DIFFERENTIAL STAINING AND PLANT CHROMOSOMES—A PROGRESS IN CYTOGENETICS

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ABSTRACT

A collective review of various banding techniques developed in the recent years and the advances made for their improvement with special attention to plant chromosomes is presented. Besides, the techniques for differential staining of sister chromatids have also been included alongwith the mechanisms involved in the various banding procedures. A due attention has been paid on a new banding technique 'O–Banding' developed in this laboratory especially suited for plant chromosomes.

CHROMOSOME Banding has revolutionized cytogenetics by allowing the precise identification of individual chromosomes and parts of chromosomes, structural and molecular organisation of chromosome, chromosome changes during evolution, studies in chromosome polymorphism, detection of structural changes in reconstructed karyotypes, aneuploid identification, genome analysis in allopolyploids, diagnosis of chromosome rearrangements in human malformation syndroms and gene mapping with the help of somatic cell hybrids, detection of interspecific translocations in mouse-human hybrids, chromosome orientation and somatic association in interphase, role of heterochromatin in pairing and in relation to breeding value in triticale.

Among the techniques which proved useful to differentiate between eu- and heterochromatin the oldest known is based on the ability of heterochromatic chromosome regions to form, in reaction with nitric acid vapours[1] and on relatively extended periods of cold treatment (cold starvation)[2], what is known as "Special Segments or H segments".

Q-banding : A breakthrough in the development of the technique of banding chromosomes came from the pioneering studies of Caspersson *et al.*[3], that fluorescent dyes such as Quinacrine Mustard (QM) affect selective, discrete fluorescent labelling in both plant and animal chromosomes. The improved procedure used by Vosa[4] for plant chromosomes is as follows:

Pretreated root tips fixed overnight are squashed in 40% acetic acid. The cover glass is removed by dry ice or inverting the slide in absolute alcohol and the preparation is air dried. Staining is carried out by immersing in 0·5% aqueous or alcoholic solution of Quinacrine Mustard (QM) for 10–15 minutes to obtain proper staining. After rinsing, the preparation is mounted in distilled water and observed under fluorescence microscope.

Vosa[4] also used ethidium bromide in place of QM and observed reduced fluorescence at the same level in *Vicia faba* where QM gives enhanced fluorescence. Sarma and Natarajan[5] used a new fluorochrome compound a *bis*-Benzimidazole derivative—"Hoechst-33258" for rye chromosomes. Filion *et al.*[6] suggested that the intensity of 'Hoechst 33258' can be significantly improved by heating the mounted sealed slides at 120°C for 6 sec. followed by rapid cooling over dry ice. Vosa *et al.*[7] observed Quinacrine-like fluorescence by the alcoholic extracts of the alaloids from fresh roots of 8 genera from papaveraceae and fumariaceae. Root extract alkaloids from *Chelidonicum majus*, *Macleaya cordata* and *Glacium flavum* showed fluorescence intensity like that of QM. Other fluorescent stains occasionally used by some workers are sarcolysinoacridine[8], berberine sulphate[9], 2, 7-di-*t*-butyl proflavine, DBP[10], .

The studies of Vosa[4] suggested for the first time, the classification of four main types of heterochromatin as defined by alocyclic DNA, namely;

(*a*) detectable by 'cold starvation' effect and showing enhanced fluorescence (*Trillium* and *Vicia faba* type);

(*b*) as (*a*) but with reduced fluoreseene (*Tulbaghia* type);

(c) not revealed by chilling and showing enhanced fluorescence (*Allium carinatum* type);

(*d*) as (*c*) but with reduced fluorescence (*Scilla sibirica* and *Allium cepa* type).

Banding with Giemsa

C-Banding : Pardue and Gall[11] observed denser centromeric regions of chromosomes of mouse compliment after *in situ* hybridization with complementary RNA of the mouse satellite DNA and subsequent Giemsa staining. Following this observation, a staining method with Giemsa was developed for the detection of repetitive DNA which is richly localised in the centromeric regions of the chromosomes[12,13]. The method followed by Vosa and Marchi[14] for plants, which is more or less identical to Sumner *et al.*[15] for centromeric heterochromatin in man, is as follows:

Air dried preparations are immersed in aqueous saturated solution of barium hydroxide at room temperature for 5 minutes (denaturation), washed thoroughly and incubated for 1–2 hrs. in 2 × SSC (0·3 M Sodium chloride + 0·03 M Sodium citrate) at 60° C (renaturation). After washing in running distilled water the

material is stained with diluted solution of Giemsa (0·5 to 5%) in M/15 phosphate buffer at pH 6·8. There is one very important difference that Giemsa method does not discriminate between segments with intense and reduced fluorescence, but stains both in the same way. The specific staining of centromeric regions of the chromosome this way is called C-banding[16], staining centromeric type of constitutive heterochromatin. However, this method besides centromeric bands also gives bands on the chromosome arms in plant systems. Various modifications of the method suggested include—*maceration* of tissue, before squashing in HCl[5,17,18], mixture of N.HCl and 45% acetic acid (2:1)[19], enzyme solution (containing 5% macerozyme +5% cellulase adjusted at *p*H 5·5 with 2 N.HCl)[20], enzyme mixture (equal parts of 5% pectinase + 5% of cellulase and 2–3 drops N.HCl for each 5 ml mixture)[21 22], 45% acetic acid[23]; *denaturation* in NaOH (0·07 M–0·1 M),[20,23,24,35] 6M urea[18], different concentrations of hot barium hydroxide solution[25], saturated hot (60° C) barium hydroxide solution[27]. Mok and Mok[29] omitted the barium step for bean chromosomes. Schweizer[23] has proposed the following schedule for standard Giemsa technique for plant chromosomes.

Conventional air dried preparations are transferred into hot 2 × SSC pH 7·0; at room temp. or McIlvaine Citric acid—Na_2HPO_4 buffer (0·2 M Na_2HOP_4 adjusted with 0·1 M citric acid pH 7·0) at 65° C and incubated for 24 hrs. Slides subsequently rinsed in alcohol grades and air dried. Preparations are stained in fresh 2% Giemsa in M/15 Sörensen phosphate buffer pH 6·9.

Fiskesjö[29,30] using *Allium* spp. has suggested that better bands particularly on chromosome arms can be obtained by storing the air dried preparations for 2 weeks to 2 months (maturation) before following denaturation and renaturation process. Limin and Dvorak[31] has shown that C-bands on rye chromosomes can be obtained by SSC treatment at temperatures as low as 0 ° C for periods as short as 1 minute. However, the barium step was essential. Kordan and Uncle[32] obtained bands merely by hydrolysis in N·HCl for 5–10 min. at 60° C followed by Giemsa staining in *Lens culinaris* and *Allium cepa*. The technique developed in this laboratory with plants involves treatment of air dried preparations by 0·2% trypsin solution in posphate buffer pH 7·0 at 0° C for few minutes, rinsing in water and stained in 2% Giemsa in phosphate buffer pH 6·8 (author, unpublished).

G-Banding : Giemsa stained bands following mere saline treatment noted in intercalary segments are referred to as 'G' bands[33, 34, 35]. Since then, the G-banding patterns have been obtained following a variety of treatments such as standard saline, NaOH/HCl prior to SSC, NaOH, trypsin, etc.,[36, 37, 38] heating[39, 40] and even by equilibrating in phosphate buffer[41]. The technique is confined mainly to animal systems.

Dotted chromosomes[42] are consistently produced in both BrdU and non BrdU substituted Chinese hamster cells after treatment with 1M Na–phosphate solution, adjusted to pH 9·0 with a supersaturating amount of $NaHCO_3$, and a temperature of 80–95° C. A series of changes in chromosome morphology are produced as the temperature of solution is progressively increased. In BrdU treated cells, only G-banding and differentially stained sister chromatids are sequentially produced prior to the appearance of dots. In non-BrdU treated cells, only G-banding is produced before dot formation. In general, the patterns of dots correspond to the G-banding patterns. Chromatids with uni or bifilarly BrdU substituted DNA or with normal DNA, require differential temperatures for the production of dots.

R-Banding : Air dried preparations (animal chromosomes) are placed in 0·2 M phosphate buffer (pH 6·5) for 10–12 minutes maintained at a temperature of 87° C and then allowed to cool to 70° C. The slides are then immediately plunged into Giemsa in the same buffer at room temperature for 10 minutes Staining is pale and the observation of bands is facilitated by use of phase contrast microscopy (Dutrillaux and Lejeune, 1971)[43]. The banding patterns are reverse to that obtained for G-banding.

Schweizer[44] used two DNA binding guanine-specific antibiotics, Chromomycin A_3 (CMA) and the closely related mithramycin (MM) as chromosome fluorescent dyes. Root tip metaphase chromosomes of three plants and human metaphase chromosomes were sequentially stained with CMA or MM and the DNA binding AT–specific fluorochrome 4′-6-diamidino-2-phenylindole (DAPI). In some cases a non-fluorescent counterstain was used as cohtrasting agent: methyl green in conjunction with CMA, and actinomycin D (AMD) in combination with DAPI.

van de Sande *et al.*[45] introduced another G–C. specific DNA binding antibiotic– Olivomycin and produced characteristic reverse fluorescence banding patterns (R-bands) on human, bovine and mouse metaphase chromosomes.

Banding with Orcein

Yamasaki[46, 47] obtained differential staining of chromosomes in *Cypripedium debile* by dividing the root tip longitudinally in 4 parts and heating at 60° C–80° C for 5–15 min. in a staining mixture of 0·25% orcein, + N·HCl and squashing after cooling.

Vosa[48] substituted Giemsa stain with 1·5% orcein in the conventional Giemsa C-banding procedure[14].

O-Banding Procedure : In several systems as in plants, the banding technique has been found to be

of limited application because of the inherent technical limitation for air drying of solid tissue, one of the protocols for Giemsa staining. Moreover, plant tissue as a whole is less responsive for Giemsa reaction. To meet this need certain authors have tried Feulgen staining for banding with limited success[49]. In order to overcome these limitations, a new banding technique termed O-banding has been developed by Sharma[50,51] and extended by Lavania[52] with orcein staining specially suitable for plant chromosomes. The method involves pretreatment in colchicine, fixation in acetic ethanol, treatment in a mixture of sodium chloride (1M) and sodium citrate (0·1 M) for 2–2½ hr. at 27–28°C, staining in aceto-orcein mixture and mounting in 45% acetic acid. Similar effect is also observed if salt treatment is replaced by tryptic digestion at low temperature (author, unpublished). O-bands appear on both sides of the centromere similar to C-bands as well as on intercalary segments, similar to G-bands depending on period and concentration of salt treatment.

T-Banding : Two new techniques of controlled thermic denaturation at 87°C using Giemsa and acridine orange in diluted buffer are decribed[53]. They especially show a staining of some terminal regions of chromosomes (terminal band or T-bands). Application of these techniques to translocations, hardly analysable by other banding techniques, allows the precise location of juxtatelomeric break points in man.

N-Banding : Nucleous organizers classically have been recognized as secondary constrictions, although some nucleolus organizers by observing the number of nuclei and secondary constrictions have been notoriously unreliable. Because the association of nucleolus organizers with constitutive hetrochromatin seem to be a reasonable generalization, the techniques reported here may be more reliable means of determining the total number of nucleolus organizers in chromosome compliment.

The various banding procedures have been employed using Giemsa staining which involve treatment of conventional air dired preparations with 5% tri-chloroacetic acid at 90°C for 15–30 min. followed by incubation in 0·1 N. HCl at 60°C for 15–90 min. or digestion of chromosomes with DNase and RNase[54,55], incubation in 1 M NaH_2PO_4 (pH 4·2 adjusted with 1N· NaOH) at 96°C for 15 min.[56] and more specifically for plant chromosomes incubating in 90°C SSC or phosphate buffer followed by incubation in saline at 0°C or strong treatment with barium hydroxide solution followed by incubation in phosphate buffer at 60°C or 2 × SSC[57] and lastly the Ag-As technique (silver staining)[58] confined to mammalian systems only.

Pericentric chromosome banding : Stack and Clarke[59] have proposed a modification of Giemsa technique of plants for obtaining pericentric chromosome bands, which involves treatment of flame dried slides in 0·12 M phosphate buffer (pH 6·8) at 94°C for 10 minutes, followed by 22 hrs. in the same buffer at 60°C and finally staining at room temperature.

Cd-*Banding* : A new banding technique[60] for animal chromosomes which reveals two identical dots (centromeric dots Cd) at the place of centromere, one on each chromatid is described.

Conventional air dired preparations which have been stored for 1 week at room temperature are incubated in Earle's BSS medium (pH 8·5–9·0) at 85°C for 45 min. The slides are then stained in 4% Giemsa in 1/300 M phosphate buffer (pH 6·5).

Hy-Banding : Greilhuber[61,62] achieved differentiation of preferentially staining heterochromatic segments in somatic chromosomes of *Allium cepa*, *A. carinatum*, *A. flavum*, *Scilla sibirica* and *Fritilaria meleagris*, when acetic ethanol fixed meristems were subjected to 0·1 or 0·2 N· HCl at temperature between 60 and 80°C and stained with acetocarmine. For the heavily and weakly staining bands the abbreviations "Hy+ bands' and "Hy−bands" respectively have been suggested.

Other Methods

Takehisa[63] observed reduced stainability of H segments, especially in the M chromosomes of *Vicia faba* after 3–4 min treatment at 90°C with 1 : 9 mixture of 1N· HCl and 2% orcein in 45% acetic acid Treatment with HCl–acetic acid followed by feulgen staining was preferable to treatment with HCl-orcein and the best results were obtained after 3 min. treatment at 90°C.

Merritt[64] in various spp. of *Nicotiana* obtained differential staining of late prophase chromosomes in the following way. Pretreated fixed roots were stored in 70% ethanol at 4°C for at least 3 days. The roots after hydrolysis in 10% HCl at 60°C, washed thoroughly and stained in leuco-basic fuschin until the meristem turns pink. After washing and staining in aceto-carmine for 5 min. the roots were squashed in a drop of acetocarmine and heated over steam bath for 1–2 min.

Differential staining of sister chromatids

Techniques have been developed where Hoechst '33258' is combined with Giemsa staining (fluorescent plus Giemsa or FPG) to make permanent Giemsa stained cytological preparations of differentially labelled chromatids. Techniques are useful for the study of sister chromatid exchanges and support the idea that the chromatid is a DNA mononeme. The method developed by Perry and Wolff[65] for animal materials

involves growing the cells in the solution of BrdU (Bromodeoxyuredine) for two rounds of DNA synthetic phase followed by proper pretreatment for chromosome analysis and fixed in suitable fixative. Air dried preparations of the material are stained in Hoechst '33258' aqueous solution and processed as usual for observation in a fluorescence microscope. Sister chromatids now fluoresce differentially. The same preparations are then allowed to age for 24 hr., cover slips removed and the preparation incubated at 60° C for 2 hours either in 2 × SSC or in water, stained with Giemsa as usual. The chromatid that incorporated BrdU into both the strands of DNA helix fluoresced dimly and now stains weakly with Giemsa whereas the chromatid that incorporated BrdU into only one strand fluoresced brightly and now stains darkly with Giemsa.

Independently of Perry and Wolff[65], Kim[66] developed a FPG technique in which the heating was omitted. After staining with "33258" Hoechst" and observing in the fluorescence microscope, the preparations were briefly treated with alcohol, rinsed in tap water and stained with Giemsa. Korenberg and Freedlender[67] have reported that an excellent differentiation with Giemsa can be obtained without a previous fluorescent staining if the preparations are heated for 10 minutes at 87–89° C in 1 M phosphate buffer, pH 8.

Sugiyama *et al.*[68] proposed that other photosensitive dyes like Thionin can be substituted for 'Hoechst 33258'. Lim and Alfi[69] introduced another new fluorescent dye 4′-6-diamidino-2-phenylindole (DAPI) replacing 'Hoechst 33258'.

For differential staining of sister chromatids or for the study of artificially induced sister chromatid exchanges in plant materials Kihlman and Kronborg[70], Schvartzman and Cortes[71] have found that sufficiently good differentiation between sister chromatids could not be obtained by growing the roots in the presence of BrdU alone and have suggested that the incorporation of BrdU had to be stimulated by suppressing the cellular synthesis of thymidilic acid with FdU (fluorodeoxyuredine) an inhibitor of thymidylate synthetase.

Miller, Aronson and Nichols[72] have described a method based on the work of Parry and Wolff[56], Korenberg and Freedlender[67], which yields 3-way Giemsa differentiation in metaphase chromosomes exposed to BrdU for three S-periods (or exposed for two S-periods and removed from exposure from the third S-period) by means of which all the SCEs occurring S_1, S_2 and S_3 can be accurately counted and distinguished from one another.

Mechanisms of chromosome banding

Experiments with acid-treated chromosomes indicate that nonhistone rather than histone proteins are primarily involved in banding[73]. It has been proposed that quinacrine or Q-banding is due to a difference in base composition of DNA along the chromosome. DNA containing G–C sequences quenches fluorescence and highly A–T rich DNA enhances fluorescence[74, 75, 76]. Comings *et al.*[76] have suggested that quinacrine binds to DNA by intercalation with the large side chain at position 9 resting in the small groove of DNA.

The thiazin dyes (methylene blue azure A, B and C, thionine) of Giemsa bind to DNA by intercalation with the phosphate groups. Associated with this binding is a metachromatic shift in the absorption spectra of the dye due to stacking (dye-monomer—dye polymer)[77]. The weight of evidence, however, suggests that both Q- and G-bands are the result of dye binding to adjacent sites on folded molecules of native DNA, the appropriate conditions necessary for such binding depending to a great extent on the relationship between DNA and its associated nonshistone protein. The major factor in C-banding appears to be the presence of non-histone proteins that bind specifically to centromeric heterochromatin and protect it from extraction by sodium hydroxide and salts. This results in intense staining of the C-bands and poor staining of the rest of the chromosome[78, 78]. Limin and Dvořák[31] have suggested that $Ba(OH)_2$ alters the chemical structure of heterochromatin, rendering them insoluble in SSC.

The importance of DNA protein linkage, as in C- and G-banding, is noted in orcein-banding as well. Treatment with mixture of sodium chloride and sodium citrate involves removal of proteins from certain sites, where due to the nature of the DNA, possibly because of the unique sequences, the binding is comparatively weak. Similarly, it allows retention of the dye represented by bands at sites where the binding is comparatively strong due to compact and homogeneous nature of DNA of repetitive sequenes. With the increase in the duration of treatment, there is stepwise disappearance of the bands, except C-bands which disappear only after a prolonged treatment. This is also borne out by trypsin treatment as well. It appears therefore that gradual removal of non-histone proteins is principally responsible for O-banding[50,51].

A similar mechanism is probably involved in staining of the nucleolar organizer regions (N-bands)[76]. From the available cytological and biochemical data it has been suggested that the N-bands represent certain structural non-histone proteins specifically linked to nucleolar organizers in various eukaryotic chromosomes[56].

The mechanism involved in the differential staining of sister chromatids by FPG technique is that replacement of thymine in chromosomal DNA with 5-bromo-

uracil strongly reduces the fluorescence of fluorochrome dyes[65, 80, 81, 82]. The reduction of fluorescence is proportional to the amount of BrdU incoporated. Chromotids containing unsubstituted DNA fluoresce with the highest efficiency, an intermediate fluorescence is observed when the chromatids have one of the strands in their DNA substituted, and chromatids having both strands of their DNA substituted fluoresce most weakly. When this fluorescence is combined with Giemsa staining, the chromatids which fluoresced dimly (bifilarly substituted) now stains weakly with Giemsa, whereas those with a bright fluorescence (unifilarly substituted) stains darkly[65, 69]. The photolysis of the BrdU substituted DNA causes dim fluorescence and subsequent weaker Giemsa stainability[68].

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FIRST RECORD OF ALGAL REMAINS (FILAMENTOUS, SPHEROIDAL) AND ACRITARCHS FROM THE PRECAMBRIAN GANGOLIHAT DOLOMITES FORMATION OF PITHORAGARH, KUMAUN HIMALAYA, INDIA

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ABSTRACT

The Precambrian Gangolihat Dolomites Formation (Calc Zone) of Pithoragrah appreciably yielded algal remains (filamentous, spheroidal) of living cyanophycean (Oscillatoriaceae, Nostocaceae, Chroococcaceae) affinities. It also revealed acritarchs (organic–walled microplanktons) in common to sporadic distribution. The general morphological characters of the ancient algal and fungal (,) microfossils are described. In addition, two new species of *Baltisphaeridium* and *Schismatosphaeridium* (*Baltisphaeridium gangolihatensis* sp. nov., *Schismatosphaeridium kumauni* sp. nov.) are incorporated. Their discovery in the Calc Zone of the area is the first find of the same known to-date. The presence of these microorganisms (algal and fungal remains, incertae sedis) in the sediments assists in the reconstruction of paleoenvironments during the ancient Gangolihat oceans and dating of the Precambrian rocks.

INTRODUCTION

WITHIN the last decade substantial data on the presence of microscopic algal remains (filamentous and spheroidal) from the Precambrian sediments of the U.S.A., Canada, Australia, Russia, Africa and India have been published (Barghoorn and Tyler[1]; Vologdin and Drozdova[30]; Vologdin, 1966; Schopf[19]; Gutstadt and Schopf, 1969 ; Hofmann and Jackson[9]; Schopf, 1970; Schopf & Blacic[20] ; Schopf, 1972; Schopf *et al.*, 1973; McConnell[15]; Hofmann[6]; Maithy[13]; Nautiyal[17]; Cloud[2]; Walter *et al.*,[31]; Hofmann[8]; Maithy and Shukla[14]). In India, however, reports on the algal microbiota are still scarce (Maithy, 1968; Srivastava, 1971; Sajujha *et al.*, 1971; Venkatachala *et al.*[25]; Maithy and Shukla[12]). These microorganisms from the Precambrian sequences of the Kumaun Himalaya, have not been reported so far.

In the course of investigation on the presence of microorganisms in the Precambrian Gangolihat Dolomites Formation (Calc Zone) of Pithoragarh (Kumaun Himalaya), the calcareous sediments and non-calcareous phyllites yielded microscopic algal (filamentous, spheroidal) and fungal (?) remains and acritarchs in common to sporadic distribution. However, these