GEORGE FEDAK[1], NAM-SOO KIM[2]
[1]Eastern Cereals and Oilseeds Research Centre
Agriculture and Agri-Food Canada
960 Carling Avenue
Ottawa, Notario, K1A 0C6
Canada
[2]Department of Molecular Biosciences
Kangwon National University
Chunchon, Korea 200–701

TOOLS AND METHODOLOGIES FOR CYTOGENETIC STUDIES OF PLANT CHROMOSOMES

A brief overview is presented in advances in cytogenetic methodology and development of aneuploid stocks since the 1920s. The methodologies range from first reports of chromosome numbers of major organisms, the development of chromosome karyotypes, then aneuploid stocks in the major crop plants. Molecular inputs included chromosome banding techniques, molecular marker maps and in situ hybridization methodologies. All of the new techniques greatly increased the degree of resolution obtained from cytogenetic studies.

Historical perspectives

Prior to the 1920s, cytological studies were carried out on biological tissues that were embedded in paraffin, sectioned, and stained (Wilson, 1925; Darlington, 1937). The methods that were in vogue were not sufficiently refined to allow for the detection of such gross morphological features as centromeres, secondary constrictions, and satellites of chromosomes. They merely facilitated the determination of chromosome numbers and permitted detection of approximate size differences among the chromosomes in somatic and meiotic cells of many eukaryotic species. For example, in the genus *Triticum,* Sakamura (1918) showed that the somatic cells of the species *monococcum, turgidum,* and *aestivum* possessed 14, 28, and 42 similar-sized chromosomes, respectively.

During the 1920s and 1930s innovations were introduced which facilitated cytological and karyotypic analyses. In 1921, Belling described a technique for studying meiosis in plant species that involved the squashing of anthers. This method permitted the separation of PMCs and facilitated the spreading of their chromosomes. In 1929, Kagawa, working with *Triticum* and *Aegilops* species, demonstrated that treatment with chloral hydrate before fixing and staining the cells shortened the chromosomes. This made it easier to separate them and study their gross morphological features: centromeres, secondary constrictions, and satellites. Pretreatment with other agents such as alpha bromonaphthalene (Schmuck and Kostoff, 1935), colchicine (O'Mara, 1939), paradichlorobenzene (Meyers, 1945) and cold water (Hill and Myers, 1945) also permitted identification of these chromosomal substructures. By the early 1940s the squashing technique, concomitant with appropriate modifications and pre-treatment, completely replaced the method of microtome sectioning of tissues in chromosome studies using somatic and meiotic tissues of most species (Hillary, 1938; Aase, 1935; O'Mara, 1939).

The squash technique, with appropriate modifications, was also used successfully in chromosome studies of insects, amphibians, and other animals (White, 1954), excluding mammals (Hsu, 1979). Mammalian cytology had to await the innovations of hypotonic solutions (Hsu, 1952), *in vitro* culturing of tissues and cells (Hsu and Pomerat, 1953), and their colchicine pretreatment (Hsu and Pomerat, 1953) for obtaining good spreads of somatic chromosome complements.

Using the innovative procedures the chromosome number in man was first correctly determined to be $2n = 46$ by both Tjio and Levan, and Ford and Hamerton in 1956. In the ensuing years this technique, combined with the use of phytohemagglutinin (Nowell, 1960) to stimulate cell divisions, was applied to karyotyping euploids, aneuploids and individuals with chromosomal abnormalities in numerous mammalian species (see Hsu and Benirschke 1967–1977). The various techniques that have been used to this day for karyotyping hundreds of species using gross chromosomal morphological features are detailed in La Cour (1947), Darlington and La Cour (1960), Sharma and Sharma (1965), and Haskell and Wills (1968).

Standard somatic karyotypes in plant species

In plant species, such as maize (Rhoades and McClintock, 1935), tomato (Rick and Barton, 1954; Rick et al., 1964) and rice (Chu, 1967; Kurata and Omura, 1978) the individual chromosomes (and therefore trisomics) could not be identified in somatic cells using standard straining procedures because the chromosomes were either too small and/or similar in morphology. In most of the plant species, however, at least a few of the chromosomes and trisomics could be identified in standard somatic karyotypes. For example, in barley (Tsuchiya, 1960) and *Petunia axiillaris* (Reddi and Padmaja, 1982), three of the chromosome pairs and trisomics could be identified using standard procedures. In beets (Romagosa et al., 1986) eight of nine chromosomes and trisomics could be identified in the standard fashion and in A. *strigosa,* Rajhathy (1975) was able to distinguish all chromosomes and identify all trisomics from standard somatic karyotypes.

Although numerous karyotypic studies have been carried out since 1939 in several species of the genus *Triticum,* only the species *T. monococcum (AA), T. turgidum (AABB),* and *T. aestivum* (AABBDD) will be reviewed here. Studies by Camara (1943), Coucoli and Skorda (1966), Giorgi and Bozzini (1969b) and Kerby and Kuspira (1988) have shown that there are 14 similar chromosomes in the diploid complement of *T. monococcum*; one ST pair, two M pairs, and four SM pairs. Depending on the accession line studied, either one or two chromosome pairs were found to possess satellites (Camara, 1943; Riley et al., 1958; Coucoli and Skorda, 1966). The karyotype of *T. turgidum* consists of two SAT pairs, two ST pairs, seven SM pairs, and three M pairs of chromosomes (Giorgi and Bozzini, 1969a; Kerby and Kuspira, 1988). Since the A genome has the chromosome constitution given above, the karyotype of the B genome in *T. turgidum* must consist of two SAT pairs, one ST pair, three SM pairs, and one M pair of homologues. Two of these chromosome pairs possess large satellites (Pathak, 1940; Riley et al., 1958) which belong to the B genome (Okamoto, 1957). The satellites in the A genome in *T. turgidum* are suppressed (Riley et al., 1958). At most, four to six of the chromosomes in the somatic complement of this species can be distinguished using standard procedures.

Depending on the genotype studied, either one (Sears, 1954), two (Morrison, 1953), three (Pathak, 1940, Camara, 1943) or four (Kagawa, 1929, Schulz-Schaeffer and Haun, 1961) satellited chromosomes are observed in *T. aestivum*. These belong to the B and D genome (Sears, 1954; Schulz-Schaeffer and Haun, 1961). Camara (1943), Morrison (1953), Sears (1954, 1958), Schulz-Schaeffer and Haun (1961), and Gill (1987) have shown that the chromosomes in the somatic complement of bread wheat are similar in size. For example, Gill (1987) reported that they range in length from 8.4 um for chromosome ID to 13.8 *um* for chromosome 3D. The latter observations also show that (i) except for the chromosome 4A pair, all other pairs in homoeologous groups 1, 4 and 5 are highly heterobrachial (ST), (ii) except for the chomosome 7B pair, all other pairs in homoeologous groups 6 and 7 are M, and (iii) chromosome pairs in homoeologous groups 2 and 3 as well as chromosome pairs 4A and 7B are SM. Even in the best somatic metaphase spreads, only a limited number of chromosomes and chromosome pairs can be distinguished by standard methods.

Chromosome identification during meiosis

Standard staining procedures render nucleoli to be clearly visible at pachytene stage and permit the detection of chromosomes that carry NORs. Moreover, in corn (Rhoades and McClintock, 1935), tomato (Rick and Barton, 1954), and rice (Khush et al., 1984) it is possible to identify each univalent, bivalent, and multivalent association on the basis of its length and chromomere pattern

during pachytene. Thus a chromosome in triplicate in these species is easily identified by examination of the trivalent configuration at pachytene using standard staining techniques.

Banding techniques. In almost all species the usefulness of standard staining procedures, however, has been limited. Although they have facilitated the ascertainment of chromosome numbers and gross morphological features of chromosomes, they have not permitted an accurate and unequivocal identification of all the chromosomes, and therefore the aneuploids, of a species. An exhaustive analysis of the karyotype requires the use of staining procedures that can reveal each chromosome as a specific, unique, and constant pattern of alternating dark and light banding regions, topologically equivalent to the bands in the polytene chromosomes in salivary gland cells of *D. melanogaster.*

Darlington and La Cour (1940) demonstrated that with cold treatment of somatic cells of *Trillium erectum* some regions of chromosomes revealed unique patterns by appearing thinner and less intensely stained than the rest of the chromosomes. The utilization of fluorescent and other dyes together with various modifications in pretreatment of cytological material in the late 1960s heralded a new era of cytogenetics. New and reliable staining procedures were introduced, each of which was capable of revealing a unique banding pattern of the chromosomes of a given species. By 1972 the application of one or another of five major banding techniques (Q, G, R, C, and N) for the purpose of karyotypic analysis came into vogue. These have led to a more precise cytogenetic and phylogenetic analysis of various eukaryotes.

Q-banding

Caspersson and his colleagues in 1968 were first to demonstrate that fluorescent dyes such as quinacrine and quinacrine mustard bind preferentially to certain regions of normal mitotic chromosomes of *Cricetulus griseus, V. faba* and *T. erectum.* As a result, unique patterns of brightly fluorescent regions alternating with non-fluorescent (dark) regions were produced in each chromosome. Weisblum and de Haseth (1972) and Burkholder (1988) have shown that fluorescent dyes interact with AT base pairs and those regions of DNA that are sufficiently AT-rich (70–100 %) fluoresce and appear as bright bands (Q bands). Q-banding permits an identification of all the chromosomes and their homologues in most species. For example, in man all 23 pairs of homologous chromosomes can be distinguished on the basis of their Q-banding patterns (Caspersson et al., 1971). In *Scilla sibirica* all eight chromosome pairs can be identified (Caspersson et al., 1969). Q-banding does not require any pretreatment and is the simplest of all the banding methods. Compared to other banding techniques, it has several disadvantages; the fluorescent bands are not permanent; the technique requires the use of ultraviolet light, and does not stain the ends of chromosomes. As a consequence Q-banding has been used to a limited extent, and since the late 1970s (Pinkel et al., 1988) has been largely replaced by other banding methods. In plants, Q-banding studies have been limited to a few in *Trillium, Scilla, Allium, Crepis, Lilium, Secale,* and *Vicia* (Caspersson et al., 1969; Vosa and Marchi, 1972a; Kongsuwan and Smyth, 1977; Schweizer, 1980; Rowland, 1981).

G-banding

In 1971, Drets and Shaw, Patil *et al.,* Seabright, and Sumner *et al.* independently developed a protocol for animal species whereby each chromosome segment and chromosome revealed a unique pattern of bands. Each of the protocols, by using a variety of treatments before fixing chromosomes and staining with Giemsa, yielded a banding pattern in normal mitotic chromosomes that was similar to the one revealed by the Q-banding technique. The dark regions are the topological equivalents to Q-bands and are called G-bands whereas the light regions are equivalent to the nonfluorescent dark ones revealed with the use of fluorescent dyes (Drets and Shaw, 1971; Dutrillaux and Lejeune, 1975). Application of G-banding methods to prophase and prometaphase chromosomes in animals revealed a larger number of bands than at metaphase which permited more precise karyotyping and cytogenetic analysis (Yunis, 1981; Iannuzzi, 1990).

The basis for G-banding is currently unknown. One plausible explanation is that of Comings (1978) who postulated that prophase and metaphase chromosomes contain a basic chromomeric structure that can be enhanced. This enhancement occurs by inducing some rearrangement of the fibers away from the light bands toward the G-bands, possibly some extraction of light-band DNA with denatured

nonhistone proteins, followed by the marked enhancement of this pattern through the ability of thiazin dyes in Giemsa to side stack on available DNA. Sumner (1982) and Burkholder (1988) have proposed alternate mechanisms.

Although the technique has been attempted in many plant species, G-bands have been generated in the chromosomes of only a few species: *Tulipa gesneriana* (Filion and Blakey, 1979), *Pinus resinosa* (Drewry, 1982), and *Vicia hajsatana* (Wang and Kao, 1988). The failure to produce G-bands in the chromosomes of most plant species, including those in the *Triticeae,* has been attributed to the increased condensation of the plant chromosomes (Greilhuber, 1977; Drewry, 1982). Anderson, et al. (1982), however, failed to show consistent differences in the degree of compaction, based on measurements of lengths and volumes of chromosomes from several plant and animal species. Wang and Kao (1988) demonstrated that improper pretreatment of plant chromosomes alters the organization of their chemical constituents and renders them unresponsive to the *G*-banding procedure.

Reverse (R)-banding

This banding technique was developed by Dutrillaux and Lejeune in 1971. Mild denaturation by heat and subsequent staining of chromosomes with Giemsa or a fluorochrome dye revealed a banding pattern that is the reverse of the patterns produced by the G-and Q-banding methods (Bobrow et al*.,* 1972; Comings, 1973; Dutrillaux et al*.,* 1973). Specifically, if the chromosomes are stained with Giemsa, the dark bands (R bands) produced with this technique are equivalent to the light bands produced by the G-banding technique and vice-versa (Dutrillaux and Lejeune, 1971, 1975). If a flurochrome dye such as acridine orange or olivomycin is used, fluorescent R-banding is the reverse of Q-banding in that the R-bands fluoresce bright green and the non-R-bands show a faint red color (Schweizer 1976; Lin et al. 1980; Schmid and Guttenback 1988). R-banding is particularly useful in the detection of structural rearrangements involving ends of chromosomes in that it stains telomeres as T-bands (Dutrillaux et al. 1973).

R-bands have been detected in only a few plant species e.g., S. *sibirica, V. fava, Allium* spp., none of which belong to the *Tririceae* tribe (Schweizer, 1980; DeumIing and Greilhuber, 1982; Loidl, 1983). Moreover, since the R-bands in these species are few in number and faint in expression, they have not been used for karyotyping and cytogenetic studies.

R-bands can be produced by GC-specific fluorochromes (Schweizer, 1976; Van de Sande et al*.,* 1977; Holmquist et al*.,* 1982), although the mechanism of R-banding is unknown (Burkholder, 1988). The mechanism proposed by Comings (1978) may also explain R-banding if the DNA and proteins in the G-and R-bands are selectively denatured under different conditions of pH, salt concentrations, and temperature.

C-banding

Pardue and Gall (1970) and Jones (1970) independently demonstrated a procedure which with stringent treatment of chromosomes prior to fixation and staining with Giemsa, stained only the regions of constitutive heterochromatin in chromosomes of *Mus musculus*. The regions, now referred to as C-bands, were observed to be proximal to the centromeres of all the chromosomes in this species and have since been demonstrated in chromosomes of the guinea pig (Yasmineh and Yunis, 1975), *Drosophila* spp. (Gall and Atherton, 1974; Brutlag et at., 1977), *Rattus rattus* (Yosida and Sagai, 1975) and many other animal species. Constitutive heterochromatin usually appears as satellite-DNA when nuclear chromosomal DNA is fragmented and centrifuged (Kit 1961). It consists of short, highly repeated base pair sequences in tandem (Southern 1970; Corneo et al. 1970; Gall and Atherton, 1974; Brutlag et al., 1977) in one or more regions of all or more chromosomes in most species. Arrighi and Hsu (1971) showed that C-bands are located next to the centromeres of each chromosome, next to the secondary constrictions of chromosomes 1,9, and 16 as well as the satellites of acrocentric chromosomes in man. With few exceptions, constitutive heterochromatin in animal species reveals a consistent pattern of distribution. Therefore, C-banding in animal species does not correspond to a banding pattern in a strict sense. It's application in these species is limited because it does not allow precise recognition of individual chromosomes.

Several lines of evidence indicate that the production of C-banding is due to the extraction of

non-C-band DNA and denaturation of proteins in these regions. The DNA in constitutive heterochromatin is resistant to extraction, remains within the chromosomes and is therefore stainable by Giemsa (Pathak and Arrighi, 1973; Dille et al., 1987; Burkholder, 1988).

Since the initial studies in *V. faba* by Vosa and Marchi in 1972, chromosomes of many species of *Aegilops, Agropyron, Elymus, Hordeum, Secale, and Triticum* (Gill and Kimber, 1974a and b; Linde-Laursen, 1975; Vosa, 1976; Gerlach and Peacock, 1980; Singh and Tsuchiya, 1981b; Seal, 1982; Teoh and Hutchinson, 1983; Endo, 1986; Morris and Gill, 1987) and other plant species (Linde-Laursen et al., 1980; Loidl, 1983) have revealed C-bands. These studies show that there is a fundamental difference in the distribution of constitutive heterochromatin within chromosomes of animals and plants. C-bands in the chromosomes of plants can be located at various sites including the regions they characteristically occupy in the chromosomes of animals. Moreover, in most of the plant species e.g., *Allium carinatum* (Loidl, I983), *Hordem* spp. (Linde-Laursen et al., 1980) and *Agropyron elongatum* (Endo et al., 1984a), some of the chromosomes do not reveal C-bands next to their centromeres. Thus, in many plant species a unique C-banding pattern occurs in each arm of each chromosome in the genome. This allows individual chromosomes in the somatic cells to be identified on the basis of their patterns (loc. cit.).

Gill and Kimber (1974a) published the first report on C-banding patterns of chromosomes of *T. aestivum*. Despite the efforts of many investigators in the interim, Endo (1986), using an improved C-banding technique, was able to unequivocally identify all 21 chromosomes in the genome of cvs. Chinese Spring and Norin 61 of *T. aestivum*. These banding patterns are currently accepted as standard patterns for the chromosomes of common wheat (Gill, 1987; Gill et al., 1988). Ferrer et al. (1984) applied the C-banding protocol to the study of chromosomes in meiocytes and clearly identified nine of the 21 chromosome pairs.

Except for the work of Simeone et al. (1988), the efforts of other investigators (Zurabishibili et al., 1978; Seal, 1982; Lukaszewski and Gustafson, 1983) to identify the A and B genomes of *T. turgidum* on the basis of their C-banding patterns have been inconsistent. Simeone et al. (1988) reported C-banding patterns for chromosomes of T. *turgidum* that were equivalent to those of their homologues in the A and B genomes of *T. aestivum* and therefore permitted their unequivocal identification. Shang et al. (1989), used the HKG (HCI-KOH-Giemsa) method to produce banding patterns in chromosomes of *T. turgidum* that were in part similar to their C-banding patterns, thus allowing identification of some of the chromosomes.

C-banding of the chromosomes of *T. monococcum* has been reported by both Gill and Kimber (1974a) and Kuz'menko et al. (1987). The banding patterns of the chromosomes in these two investigations were partially dissimilar. Moreover, the C-banding patterns of some of the chromosomes in these publications were different from those for the A-genome chromosomes in *T. aestivum,* thus precluding their identification. Using the HKG method, Shang et al. (1988 and 1989) reported banding patterns for chromosomes of *T. monococcum*, partially, resembled their C-banding patterns. Although the banding patterns revealed by the HKG method rendered some of the chromosomes distinguishable from the others, they did not allow for their unequivocal identification.

C-banding has been used in the identification of aneuploids (Linde-Laursen, 1978b, 1982; Zeller et al., 1987), translocations and other structural rearrangements (Gill and Kimber, 1977; Lukaszewski and Gustafson, 1983; Lapitan et al., 1984) and the precise physical mapping of genes (Kota and Dvorak, 1986; Jampates and Dvorak, 1986). C-banding analysis of *durum-timopheevi* and *durum-speltoides* hybrids by Chen and Gill (1983) has supported Dvorak's suggestion (1983) that chromosomes 4A and 4B should be reassigned to the B and A genomes, respectively. Moreover, C-banding has clarified and further substantiated phylogenetic conclusions based on chromosome pairing in interspecific and intergeneric hybrids (Gill and Kimber, 1974a; Hutchinson and Miller, 1982; Chen and Gill, 1983; Morris and Gill, 1987).

N-banding

In 1973 Matsui and Sasaki developed a technique they called N-banding, which selectively stained NORs in the chromosomes of mammalian species. Funaki et al. (1975) improved this proce-

dure and demonstrated that N-bands were confined to the NORs of the chromosomes of 27 eukaryotic species that they studied. Faust and Vogel (1974) and Pimpinelli et al. (1976) observed that the bands obtained with this procedure are not NOR-specific in *D. melanogaster* and the mammalian species studied. Nevertheless, these non-NOR bands were and continue to be referred to as N-bands. Using the method of Funaki et al. (1975), with slight modifications, Gerlach (1977) and Jewel1 (1979), working with *Triticum* and *Aegilops* species respectively, clearly demonstrated that N-bands do not necessarily correspond to NORs. Moreover, at least some of the chromosomes in the species analyzed had unique N-banding patterns, permitting their identification. Gerlach (1977) identified nine of the 21 chromosomes of common wheat on the basis of their N-banding patterns. Subsequently, Endo and Gill (1984a) identified 16 of the 21 chromosomes of common wheat, including five in the A genome, using an improved N-banding protocol. Jewell (1979) identified all 14 chromosomes of *Aegilops variabilis* on the basis of their N-banding patterns. The technique has also been used to identify chromosomes in barley (Singh and Tsuchiya, 1982b), rye (Jewell, 1981; Schlegel and Gill, 1984), lentils (Mehra et al., 1986) and *Elymus* spp. (Morris and Gill, 1987). N-banding has also been used to identify various types of aneuploids (Singh and Tsuchiya, 198Zb; Zeller et al., 1987), alien addition and substitution lines (Islam, 1980), and translocations and deletions (Jewell, 1978). It should be noted that N-banding has been attempted in *T. monococcum* by B.S. Gill (personal communication) and in our laboratory without success. Why this should be, since some of the A genome chromosomes in *T. aestivum* contain N-bands, is unknown.

Gerlach (1977), and subsequently others, noted that many of the N-bands occupy the same positions as C-bands, implying that the N-banding technique, like the C-banding one, identifies constitutive heterochromatin and that at least two classes of heterochromatin occur in wheat, rye and other species. This was confirmed by Schlegel and Gill (1984) and Endo and Gill (1984a). Some heterochromatic regions in each chromosome stain positively using both C- and N-banding procedures. These regions are referred to as C+N-bands. Other such regions stain positively only with C-banding techniques. These heterochromatic segments are called C+N-bands. Schlegel and Gill (1984) have shown that only N-bands ($C+N^+$ bands) possess multiple copies of the (GAA) n (GAG) n sequence DNA. The base pair sequences in C+N–bands have not been identified.

Gill (1987) and Gill et al. (1988) have proposed banding nomenclatures for the chromosomes of T. *aestivum* cv. Chinese Spring.

Identification of NORs and Nucleoli. Nucleolus organizer regions (NORs) are the sites of rRNA genes in the chromosomes of animal (Ritossa and Spiegelman, 1965; Wallace and Birnstiel, 1966; Henderson et al., 1972, 1974) and plant species (Phillips et al., 1971; Flavell and O'Dell, 1975; Hutchinson and Miller, 1982). Methods have been developed for the selective staining of these chromosomal regions both in animals (Goodpasture and Bloom, 1975; Howell et al., 1975; Verma and Babu, 1984) and plants (Hizume et al., 1980; Lacadena et al., 1984; Mehra et al., 1985; Cunado et al., 1986). The Ag-As (ammoniacal silver) method selectively stains those sites on chromosomes which correspond exactly to regions that can be detected by *in situ* hybridization with rDNA probes (Howell et al., 1975; Miller et al., 1976a and b). It seems that this procedure stains only the NORs that are functionally active during the preceding interphase (Howell, 1977; Schmiady et a1. 1979). There is evidence to suggest acidic or nonhistone proteins associated with the rDNA regions are responsible for the selective staining of NORs (Howell et a1., 1975; Wang and Juurlink, 1979; Howell, 1985).

In situ hybridization and its application to wheat cytogenetics. Gall and Pardue (1996) and John *et al.* independently reported a procedure that facilitated the cytological detection of hybrid nucleic acid regions. This technique involves the annealing of radioactively labelled nucleic acid sequences to cytological (chromosomal) preparations *in situ* (on slides) and subsequent detection of the hybrid regions by autoradiography. Specific DNA sequences in the chromosomes of animal and plant species have been localized with this technique. These include some highly repetitive short base-pair sequences in the chromosomes of animals (Pardue and Gall, 1970; Brutlag et al., 1977) and plants (Gerlach and Peacock, 1980; Appels and

McIntyre, 1985; Ganal et al., 1988; Lapitan et al., 1989), moderately repeated sequences such as rRNA genes in the chromosomes of animals (Wimber and Steffensen, 1970, 1973; Henderson et al., 1972) and plants (Wimber et al., 1974; Gerlach and Bedbrook, 1979; Mascia et al., 1981; Clark et al., 1989), and some single copy genes in animals (Harper and Saunders, 1981; Henderson, 1982; Olsen el al., 1989) and plants (Ambros et al., 1986; Huang et al., 1988).

Gerlach and Peacock (1980) isolated a highly repetitive DNA sequence from *T. aestivum* cv. Chinese Spring and hybridized it to cytological preparations of *T. aestivum, T. dicoccoides, T. monococcum* and *Ae. squarrosa*. A number of heavily labelled chromosomes were observed in the preparations of *T. aestivum* and *T. dicoccoides,* but not in those of *T. monococcum* and *Ae. squarrosa.* On the basis of these results the authors concluded that most of the heavily labelled chromosomes belong to the B genome. C-banding studies by Endo (1986) and Gill (1987) and N-banding reports by Endo and Gill (1984b) support this conclusion. Peacock et al. (1981) demonstrated that the DNA sequence is composed of repeated (GAA) n and (GAG)n units. Rayburn and Gill (1985) showed that the major *C*-and N-bands correspond to sites which contain this satellite sequence. *In situ* hybridization studies with a highly repetitive D-genome specific DNA sequence isolated from *Ae. squarrosa* were used by Rayburn and Gill (1986) to identify D-genome chromosomes in hexaploid wheat.

At least four pairs of chromosomes (lA, 1B, 5D, and 6B) of *T. aestivum* contain NORs (Crosby, 1957; Darvey and Driscoll, 1972). If NORs are the sites of ribosomal RNA (rRNA) genes, then all these chromosomes should be expected to possess clusters of rRNA genes. Flavell and Smith (1974a, 1974b) and Flavell and O'Dell (1976, 1979) showed that in hexaploid wheat a large proportion of the rRNA genes are on chromosomes 1B and 6B, with only a small proportion of the genes residing on chromosomes 1A and 5D. Gerlach and Bedbrook (1979) cloned the 18S+26S rRNA genes of *T. aestivum* into a bacterial plasmid and showed that the probe derived from this clone hybridized to regions on chromosomes 1B and 6B. Miller et al. (1980) showed that the same rDNA probe hybridized to minor NORs on chromosomes 1A and 5D in bread wheat. A similar approach with a different rDNA probe enabled Appels *et al.* (1980) to confirm the location of rRNA genes on chromosomes lB, 5D, and 6B only. They speculated that too low a level of rRNA genes on chromosome 1A may have precluded their detection by *in situ* hybridization experiments. *In situ* hybridization experiments in *T. turgidum* and *T. timopheevi* with rDNA probes confirmed the assignment of rDNA loci to chromosomes 1B and 6B, (Appels and Dvorak, 1982; Dvorak and Appels, 1982). Miller et al. (1983) showed that, in *T. urartu,* a labelled rDNA probe hybridized in *situ* to a region on chromosome 5A that corresponds to the NOR. Some genotypes of *T. urartu,* and other diploid wheat, have been shown to have two chromosome pairs with nucleolus organizers (Gerlach et al., 1980). The second NOR must, by deduction, be located on chromosome 1A. Frankel et al. (1988), using a synthetic tetraploid AABB and a ^{3}H-labelled rDNA probe, clearly demonstrated that the NORs of two pairs of A-genome chromosomes were labelled after *in situ* hybridization. Apart from one being more heavily labelled than the other, the two chromosome pairs (1A and 5A) could not be distinguished cytologically.

Information on the location of 5S rRNA genes in wheat species is scanty. Appels et al. (1980) localized the 5S rRNA gene cluster to chromosome 1B of *T. aestivum* at a site distinct from and distal to the NOR region. Kota and Dvorak (1986) mapped these genes to a single site on the *p* arm of chromosome 5B, using a line with a spontaneous deletion. Lassner and Dvorak (1985) and Kota and Dvorak (1986) suggested that chromosomes 5A and 5D may also carry the genes for 5S rRNA. Scoles et al. (1987), using cloned 5S rDNA sequences obtained unequivocal evidence for the presence of 5S rRNA genes on chromosomes 1B, 1D, and 5B in *T. aestivum*. Studies by Dvorak *et al.* (1989) have shown that chromosomes 1A and 5A of *T. monococcum* var. *aegilopoides* carry *5S* rRNA genes. Moreover, they indicate that the *5S* rDNA on chromosome 1B is linked to the Nor-B1 locus.

Production of molecular probes. Molecular probes, are derived from cloned recombinant DNA molecules e.g., plasmids such as pBR 322 with cDNA genes. These molecules are generated using restriction and other DNA modifying enzymes and then cloned in a proper host (Mertz and Davis

1972; Watson et al., 1983). Protocols for generating recombinant DNA molecules, cloning them, excising the relevant DNA fragment e.g., *5S* rDNA genes from these molecules and subsequent use in *in situ* hybridization and other experiments are detailed in Watson et al. (1983) and Winnacker (1987). The procedures used in the cereals are given by Gerlach and Bedbrook (1979) and Lawrence and Appels (1986).

Restriction fragment length polymorphism (RFLP) for chromosome identification. RFLP markers are currently being extensively used in genetic mapping (Wyman and White 1980, Helentjaris 1987, White and Lalouel, 1988) and determining genome homologies among crop species (Bonierbale et al., 1988; Sharp et al., 1989). They have also been used in the identification of the critical chromosomes in aneuploid plants in tomato (Young et al., 1987) and bread wheat (Gale et al., 1988).

Sharp et al. (1989) isolated specific RFLP probes for each arm of the seven chromosomes of the homoeologous genomes of *Triticum*. These 14 homoeologous probes permit the identification of chromosomes in each genome of the diploid and polyploid wheats.

Aneuploid stocks

Several types of aneuploid stocks have been produced in wheat that have been used for decades for studies of physical mapping. These stocks are at the series of 21 mulli tetras produced by Ernie Sears (Sears, 1966) that have been used for assigning genes to individual chromosomes (Sears, 1954; 1966) and the 24 ditelo stocks for mapping genes to individual chromosome arms (Sears and Sears, 1978). These stocks have also been used for a number of studies; from assigning some of the original molecular markers to individual chromosomes in the construction of the first molecular marker maps (Anderson et al., 1992) and to assigning ESTs to physical positions on chromosome arms (Han et al., 2005). The trisomic series in barley and the ditelo series in barley (Tsuchiya, 1960, Fedak et al., 1971, 1972) and trisomic series in *T. monococcum* (Friebe et al., 1990) and rye (Kamanoi and Jenkins, 1962) have also been used for physical marker mapping. Another aid in wheat cytogenetics was the publication of a standard karyotype and nomenclature system for hexaploid wheat (Gill et al., 1995). The addition lines of Betzes barley in Chinese Spring wheat (Islam, 1980) have also been used in initial studies of assigning molecular markers to chromosomes in the initial phases of developing molecular maps in barley.

The deletion stocks in wheat are another useful cytogenetic tool. They were produced by the gametocidal action of a particular genotype of *Ae. speltoides* (Endo and Gill, 1996) that caused a series of terminal deletions on all wheat chromosomes followed by restoration of the telomere function. The deletion stocks consist of 101 lines with 119 chromosome segment deletions for subarm mapping. These stocks provide complete coverage of the wheat genome, subdividing it into 159 chromosome bins. These stocks were recently used to physically map 16,000 wheat ESTs (Qi et al., 2004). Such a map is having numerous applications such as a source of SNP discovery (Somers et al., 2003) microsatellite discovery (Thiel et al., 2003) comparative mapping, hastening the speed of gene discovery in wheat plus providing a framework for constructing BAC-contigs of wheat. The stocks have also been useful on a smaller scale, as for example the physical mapping of ESTs upregulated in wheat following fungal attack (Han et al., 2005). Another tool for cytogenetic analysis of genomes, cloning and tagging of genes is the BAC libraries that have been constructed in diploid (Lyavetsky et al., 1999) and hexaploid wheats.

Chromosome Addition Lines

Another set of cytogenetic stocks that permit analysis of single donor chromosomes are the addition lines of maize in oat (Riera-Lizarazu et al., 2000) which were produced by means of oat by maize pollination (Riera-Lizarazu et al., 1999). Yet another cytogenetic tool is that of chromosome sorting. Chromosome sorting is a technique to provide aliquots enriched in particular wheat chromosomes (Kubalakova et al., 2005) that can be used for molecular cytogenetic studies.

Complete series of addition lines from numerous alien species have been produced in a wheat background. Chromosomes from more than 20 alien species have been added to wheat; in some cases a complete set of addition lines has been produced. From 18 of these combinations, chromosome substitution lines have also been produced. In many cases, translocations have been induced between the chromosomes of the crop plant and

the alien species. A compendium of such cytogenetic stocks appears as an appendix in the proceedings of international wheat genetics symposia (e.g. Proceedings of the Seventh International Wheat Genetics Symposium, Cambridge E, July 13–19,1998. pp1374–1387). Various molecular methods have been devised to monitor the transfer of traits from alien species into crop plant chromosomes (Fedak,1999). Numerous translocations have been induced between alien and wheat chromosomes from the alien addition lines as a means of transferring disease resistance to wheat (Friebe et al., 1996).

Molecular Marker Maps

Another class of fairly recent genetic tools are the molecular linkage maps. These are not physical chromosome tools but are very useful in chromosome and genome identification and monitoring alien introgressions. RAPD markers were basically random base sequences that were extensively used to tag genes controlling agronomic traits (Williams et al., 1990). The next class of markers were RFLPs. Extensive maps consisting of 1000 RFLP markers were generated in wheat (Gale et al., 1995). These markers were characterized as too expensive and cumbersome for application to marker assisted selection but played a significant role in uncovering relationships between diverse grass genomes (Gale and Devos, 1998). For example overall colinearity between wheat, barley, maize and rice genomes was established (Feuillet and Keller, 1999) which permitted the sharing of markers across the maps. Because the rice genome was fully sequenced (Goff et al., 2002), this permitted the extensive use of rice sequences to perform fine mapping in wheat genetic studies (Liu et al., 2006). RFLP technology, particularly the use of cDNA probes has been and still is a valuable method of monitoring alien chromatin introgression into the wheat genome, mainly because of sequence conservation and systemic relationship among the Triticeae. In more recent fine mapping experiments it is being discovered that the collinearity between chromosomes of species such as wheat and rice is not as close as initially assumed (La Rota and Sorrels, 2004).

Other molecular maps were developed with PCR-based markers including RAPDs (William et al., 1990) AFLPs (Vos et al., 1995) and microsatellites (SSRs) (Roder et al., 1998). High density microsatellite maps coupled with high throughput capillary electrophoresis are the essential components of a marker assisted breeding program. The high density consensus map of Somers et al., 2004 is adequate for QTL detection.

High density maps are essential for map based cloning by increasing the probability of discovering markers flanking the gene in question and by reducing the number of BAC clones containing the gene.

FISH and GISH analysis

Another class of methodologies that facilitate the identification of specific genomes, individual chromosomes or chromosomal segments is the use of fluorescent signals. The first procedure to use fluorescent labels to distinguish plant chromosomes was the process of genomic *in situ* hybridization (GISH) (Shwarzacher et al., 1989). This technique was then used to identify parental genomes and genome organization, plus alien genome/chromosome introgression (Jiang and Gill, 1994). The identification of the three genomes of hexaploid wheat has been achieved, but proved to be difficult to repeat consistently (Mukai et al., 1993; Sanchez-Moran et al., 1999). A modification of the multicolour GISH technique (Han et al., 2003, 2004) permitted the unequivocal identification of all three wheat genomes plus the presence of alien chromosomes and translocations. Other cytogenetic tools such as the Afa family, a repetitive sequence, cloned from *Ae. squarossa* (Rayburn and Gill, 1986); PSc119.2-a rye subtelomeric heterochromatic sequence (Bedbrook et al., 1980); and PTa7l, an rDNA clone of the 18 S-8.5S-26S ribosomal sequence (Gerald and Bedroock, 1979) can be used singly, in combination or in combination with FISH to identify individual chromosomes of wheat or barley. For example, using a combination of FISH, Afa and PSc119.2. Kubalakova et al. (2005) were able to identify all chromosomes of durum wheat.

Additional tools that complement the use of FISH technology for cytogenetic analysis of individual chromosomes are 5S and 25S genes plus BAC clones that can be differentially stained to identify individual chromosomes (Zhang et al., 2004). The fiber FISH technique, using appropriate probes which is applied to stretched somatic chromatin can provide better resolution of gene

and repeated sequence arrangements on wheat and bareley chromosomes (Valanik et al., 2004).

It is obvious from this review that in recent years classic cytogenetic analysis has been replaced by molecular methods. In recent years, however, there is a renewed interest in minichromosomes. These are structures that consist of a centromere and minimal proximal chromatin into which genes of interest will potentially be integrated. The minichromosomes will not pair with those of the regular complement, will behave normally at meiosis and provide stable transmission of the introgressed trait. This technology is most advanced in mamalian cells. In plant cells it is being developed in maize from B chromosomes (Yu et al., 2007).

It is interesting to note that Gus Wiebe, a Research Coordinator for USDA had developed lines of 16 chromosome barley in the 1960s. His theory was that removing the telomeres from the additional chromosomes would limit their pairing with normal chromosomes and would thus stably carry additional traits for the barley genome.

Another recent development is the use of rice sequence information for fine mapping in wheat, barley and maize. In many cases the colinearity of sequences between rice and the other species is not as precise as originally predicted. More recently it has been suggested that the genome of *Brochypodium* could be used as a model system for the study of grass genomes (Bossolini et al., 2007).

George Fedak, Nam-Soo Kim

TOOLS AND METHODOLOGIES FOR CYTOGENETIC STUDIES OF PLANT CHROMOSOMES

Представлен краткий обзор развития методов цитогенетики и анеуплоидов с 20-х годов. Методология варьирует от первых сообщений о хромосомных наборах большинства организмов, развития хромосомных кариотипов, а затем анеуплоидов для большинства возделываемых растений. Вклад молекулярной биологии включает технологии исчерченности хромосом, карты молекулярных маркеров и методы гибридизации *in situ*. Все эти новые методики значительно увеличили точность цитогенетических исследований.

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