent hypersensitive response by the tomato *Cf-4* and *Cf-9* Proteins. Plant Cell, **13**: 255-272.

Yoshimura, S., Yamanouchi, U., Katayose, Y., Toki, S., Wang, Z.X., Kono, I., Kurata, N., Yano, M., Iwata, N., and Sasaki, T. 1998. Expression of *Xa1*, a bacterial blight-resistance gene in rice, is induced by bacterial inoculation. Proc. Natl. Acad. Sci. U.S.A. **95**: 1663-1668.

Yu, Y.G., Buss, G.R., and Saghai Maroof, M.A. 1996. Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. Proc. Natl. Acad. Sci. U.S.A. **93**: 22751-11756.

Yu, Y., Tomkins, J.P., Waugh, R., Frisch, D.A., Kudrna, D., Kleinhofs, A., Brueggeman, R.S., Muehlbauer, G.J., Wise, R.P., and Wing, R.A. 2000. A bacterial artificial chromosome library for barley (*Hordeum vulgare* L.) and the identification of clones containing putative resistance genes. Theor. Appl. Genet. **101**: 1093-1099.

Zhou, T., Wang, Y., Chen, J.-Q., Araki, H., Jing, Z., Shen, J., and Tian, D. 2004. Genome-wide identification of NBS genes in japonica rice reveals significant

expansion of divergent non-TIR-NBS-LRR genes. Mol. Genet. Genomics. [Online]. Available from http://springerlink.metapress.com/media/F01690QGQGCRWD6VKYVM/Contributions/G/N/Q/X/GNQXGUKBK0MBV2PR_html/fulltext.html

VITAE

Jafar Mammadov was born on January 19, 1967 to parents Akif and Izabella Mammadov. Jafar graduated from Baku High School # 174 in May 1984. In September 1984, he began college at Azerbaijan Agricultural Academy, Ganja, Azerbaijan. After spending 5 years at the Department of Agronomy, Jafar earned a Bachelor of Science and Master of Science in Agronomy. In 1989, Mr. Mammadov joined Ph.D. program in Genetics and Plant Breeding at Azerbaijan Academy of Sciences and after 5 years successfully earned his Kandidat Nauk degree (Ph.D. equivalent in the former Soviet Union) in Genetics and Plant Breeding. In 1999, Jafar earned his second MS degree, namely, Master of Science in Environmental Sciences and Policy from Central European University, Budapest, Hungary. He joined the Ph.D. program in Crop and Soil Environmental Sciences at the Crop and Soil Environmental Sciences Department, Virginia Tech, Blacksburg, VA in August 1999.