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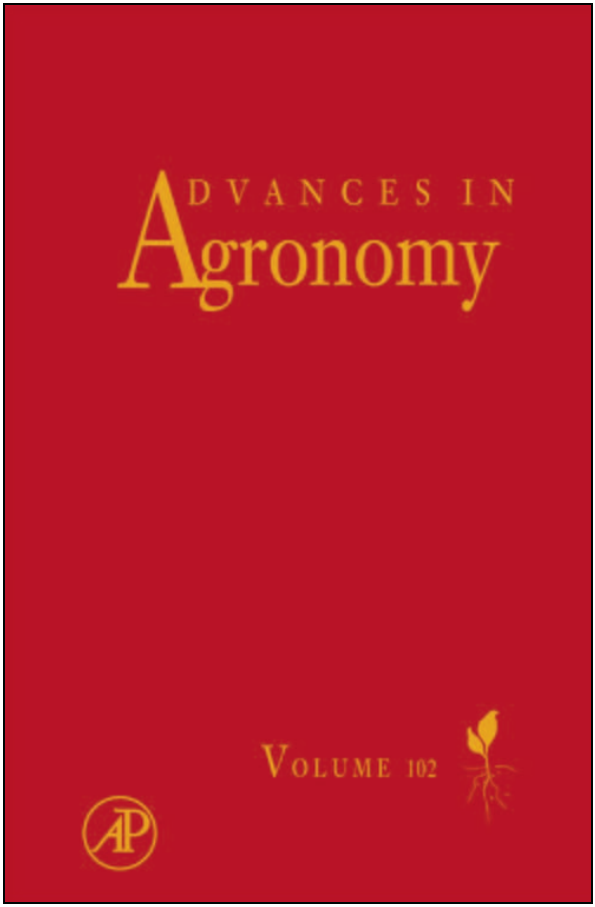

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CHAPTER SIX

Radiation Hybrid Mapping in Crop Plants

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Abstract

A number of recombination-based and physical mapping methods have been developed in order to study and understand the genomic organization of plant species. Of these methods, physical mapping methods provide the best correlation between position on a map and actual physical location on the chromosome. In this review, we discuss differences between maps developed on the basis of recombination and those developed independent of recombination. In the latter method, termed commonly as physical mapping, we discuss methods that have been employed in a number of agriculturally important plant species including rice, the legume model *Medicago*, tomato, soybean, barley and wheat, and then focus on the radiation hybrid method of physical mapping. After a brief overview of the radiation hybrid methods employed in mammalian species, we discuss radiation hybrid mapping for maize, barley, cotton and wheat in detail.

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1. Introduction and Overview

Mapping determines the linear order of genes and/or molecular markers in the material at hand, and can be performed at several levels of detail (resolution) that traditionally falls into two broad categories: genetic or linkage mapping and more detailed physical mapping. A linkage or genetic map of any plant or animal species denotes the linear order of markers on a particular chromosome, which is determined by the number of crossover events and recombination frequencies between markers (Sturtevant, 1913). During segregation if a block of genes or chromosomal segments do not assort independently, they are described as being linked. This is the basis on which a genetic map is developed. Recombination among polymorphic loci and the likelihood that recombination events occur between two points of a chromosome depends in general on their physical distance: the closer they are located to each other, the more they tend to stay together after meiosis. With the increase of the distance between these points on the chromosome, the probability for recombination increases and genetic linkage tends to disappear. This is why genetic linkage can be interpreted as a measure of physical distance. However, across the whole genome, the frequency of recombination is not constant as it is influenced by chromosome structure which varies across the length of chromosomes.

There have been two basic requirements for genetic mapping: a population of individuals or gametes exhibiting marker segregation, and markers that can identify segregating components. Mapping population development is dependent on the modes of reproduction of the species under study. In self-fertilizing plants, mapping populations can consist of F_2 plants, recombinant inbred lines (RILs), backcross (BC)-derived populations, introgression lines assembled in different individuals, or doubled haploid (DH) lines. In the case of crossfertilizing species, mapping populations can be derived from heterozygous parental plants such as F_1, intermated F_2, or BC lines. DNA polymorphism is usually sufficient to function as a molecular marker in mapping. These DNA sequence differences are revealed in the form of restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), microsatellite or simple sequence repeat polymorphisms (SSRs), single-strand conformation polymorphisms (SSCPs), and single nucleotide polymorphisms (SNPs). Genetic maps have been constructed in many crop plants using a single or a combination of marker systems (Phillips and Vasil, 2001).

Although genetic mapping can be used to ascertain the relative location of genes/markers along a chromosome, frequencies of recombination can vary along a chromosome. Thus, it is important to remember that linkage maps are not always the best possible representation of the physical distance among

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genes or markers. Increasingly, genomics research has necessitated the development of physical maps that more accurately reflect the actual position of molecular markers and/or genes on the chromosome(s) and the actual distance in nucleotides between them. In this sense, physical maps provide a high-resolution view of the organization of a given chromosome. In addition, independently derived physical and/or genetic maps can be used to assess the completeness and the integrity of whole-genome sequences. Therefore, the use of a combination of genetic and physical mapping methods has been very useful for advancing knowledge on the number and distance between genes of interest as well as to better understand the topography and organization of the genome.

2. Physical Mapping

Genetic maps of moderate resolution can be used to follow the inheritance of any chromosomal region. With an adequate number of markers, it should be possible to survey the entire genome of a species, segment by segment. There are several basic and applied uses of genetic maps, for example, (a) study genomes of related species with common markers, (b) tag important traits of interest with associated markers and follow the transmission and selection of the former in breeding cycles, and (c) with the advent of recombinant DNA technologies, genetic mapping can be carried to its logical conclusion, positional cloning (isolation) of a gene solely on the basis of its chromosomal location without regard to its biological function. On the other hand, a map generated by recombination is rarely sufficient for starting the sequencing phase of a genome project for two main reasons. Firstly, genetic maps have limited resolution because they depend on the number of crossovers. In most eukaryotes, it is simply not possible to obtain a sufficiently large number of meioses to attain resolutions that are greater than 1 Mb. Secondly, genetic maps often have limited accuracy in areas of reduced recombination. Thus, it is difficult to order closely linked markers. These limitations of genetic mapping may be overcome by analyzing more meioses and/or adding more markers. Still, the use of some form of physical mapping in addition to genetic mapping will be necessary before the start of large-scale DNA sequencing.

Physical mapping can be performed at various levels of resolution. Classically, physical mapping involved determining the location of a gene or a marker relative to features of a chromosome such as heterochromatic knobs (McClintock, 1929), polytene chromosome bands (Bridges, 1935), or a specific chromosome segment (Jiang *et al.*, 1993). In wheat, for example, genes or markers have been mapped to chromosomes, chromosome arms, and chromosome segments or bins using aneuploids (e.g., nullisomics,

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monosomics, trisomics, ditelosomics) and a collection of deletion lines (Endo, 1990; Qi and Gill, 2001). Deletion lines have been extensively used in wheat for localizing a large number of genes onto different chromosome bins (Hossain *et al.*, 2004a; Linkiewicz *et al.*, 2004; Miftahudin *et al.*, 2004; Munkvold *et al.*, 2004; Peng *et al.*, 2004; Qi and Gill, 2001; Qi *et al.*, 2004; Randhawa *et al.*, 2004). A more direct way to localize a gene/marker to a chromosomal region involves the use of fluorescent *in situ* hybridization or FISH (Albini and Schwarzacher, 1992), and chromatin fiber FISH (Florijn *et al.*, 1995). Although these methods have been useful to place genes onto chromosome regions, their resolution has been limited to several Mb of DNA.

To attain physical maps of greater resolution, maps based on large-insert DNA clones have been constructed. The reconstruction of chromosomes or genomes from large-DNA insert libraries has been an active area of genomic studies in many species. In humans (McPherson *et al.*, 2001) and several model species (Chang and Karin, 2001; Chen *et al.*, 2002; Gregory *et al.*, 2002; Hoskins *et al.*, 2000; Marra *et al.*, 1997; Tao *et al.*, 2001), the importance of physical maps in large-scale genome sequencing as well as transcript mapping, positional cloning, comparative genome analysis, and geneknockout studies have been well documented (Chaveroche *et al.*, 2000). Physical mapping by fingerprint analysis and end sequencing of large-DNA insert clones does not require a segregating population, and hence allows the construction of genome maps even for species in which development of genetic maps is impossible owing to the absence of appropriate mapping populations.

2.1. Examples of physical mapping in plant species

A wealth of physical mapping resources has been generated in major plant species of which several examples are discussed below. Rice has emerged as a model species for studying cereal genomics because of its relatively small genome size (~400 Mb) (Arumuganthan and Earle, 1991). In rice, *Oryza sativa* subspecies *japonica* and *indica* are both widely cultivated and equally important to human food supplies. The genomes of both subspecies have been sequenced. The complete sequencing of the *japonica* genome was the objective of the International Rice Genome Sequencing Project (IRGSP) (Takuji and Burr, 2000). IRGSP (2005) took a clone-by-clone strategy to sequence the *japonica* genome, while a whole-genome shotgun approach was used in sequencing the *indica* genome (Yu *et al.*, 2002). To sequence the rice genome using a clone-by-clone approach, a BAC-based physical map for the rice genome was a prerequisite. A total of 73,728 BAC clones were fingerprinted using a single restriction enzyme, with 88% clones successfully fingerprinted, representing 20× genome coverage (Chen *et al.*, 2002). To develop an integrated physical and genetic map of the rice genome, BAC

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contigs were anchored to the rice genetic map. This was accomplished with a combination of approaches, including anchoring genetic markers into BAC clones, contig end walking with overgo primer pairs, and integration of a portion of the Monsanto draft rice genome sequence data into the physical map (Chen *et al.*, 2002). Such analyses allowed the verification of the physical map to enhance its accuracy.

The legume model *Medicago truncatula* which is a relative of the forage crop alfalfa (*M. sativa*) is currently being sequenced. This species with a 450 Mb genome is autogamous, demonstrates prolific seed production, and has a number of tools that serves as a good model for legume molecular genetics and genomics. Kulikova *et al.* (2001) constructed a molecular cytogenetic map of *M. truncatula* based on a karyogram of pachytene chromosomes. This map is especially important because it integrated genetic maps with FISH-based mapping of BAC clones. At least two to five BAC clones were mapped onto each genetic linkage group, thus providing a link between the genetic and physical maps developed via FISH. The resolution for the euchromatic portion of linkage group 5 was estimated to be about 60 kb for its cytological counterpart. This work also demonstrated the use of chromosomes at the meiotic prophase stage for karyotyping rather than chromosomes in meiotic metaphase. Over 190,000 *Medicago* expressed sequence tags (ESTs) have been generated to date (http://www.medicago.org/MtDB2/ and http://www.tigr.org/tdb/tgi/plant.shtml), and hybridization of ESTs to BAC clones have helped in the linking of genes to specific BAC clones. These include the hybridization of resistance gene analogs (RGAs) and RFLP clones from related legumes. BAC end sequences (BES) and subsequent contiging in *Medicago* (ftp.tigr.org/pub/data/m_truncatula) with detailed links to genetic and physical maps are very useful to eventually aid in the linking of genomic sequence data that is currently being generated.

Tomato (*Solanum lycopersicum*) is a very important solanaceous crop second only to potato (*S. tuberosum*) (Gould, 1992). With a chromosome number of 12, it carries the smallest haploid genome size (953 Mb) in the Solanacea family (Arumuganthan and Earle, 1991). Tomato has been a subject for classical genetic researchers from the beginning of the twentieth century and has excellent morphological maps and high-density molecular maps that contain more than a thousand markers (Broun and Tanksley, 1996; Rick and Yoder, 1988; Tanksley *et al.*, 1992). A large collection of well-characterized phenotypic mutants and near-isogenic lines are available in tomato to help in gene identification and characterization, and to connect phenotypes to sequence data. Muhammad *et al.* (2000) developed a 15× BAC library from tomato with 129,024 clones and an average insert size of 117.5 kb. BES from 1490 clones were also generated so as to develop a framework of sequence-tagged connectors (STCs) that would aid in whole-genome sequencing.

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Soybean is one of the world's most important legume crops with a genome size of 1115 Mb/1C (Arumuganthan and Earle, 1991). Approximately 40–60% of its genome contains repetitive sequences and is heterochromatic (Goldberg, 1978; Gurley *et al.*, 1979). Soybean has been classified as a recently diploidized tetraploid species and it has been determined that there are duplicated segments with an estimated six copies per gene (Shoemaker *et al.*, 1996). A number of genetic and physical mapping resources have been developed for this species which include linkage (Cregan *et al.*, 1999a,b; Iqbal *et al.*, 2001; Keim *et al.*, 1997; Lark *et al.*, 1993; Shoemaker and Specht, 1995) (http://soybase.agron.iastate.edu) and physical maps (Marek *et al.*, 2001). Wu *et al.* (2004) developed an integrated physical map derived from approximately 78,001 clones which represents approximately 9.6 haploid genomes. This map integrated large-insert BAC and BIBAC libraries (developed from three soybean cultivars and high-throughput fingerprinting), with the existing soybean composite genetic map (Marek *et al.*, 2001) and included 781 physical map contigs spanning approximately 59.5% of the total genome size equivalent to 663 Mb.

Cultivated barley (*Hordeum vulgare* L.) is a diploid crop species with an estimated haploid genome size of 5000 Mb (Arumuganthan and Earle, 1991). During the past few years, considerable progress has been made in the establishment of public genomic resources. These include large-insert BAC libraries (Yu *et al.*, 2000), several widely used genetic maps (Behn *et al.*, 2004; Cooper *et al.*, 2004; Hearnden *et al.*, 2007), a large EST collection (Zheng *et al.*, 2004), and Affymetrix arrays to examine genome-wide profiling of gene expression (Shen *et al.*, 2005). A barley BAC library has been constructed from the cultivar Morex containing 313,344 clones with average insert size of 106 kb, representing 6.3 haploid genome equivalents (Yu *et al.*, 2000). The BAC clones from this library have been widely used for a wide range of genomics research, including sequencing large-insert genomic regions for comparative analysis and characterization of genome organization. Despite these efforts, genome-wide physical mapping of the barley genome has not been reported. However, the Morex BAC library has been used to develop physical maps for specific regions. For example, screening the BAC clones using a set of RGA identified 121 RGA-containing clones representing 20 different regions of the genome with an average of 6.1 clones per locus (Yu *et al.*, 2000).

Common wheat (*Triticum aestivum L.*) is an allohexaploid crop species with an estimated haploid genome size of 16,000 Mb (Arumuganthan and Earle, 1991). To develop a physical map of the wheat genome, two approaches have been pursued. In one, a global fingerprinting method (fingerprinted contigs or FPC) is being utilized to construct a physical map of one of wheat's genomes (the D-genome) derived from *Ae. tauschii* (Luo *et al.*, 2003). To date, 7447 contigs and 5304 singletons associated with 251,586 BAC/BIBAC clones, and 520 RFLP and 1700 EST markers have

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been assembled (http://wheat.pw.usda.gov/PhysicalMapping). Another approach, utilizing the unique collection of wheat cytogenetic stocks, aims at dissecting the genome one chromosome at a time (Safar *et al.*, 2004). Through this latter approach, individual wheat chromosomes are sorted by flow cytometry and cloned into chromosome enriched BAC libraries. These individual chromosome BAC libraries can then be fingerprinted to develop contig maps of individual chromosomes. As of August 2006, 1997 contigs associated with 67,968 BAC clones for chromosome 3B, with an estimated coverage of 800 Mb (80% coverage), had been developed. Nearly 20,000 BES had also been derived from this library with 2000 BES providing unique retroelement junctions for mapping (10% success rate) (Feuillet, 2006). Only a fraction of these BES (700) yielded a polymorphism and could be mapped genetically. Repetitive and middle repetitive sequences, being significant components of most plant genomes, tend to complicate assembly of BAC contigs by FPC and limit progress toward development of a complete BAC-based physical map. Therefore, other methods that can aid in tackling the large genome size and repetitive nature of most plant genomes are needed.

As seen above, a number of physical mapping resources in plants have been developed that have greatly aided in the ability to correlate genetic to actual distances. Since development of physical mapping resources like the BAC- or BIBAC-based libraries requires the random shearing or digestion of genomic DNA and subsequent assembly, and because the assembly of overlapping sequences can be limited in most plant genomes by the presence of repetitive sequences there is a need for another method that will facilitate assembly. To avoid fragmentary, segmented and therefore incomplete physical maps, a recombination-independent strategy can be used to order BAC clones via molecular markers. One such method that has been discussed earlier is deletion-based mapping of molecular markers to large chromosomal bins. A limitation of this latter approach is the verification of order of molecular markers within large chromosomal bins. A very successful approach that has been used in a number of nonplant and a few plant systems is radiation hybrid (RH) mapping which has been effective in the high-resolution ordering of molecular markers.

3. Radiation Hybrid Mapping in Nonplant Species

Radiation hybrid mapping involves the use of radiation-induced chromosomal breakage and marker segregation to reconstruct marker order. Physical distance is calculated based on coretention frequencies between chromosomal fragments and molecular markers. RH mapping is

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recombination independent and was first described by Goss and Harris (1975, 1977) and subsequently by Benham *et al.* (1989) and Cox *et al.* (1990). In this approach, donor cells are irradiated and fused with recipient cells and irradiated chromosomes were studied in the background of the recipient's cell (radiation hybrids). A panel of radiation hybrids with random chromosome breaks in the donor genome can be used to precisely place and order molecular markers or genes on a particular chromosome or entire genome (Cox *et al.*, 1990). Markers that are physically close on the chromosome experience less chromosomal breakages during irradiation and will tend to be present together on the same fragment within the same hybrid cells of a panel than the distantly positioned markers (Cox *et al.*, 1990). In this process, a test genome is fragmented into desired resolution and fused with recipient cells to construct an RH mapping panel. The RH mapping panel is assayed usually with PCR-based molecular markers and markers that are present or absent are recorded. Subsequently, the resulting data set is used to determine the order and spacing of markers along each chromosome (Boehnke *et al.*, 1991). In RH mapping, different doses of radiation can be used to construct maps with varying levels of resolution. Although RH mapping is conceptually similar to meiotic mapping, the later cannot easily be used to construct high-resolution maps and is dependent on the frequency of recombination and use of polymorphic markers.

Goss and Harris (1975) used RH mapping to localize genes on the human X chromosome. X-ray irradiation of a human cell line containing a single X chromosome resulted in random breakage. The fragmented X chromosomes are reassembled during DNA repair, and the resulting segmented chromosomes are recovered by fusion of the irradiated cell line with a nonirradiated rodent cell line. Although a specific gene in the starting cell line may be used to facilitate hybrid selection, the majority of the radiation-induced fragments are transferred nonselectively. Somatic cell hybrids with human subchromosome fragments were used to develop a high-density map of the human genome using the RH mapping approach (Olivier *et al.*, 2001). Given that a single subchromosome fragment stock may contain multiple noncontiguous pieces of a chromosome, it is not possible to use any single stock as a mapping reagent. However, the frequency of radiation-induced breakage between two markers or loci can be used as a measure of the distance between these markers or loci. Physically close markers will show little breakage and more distant markers will show greater breakage. Thus, a population of subchromosome fragment stocks can be used to calculate the frequency of breakage between all pairwise combination of markers or loci to construct a map analogous to a meiotic linkage map (Cox *et al.*, 1990).

RH mapping has been used to develop a high-resolution (100 kb) contiguous map of human chromosomes with ~41,000 ordered sequence tagged sites (STSs) that includes 20,000 unique human genes (Schuler *et al.*, 1996;

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Stewart *et al.*, 1997). Subchromosome fragments in radiation hybrids have been excellent vehicles for the production and characterization of libraries enriched in DNA markers and genes for a particular segment (Ledbetter *et al.*, 1990). The success of RH mapping and its application to the human genome has expanded its application in resolving the order of tightly linked loci in mouse (McCarthy *et al.*, 1997), pig (Hawken *et al.*, 1999), dog (Vignaux *et al.*, 1999), zebrafish (Kwok *et al.*, 1999), and rat (Watanabe *et al.*, 1999).

4. Radiation Hybrid Mapping in Crop Plants

The increasing repository of genomic resources such as genetic markers, increasing number of molecular markers; integrated genetic and physical maps; large-insert libraries and BAC contigs; and the availability of genomic sequences have expanded the application of RH mapping for localizing and identifying genes with potential application in plant improvement. The potential of this technology has been explored in maize (Kynast *et al.*, 2002; Riera-Lizarazu *et al.*, 2000), barley (Wardrop *et al.*, 2002, 2004), and cotton (Gao *et al.*, 2004). In wheat, this technology has been successfully used for mapping a specific gene (Hossain *et al.*, 2004c) and developing a high-resolution map of a particular chromosomal region in durum wheat (Kalavacharla *et al.*, 2006).

4.1. Radiation hybrid mapping in maize

The first attempt to implement RH mapping in plants was described by Riera-Lizarazu *et al.* (2000) where a genetically buffered (allohexaploid) oat (*Avena sativa*) line carrying a single maize chromosome 9 (monosomic maize chromosome 9 addition line) was used as source material for the production of chromosome-specific radiation hybrids. A monosomic maize chromosome 9 addition line seed was treated with γ-rays (30–50 krad), subsequently planted, and surviving plants were self-pollinated. Progeny from self-pollination (the radiation hybrids) possessing maize chromatin including plants with an apparently normal maize chromosome 9 were recovered. Plants with various maize chromosome 9 rearrangements (intergenomic translocations, deletions, and a combination of both) were selected for further analysis. Radiation hybrids with a range of 1–10 radiation-induced chromosome breaks were identified. Since the average number of chromosome breaks per line was 3, it was estimated that a panel of 100 radiation hybrids would permit mapping this 191-Mb maize chromosome at the 0.6-Mb level of resolution.

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The average marker retention frequencies in the oat–maize radiation hybrids ranged from 75% to 85% (Riera-Lizarazu *et al.*, 2000). These marker retention frequencies were higher than the 10–50% of donor genome retention that are typically cited as being best suited for RH mapping in mammalian systems (Stewart *et al.*, 1997). Thus, the level of chromosome breakage and mapping resolution in the oat–maize radiation hybrid system may be lower than that attainable in reports using human/rodent radiation hybrids. This difference has been attributed to the way mapping panels are developed. Human/rodent radiation hybrids are the product of *in vitro* rescue by cell fusion of lethally irradiated donor cells with nonirradiated recipient cells whereas the oat–maize radiation hybrid system involves live self-propagating plants derived from irradiated seed. Thus, the need for morphogenetic capacity and fertility may limit the level or mode of chromosome breakage that is tolerated and sexually transmitted. On the other hand, a recent study with durum wheat, *Triticum turgidum*, that is discussed later (Kalavacharla *et al.*, 2006), suggests that high mapping resolutions can be obtained. Another benefit of working with live and fertile plants is the availability of material for comprehensive mapping and future use. Vales *et al.* (2004) showed that most maize chromosome 9 rearrangements (simple or complex) can be maintained in subsequent generations by self-fertilization.

4.2. Radiation hybrid mapping in barley

Barley RH panels were developed by fusing irradiated transgenic barley protoplasts, harboring the bar transgene as selectable marker, and conferring resistance to bialaphos, with untreated tobacco protoplasts (Wardrop *et al.*, 2002). In this strategy, the barley protoplasts were first irradiated with X-rays with different dosages, prior to electrofusion, to induce varying amounts of chromosome fragmentation. Putative fusion hybrids were selected by culture in a medium containing bialaphos. Hybrid calli from fusion protoplasts carrying the bar transgene were maintained under selection up to 12 weeks to yield enough tissue for DNA preparation. Although hybrid production was feasible, the efficiency was low compared to the technique used to generate mammalian RH panels (Olivier *et al.*, 2001). It was suggested that multiple fusion experiments be conducted to generate a suitable number of putative hybrids for RH panel construction. The experimental results showed that exposure of cells to an X-ray irradiation dose of 5 krad generated the largest number of putative hybrids. About 200 putative calli exhibiting resistance to bialaphos were generated from 10 fusion experiments. Among them, 40 calli were verified to be of RH status as assessed by PCR for the presence of the bar gene. Validation of the RH panel was also carried out by studying coretention of a marker, Xpsr145, which was genetically linked (3.7 cM) to the bar gene on chromosome 5H. This

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marker was retained in fusion hybrids at a frequency of 77%, confirming the physical linkage of Xpsr145 to the bar gene.

To evaluate the genome coverage of the RH panel, somatic hybrids were characterized using 35 barley-specific microsatellite markers, which offered genome-wide coverage and gave no amplification with recipient tobacco DNA. The marker retention frequency (%) was calculated based on the presence and absence of individual markers in the RH panel. In the barley RH panel, the average marker retention frequency, which is the number of hybrids retaining individual markers, was ~26%. Although no statistically significant differences in marker retention were found between the different linkage groups, a number of individual loci did appear to be retained at both very low and very high frequencies. Those with high retention frequency could be explained by the possibility that they might be responsible for the specific biological functions associated with the survival of hybrid calli. In addition, it is not surprising that markers that were tightly linked to the bar gene exhibited a high retention rate.

The first barley RH panel consisting of 40 hybrids provided a new resource in terms of physical mapping of the whole barley genome. One technical challenge of RH panels generated by protoplast fusion is the low efficiency associated with hybrid production. Furthermore, the technique can only been applied to the plant species for which efficient protoplast isolation and fusion have been well developed.

4.3. Radiation hybrid mapping in cotton

There have been numerous efforts devoted to the development of genetic linkage maps in cotton, *Gossypium hirsutum* (Lacape *et al.*, 2003; Mei *et al.*, 2004; Reinisch *et al.*, 1994; Shappley *et al.*, 1998; Ulloa and Meredith, 2000; Yu *et al.*, 1998; Zhang *et al.*, 2002). Since linkage groups in cotton exceed the chromosome number ($n = 26$), there is a need for the integration of linkage groups to actual physical locations, and thus chromosomes in the genome. Additionally, since there is a difficulty in correlating information provided by various genetic linkage maps, there is a need for the development of detailed physical maps that would aid in this effort. Traditional RH mapping has dealt with one single chromosome at a time, but to overcome this limitation, Gao *et al.* (2004) used whole-genome radiation hybrid mapping (WGRH) in cotton, which is a modified method of RH mapping, first used by Walter *et al.* (1994). In this method, the RH material is generated from a diploid cell line instead of a somatic single-chromosome addition line. This is especially useful if the intent is to construct maps of all of the chromosomes of the species of interest. Although useful, this approach is limited by the ability to rescue the irradiated material. In cotton, Gao *et al.* (2004) irradiated pollen from cultivated cotton (*G. hirsutum*) with 5 krad of γ-rays, and then rescued by *in vivo* fertilization with egg cells from

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the related species *G. barbadense*. This method termed wide-cross RH (WWRH) mapping demonstrated the first use of RH mapping in cotton and permitted the placement of 102 SSR markers to chromosome segments. These researchers tested a wide range of radiation dosages and found that the F_1 seeds that were obtained from the wide cross after irradiation were viable at the lower doses (1.5 and 5 krad) but were unviable at the higher dosages (15 and 30 krad). To assay for deletions, 22 plants each were randomly selected from the two lower doses and screened with 33 SSR markers. It was seen that the 5-krad panel showed greater number of deletions as well as more variation in the type of deletion (with respect to size or chromosomal locations). The authors also found that the marker retention frequency was high in the 1.5-krad lines which coincided with the lower incidence of deleted markers. The retention frequency for individual molecular markers ranged from 77% to 100% with an average across all assayed loci at 93%. Therefore, in this work, the 5-krad panel performed best, and the authors concluded that the optimal RH dosage could be between 5 and 15 krad. Given these results, the researchers generated additional 5-krad RH lines and used a total of 93 of the 101 individual lines to use as a WWRH panel. To assay the lines for the nature and amount of deletions, the authors used markers from cotton linkage group 9 (LG9) because of the even spread of eight SSR markers across this LG. When assayed with these eight markers, 20 of the 101 lines were identified as carrying deletions in the region represented by this LG and eight SSR markers. A clear use of the RH mapping effort in breaking linkages observed during recombination-based map construction was demonstrated by the cotton WWRH panel. The cotton SSR markers BNL0625 and BNL2805 cosegregated in the recombination-based linkage map but were separated in the two WWRH lines GH6550 and GH6707. Additionally, molecular markers that map separately onto two cotton linkage groups (LG9 and LG13) were seen to map onto the same RH syntenic group because of the use of the WWRH panel.

4.4. Radiation hybrid mapping in wheat

The availability of the appropriate cytogenetic material, which will help assay for deletions due to irradiation, is a very important component of RH mapping. The dosage that is used for RH mapping needs to be balanced and adjusted to aid in the recovery of the maximum number of viable plants containing deletions. Another critical feature that must be considered is the ability to separate out portions of the genome and assay these portion(s) in an independent fashion that will enable the construction of a high-resolution map of that area. Wheat, as indicated earlier, is uniquely suitable for RH mapping because of the vast collection of cytogenetic stocks. An alloplasmic durum wheat line which contained the nucleus from cultivated

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durum and the cytoplasm from *A. longissimum* (S. & M.) carrying a portion of hexaploid wheat chromosome 1D was identified and characterized by Hossain *et al.* (2004b) in their analysis of the scs^{ae} gene. This gene is responsible for compatibility between the nucleus and cytoplasm in wheat lines which contain a nucleus from one species and the cytoplasm from another species. Therefore, this line is uniquely suitable as a test case for RH mapping in wheat because of the presence of this D-genome fragment in a AB genome background, as well as the target of analysis of the phenotype conferred by the scs^{ae} gene (plump seed contain the gene, whereas shriveled seed do not contain the gene). Hossain *et al.* (2004c) used this line to demonstrate the feasibility of RH mapping in wheat by using γ-irradiation of seeds. The male-sterile hemizygous (lo) scs^{ae-} line was crossed with the durum wheat line LDN16. Seed from this cross segregated both plum seed and shriveled seed. Plump seed (which therefore contain the scs^{ae} gene) were then irradiated with 35-krad γ-rays, after which they were germinated and planted in the growth room. The RH_0 plants derived from these treated seed were again crossed to the euplasmic durum wheat line LDN16 to obtain RH_1 seed. Plump seed from this cross were selected and 87 RH_1 plants were grown in the greenhouse, and used for extraction of DNA and analysis using molecular markers that were specific to the homeologous group 1 of chromosomes of wheat. This set of 87 durum wheat RH lines carrying deletions in the 1D chromosome constitutes the durum wheat radiation hybrid-1D (DWRH-1D) panel. These authors used a combination of RFLP, SSR, and EST markers. The RFLP markers were selected based on the order on genetic and physical maps, while the SSR markers genetically mapped onto chromosome 1D. The EST markers were selected based on their assignment to the chromosomal deletion breakpoints of chromosome 1D as part of the wheat EST genomics project (http://wheat.pw.usda.gov/NSF). Hossain *et al.* (2004c) observed that the use of radiation doses exceeding 40 krad resulted in a dramatic reduction in survival and plant vigor. It was also observed that 60% of the RH_1 plants carried deletions of molecular markers with number of markers lost ranging from one to six, with 69% (27 of 39 markers) of the latter identifying deletion breakpoints which ranged from 1 to 11. Since the alloplasmic wheat line carries a portion of the 1DL chromosome that carries the scs^{ae} gene, Hossain *et al.* (2004c) estimated the size of this portion to be approximately 464.8 Mb (excluding the missing telomeric portion of 1DL), and therefore the average distance between the radiation-induced breaks for the 1D portion of this line is estimated to be ~5.3 Mb. The average marker retention frequencies for this population with this set of molecular markers used ranged from 87% to 100%. There were two regions in the RH population which retained all molecular markers, one of which is of particular interest to the isolation of the scs^{ae} gene. A region containing four molecular markers in the short arm of 1D (which might contain a factor that

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is important for seed plumpness) and the region of the 1DL4–1DL2 deletion breakpoint. This latter retention of molecular markers is likely due to the selection for the *scs*^{ae} gene in the scheme to develop the RH population. Therefore, this method of physical mapping in wheat allowed the localization of the *scs*^{ae} gene and serves as a model by which future wheat genes of interest may be localized.

Since Hossain *et al.* (2004c) demonstrated the initial use of RH mapping in wheat using a limited number of molecular markers, Kalavacharla *et al.* (2006) set out to develop a high-resolution RH map of the chromosome 1D region that carries the *scs*^{ae} gene using the DWRH-1D panel by expanding the number and types of molecular markers. A total of 378 markers were used for this analysis. This marker set included 209 AFLPs, 134 ESTs, and 24 RFLP and SSR markers that were previously localized onto the group 1 homeologous chromosomes. Therefore, this group of molecular markers, believed to be derived from both gene-rich and gene-poor regions, provided even more coverage of chromosome 1D.

The analysis of the DWRH-1D panel with the 378 molecular markers showed that 77% of the 87 lines used in this study lost at least one marker and that anywhere from 18 to 134 markers were missing per individual line, which meant that of the 378 total markers, ~96% were lost at least once. This study also detected the deletion of at least one marker that was determined to be retained by Hossain *et al.* (2004c). The EST marker BE444505 which was previously placed at the proximal end of the long arm of 1D. The average marker retention frequencies seen across the DWRH-1D panel was estimated to be around 74%, and is lower than the Hossain *et al.* (2004c) study since AFLP loci that were retained were excluded from analysis as they did not provide useful mapping information. The retention frequencies that were observed are similar to that observed in the cotton and maize RH panels. These high retention frequencies may be due to the use of seed or pollen as the target of irradiation in the plant systems, compared to the use of somatic cells used in the mammalian and nonmammalian systems where the frequencies of retention have ranged from 5% to 45%. Since 368 of the 378 markers used in this study detected at least one break, Kalavacharla *et al.* (2006) estimated the resolution of the RH map in terms of the average distance between breaks. Hossain *et al.* (2004c) had identified 88 breaks using 39 markers, while the subsequent study with 378 markers showed an increase in the number of obligate chromosome breaks by 26-fold, thereby detecting 2,312 breaks. In the prior study, the RH map resolution (size of chromosome/total number of breaks) was estimated to be ~5 Mb/break, while the Kalavacharla *et al.* (2006) study showed a resolution of ~199 kb/break or a 27-fold increase.

Given the size of the chromosome 1D region of interest (464 Mb) and the estimated resolution of ~199 kb/break, >2300 molecular markers would need to be assayed to completely cover the chromosome. Since only 378

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markers were used in this study (16% of total number of markers needed), the authors estimated that there would be a number of linkage groups and unlinked markers that would be seen after analysis. At the two-point LOD of 4, five major linkage groups consisting of a total of 158 markers, and 14 linkage groups with 2–4 markers totaling 33 additional markers were observed, while 187 markers were shown to be unlinked. Interestingly, four of the major linkage groups consisted entirely of AFLP markers, whereas one major linkage group consisted entirely of EST markers, and these linkage groups covered a total of 3341 $cR_{35,000}$ (defined as a unit of distance for a RH panel generated with 35 krad of radiation). The comprehensive linkage map generated at a lower confidence of a two-point LOD of 2.0 included all the markers used in the study and comprised a total length of 11,737 $cR_{35,000}$.

RH mapping can also be used to order molecular markers within large linkage groups. In the wheat deletion bin mapping method, large number of molecular markers can be effectively placed into chromosomal regions, but the order of the markers cannot be effectively determined. To determine the effectiveness of high-resolution RH mapping, Kalavacharla *et al.* (2006) demonstrated that the EST markers derived from the chromosomal deletion bin 1DL2–0.50–0.80 can be ordered effectively. Therefore, high-resolution mapping can be effectively performed for complex plant species using the RH method as demonstrated in durum wheat (Kalavacharla *et al.*, 2006).

5. Prospects of Mapping Genes and Genomes Using RH Mapping

The success of RH mapping depends on the ability of having a system where one can induce a desired level of chromosomal breakage, the ability to recover or isolate these events, and a robust genotyping platform. The ease of developing maps of varying levels of resolution and availability of genomic resources such as a diverse array of molecular markers, genomic sequences, and BAC contigs in major animal and plant species have increased the application of this technology in various ways.

Recent advances in individual chromosome sorting technology (Kubaláková *et al.*, 2005) and isolation and fusion of microprotoplasts containing one or few chromosomes (Saito and Nakano, 2002), have broadened the scope of using the RH method for chromosomal mapping. Development of individual chromosome maps and integration of these maps to dense genetic maps and BAC contigs may help in the sequencing of an individual chromosome and eventually the whole genome especially for plant species with large genomes or higher ploidy levels.

Systematic sequencing of complex genomes involves the clone-by-clone approach, the whole-genome shotgun sequencing (WGS), or a combination

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of both (Green, 2001). For efficient WGS, high-quality physical maps are essential especially for the larger genomes including wheat (Meyers *et al.*, 2004). In addition to recombination-based maps, RH mapping may be an alternative for developing a robust physical map. Molecular markers developed from ESTs usually show lower levels of polymorphism and are therefore not suitable for meiotic mapping. The advantage of the RH strategy is that this technology requires only that a molecular marker be amplified to be placed on an RH map (Bentley and Dunham, 1995). Additionally, sequences of markers used in the RH map can potentially be used to screen BAC libraries and assign contigs to chromosome regions. The potential of using this strategy of ordering BAC contigs using RH maps has recently been explored in wheat (Kalavacharla *et al.*, 2006). RH mapping technology does not depend on meiotic recombination and high-resolution maps can be generated with relatively fewer (100) lines (Green, 2001; Meyers *et al.*, 2004) compared to the thousands of individual lines to achieve a similar level of resolution by recombination-based mapping methods.

Comparative gene mapping studies provide a picture of the evolutionary history of chromosomes and it allows the extrapolation of information from one genome to another. The latter is extremely important and useful for eukaryotic species that are related to a model species with substantial genomics resources and information. Comparative mapping of human and mouse genomes with cattle genomes (Chowdhary *et al.*, 1996; Solinas-Toldo *et al.*, 1995; Womack and Moll, 1986) demonstrated that rearrangement of gene order within segments of conserved synteny is very common and must be addressed for effective trans-species shuttling of information between the species being compared (Yang and Womack, 1998). Using comparative RH mapping of 24 orthologous genes between human chromosome 17 (HSA17) and bovine chromosome 19, Yang and Womack (1998) revealed internal structural rearrangements between these chromosomes. In wheat, over 8000 ESTs have been localized into chromosome deletion bins spanning all of the chromosomes. However, the order of these ESTs is unknown which limits the use of these gene sequences in comparative gene discovery. RH mapping in wheat has recently shown that ordering of genes in a specific bin is possible (Kalavacharla *et al.*, 2006). Thus, coupling RH mapping to bin mapping information would add value to this resource.

Genome sequencing of both plants and animals involves the assembly of overlapping sequence reads generated from random genomic fragments. To accomplish most of the sequencing efforts, redundant sequencing is required. In plants and animals, reference genomes have been sequenced most extensively, with each base represented by an average of >8 reads (i.e., >8× coverage). Consequently, the genomes of species that comprise large section of phylogenetic trees of major plant and animal remained unexplored. The sequencing of all species belonging to the phylogenetic

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trees of all major plant and animal species is impractical. However, survey sequencing (1× to 2× coverage) of the unexplored plant and animal species can provide enough data to understand genomic infrastructure. It is also possible to generate molecular markers for mapping in the genome of interest, and high-resolution physical maps can be constructed by combining RH mapping technology with survey sequence data. By anchoring mapping data with BAC inserts of the related major species, it is possible to understand sequence organization and structure as well as functional integrity of the unexplored species. The prospect of this strategy has been recently investigated in dog using human and mouse genomes (Hitte *et al.*, 2005) and in the canine genome using the human genome sequence (Sidjanin *et al.*, 2003), and this is a viable strategy that can be explored in plants.

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