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Plant cytogenetics : A re-birth in twenty-first century

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Abstract

During the last few decades of the last century, emphasis on classical plant cytogenetics largely declined due to the emergence of molecular biology tools. In the realm of plant cytogenetics, this marked the end Era 1 dealing with the study of structural and numerical changes of chromosomes, and also Era 2 dealing with DNA content and composition (repetitive vs unique DNA). In Era 3, during 1980s and 1990s, molecular markers were extensively used for construction of molecular linkage maps and physical maps of chromosomes in a variety of plants. Molecular markers were also used and for the study of marker-trait associations for marker-aided selection (MAS). Later, in Era 4, starting in mid 1990s, two major areas of research took over; these were plant genomics involving whole genome sequencing (sequencing of the genomes of *Arabidopsis thaliana* and *Oryza sativa*), and plant epigenomics involving study of nuclear architecture, chromatin remodeling (chromatin remodeling complexes, and histone modifications and variants; the histone code). Some details of chromosome organization within the nucleus, and genomics/ epigenomics research are discussed in this article.

Introduction

Soon after the rediscovery of Mendel's Laws in the year 1900, the formulation of 'chromosome theory of inheritance' laid the foundation of cytogenetics, which has thus completed its first hundred glorious years in the year 2003. Subsequently, in the last few decades of the last century, the interest in plant cytogenetics that involved study and use of structural and numerical changes of chromosomes had largely declined. However, more recently, with interest in construction of molecular maps of chromosomes, and in the sequencing of whole genomes comprising an entire set of chromosomes, plant cytogenetics perhaps had a re-birth. Other areas of recent research in plant cytogenetics include study of the organization of individual chromosomes in the form of chromosome territories and location of replication and transcription territories within the nucleus. Chromosome structure has also been studied in great detail, so that the chromatin is now known to consist of nucleosome and chromatosome subunits, which undergo several levels of folding to contain it within the boundaries of the chromosomes. The structure of these nucleosome subunits has been studied at atomic resolution, and the structural relationship between adjacent nucleosomes has been recently resolved through X-ray crystallography of tetanucleosomes (Schalch *et al*., 2005). The structure of telomeres and centromeres have also been studied in several plant systems (particularly the cereals) in great detail suggesting that while structure of telomeres is conserved, but that of centromeres exhibit sufficient diversity. In recent years, it has also been shown that both DNA and histone proteins, as components of chromosomes undergo large-scale modifications, which regulate gene expression. The study of these modifications also gave birth to the science of 'epigenetics and epigenomics'. Projects like whole genome/epigenome sequencing of important plant systems are also yielding new information, giving new directions to research in plant cytogenetics. These exciting new developments in the field of plant cytogenetics will be briefly discussed in this article.

Four overlapping eras of cytogenetics research

Progress of plant cytogenetics during the last ~80 years can be broadly divided in the following four eras, which may be slightly overlapping.

Cytogenetics Era 1 (1910-1970): In this era chromosome number of a number of plant systems became known, structural changes like interachanges and inversions were studied for the first time in *Stizolobium*, *Datura* and *Oentothera*, inversions were studied in maize, and anuploids developed and cytogenetic maps constructed in several crops like maize, wheat, barley, etc. Alien addition and substitution lines were also developed in bread wheat using rye and few *Aegilops*/*Agropyron* species as the source of alien chromosomes.

Cytogenetics Era 2 (1950-1980): In this era, haploid DNA content (C-value) and composition (unique and repetitive) of nuclear DNA were determined in a large number of flowering plants using techniques of cytospectrophotometry and reassociation kinetics. This led to recognition of two versions of C-value paradox. Firstly, the DNA contents in most eukaryotes were too high for the number of genes in the corresponding taxa, as estimated on the basis of known rates of mutations, and secondly the large-scale variation in DNA contents, could not be explained with the level of difference in complexity witnessed in these different organisms. The occurrence of large proportion of repetitive DNA in each of these eukaryotic genomes partly resolved the C-value paradox (for details and references, see Gupta, 1995).

Cytogenetics Era 3 (1980-Contd.): In this era, starting in early 1980s, DNA-based molecular markers were developed and molecular maps constructed in a large number of animals and plant systems, so that these molecular markers and the corresponding maps became an important resource for a variety of research problems, including their use in diagnostics and plant breeding. During this period, another significant development was the availability of a variety of fluorescence in situ hybridization (FISH), including multicolour FISH (McFISH), chromosome orientation FISH (CO-FISH), fibre-FISH, RNA-FISH, Comparative Genomic Hybridization (CGH) and 3-D FISH(for details about molecular markers, and molecular maps, consult Gupta *et al*., 2002; Speicher and Carter, 2005)

Cytogenetics Era 4 (1995-Contd.): This era started in mid-1990s and gained momentum in the present century, with two distinct areas of cytogenetics research; first, the whole genome sequencing giving birth to 'reverse genetics', and second, the chromatin remodeling giving birth to the concept of 'histone code'. It is thus obvious that chromosome research has evolved and progressed at an incredible pace in the last two decades, more particularly during the past ten years. Since details of research done during Era 1, Era 2 and Era 3 would be widely available in text books and review articles, we mainly discuss the progress made during Era 4, in which significant progress has been made during the last few years to elucidate how the nucleosome and chromatin structure are modulated for expression of genes in time and space.

Nuclear architecture and chromosomes

Chromosome organization within the nucleus: In the past, it was believed that chromatin within the nucleus is a network, intertwined and randomly distributed within the space available in the nucleus. However, both in plants and animal systems, recent evidence has demonstrated that the nucleus is a highly compartmentalized structure (Fig. 1).

Chromosome territories and interchromatin compartment (CT-IC model): The chromatin within the nucleus is organized in the form of **chromosome territories**(**CTs**) and **interchromatin compartments** (**IC**), and hence the formulation of CT-IC model. While CTs contain individual chromosomes, IC contains macro molecular complexes that are needed for replication, transcription, splicing and repair. Newer techniques combining 3-D-FISH and computer aided deconvolution techniqueshelped in resolving the followingfeatures of chromatin organization and behaviour (i) in an interphase nucleus, each individual chromosome occupies a discrete space, called the '**chromosome territory'** or **CT** and that there is little inter twining among chromosomes; (ii)in interphase cells, each chromosome also interacts with the nuclear envelope through consistent contact points; (iii) in interphase cells, each chromosome interacts with other chromosomes through its heterochromatic regions; and (iv) in dividing cells, chromosome movements are also non-random. The above information regarding chromosome organization at the physical level has also been integrated with genetic and molecular data, to decipher the mechanisms of different nuclear processes, in which chromosomes are involved, more particularly the transcription and DNA replication.

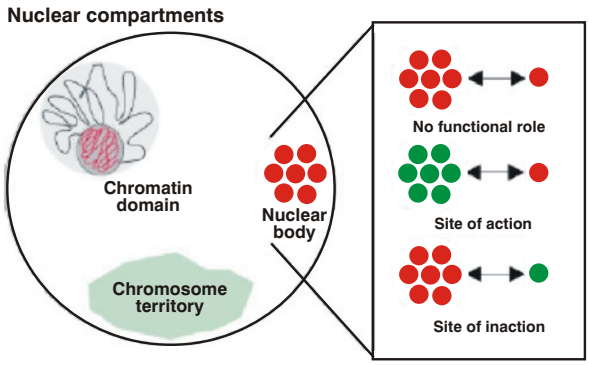

Figure 1. Compartmentalization in the mammalian nucleus. The nucleus contains proteinaceous nuclear bodies, chromatin domains including heterochromatin and euchromatin and chromosome territories. Nuclear bodies can either be non-specific aggregates, sites of nuclear processes (rRNA transcription in the nucleolus) or sites of inaction (storage of splicing components in splicing factor compartments).

Rabl organization and telomeres orientation: Chromosome segregation at anaphase results in the polarization of chromosomes because sister centromeres are pulled in opposite directions and the rest of the chromosome arms trail behind. In some instances, this anaphase arrangement of chromosomes persists into the following interphase; this is known as the **Rabl organization**, in which chromosomes have a preferentially polarized organization, with centromeres at one end of the nuclear envelope, called the **apical side**, and telomeres at the opposite end, called the **basal side**. The presence of the Rabl organization is known to vary greatly between species and among tissues or developmental stages of an organism. In plants, it is generally observed in species with bigger genomes like those of wheat, rye, barley, and oats, but not in species with smaller genomes like those of sorghum and rice. Maize with intermediate size of genome displayed neither entirely Rabl nor entirely random chromosome organization.

Bouquet and telomeres clustering: The bouquet is the clustering of chromosome ends on the nuclear envelope (NE) during meiotic prophase, coincident with the initiation of homologous chromosome synapsis. The bouquet has been extensively described in many species in all eukaryotic groups and has been

proposed as an aid to presynaptic alignment of homologous chromosomes. The similarity of the bouquet to the **Rabl conformation** has long been noted. However, it is clear that the two are not the same, although similar functions (chromosome pairing, recombination initiation and SC formation) have been assigned to both of them.

Relation between chromatin structure/ organization and transcription

Heterochromatin and euchromatin: We know that the chromosomes of eukaryotes consist of darkly stained heterochromatin and lightly stained euchromatin. According to the classical view, heterochromatin is transcriptionally inactive and euchromatin is active. This view is changing now; following are some examples: (1) there is evidence that several repeats within heterochromatic centromeres are transcribed to produce siRNA; (ii) it has been show that in each of the five chromosomes of *Arabidopsis*, heterochromatin is largely confined to the pericentromeric regions, and mainly consists of 180-bp satellite repeats, and retrotransposons (mainly from *Athila* family);(iii) NORs, mainly consisting of repetitive DNA, carries thousands of kilobases of tandemly repeated ribosomal DNA encoding rRNA.

It has now been conclusively proved that in eukaryotic chromatin, cytosine methylation in repetitive DNA and distinctive modifications of individual histone proteins are frequent and cause heterochromatin formation. There are at least three kinds of methylases causing cytosine methylation in DNA: (i) Dnmt-1 type DNA methylase adds methyl-residues to cytosines (CG or CNG?) on newly synthesized strand of a DNA duplex, by using information from the conserved parental DNA strand carrying met-C, thus making DNA methylation heritable; (ii) Dnmt-3 type DNA methylase brings about DNA methylation (CG or CNG?) *de novo*, so that unmethylated DNA becomes methylated on both strands; (iii) chromomethylases (unique to plants) in *Arabidopsis* and maize recognize CpNpG sequences and bring about cytosine methylation.

Transcription and replication factories : A new concept

In recent years, specific regions have been identified within the eukaryotic nucleus, where replication and transcription takes place. It has also been shown that transcription sites are spatially distinct from replication sites. Therefore, we need to realize thattranscription by Pol II is not homogeneously distributed throughout the nucleoplasm, but occurs at highly enriched Pol II foci, known as transcription factories, which contain most of the hyperphosphorylated, elongating form of Pol II. Similarly replication takes place in 'replication factories'. It has been demonstrated that the chromosome segments destined to be transcribed or replicated have to move physically to these regions for transcription or replication to take place. Peter Fraser and his co-workers from Cambridge described this subject in some detail (Chakalova *et al*., 2005).

Transcription factories: A transcription factory is generally 80 nm tripartate structure mainly containing three spatially contiguous regions, with the template, the RNA polymerase II (Pol II) and the newly synthesised mRNA. The Pol II is perhaps attached to the transcription site, so that the template would move along RNAP II rather than the Pol II tracking along the template. Some translation also appears to be coupled with transcription at transcription factories

There are fewer transcription factories than there are active genes and other transcription units in the nucleus, so that that more than one active gene is transcribed in each factory. And actively transcribed genes that are separated by long distances frequently co-localize in the same transcription factory. It has also been shown that actively transcribed genes co-localize with transcription factories, whereas identical, temporarily non-transcribed alleles, which can often be in the same cell, do not. Therefore, the 'on' state correlates with factory occupancy and the 'off' state with relocation away from factories. The specific Thus the specific nuclear repositioning of genes is correlated with transcriptional activation, silencing and replication timing.

The fact that different genes frequently co-occupy the same factory provides strong evidence that genes do not assemble their own transcription sites *de novo* when they become active, but instead migrate to preassembled transcription sites. A stable factory implies that genes or transcription units would essentially be pulled through a factory, rather than polymerases moving along the chromatin fibre, as is commonly believed. The finding that approximately 15% of the genome is transcribed — although probably not all at once — indicates that an extraordinarily large part of the genome passes through the limited number of transcription factories in a cell nucleus. This must have a profound effect on the nuclear organization of the genome.

Replication factories: It has been shown that a segment of DNA that needs to be replicated temporarily disengages Pol II and ceases transcription, although transcription in other regions of the genome continues uninterrupted throughout S phase. This segment of DNA needs to relocate itself in a replication factory. There seems to be no overlap between replication and transcription sites/factories.

Chromatin remodeling

The term "chromatin-remodeling" generally refers to changes in histone-DNA interactions in nucleosomes. Histone proteins are modified and non-histone chromosomal proteins (e.g. high mobility group nuclear

proteins = HMGN proteins; heterochromatin protein = HP1) are indirectly involved in the modification and activity of chromatin. Histone-DNA interactions in the nucleosomes are also modulated (remodeled) to facilitate interaction of other factors (not involved in remodeling) with DNA template. It is believed that the histone octamers of nucleosomes are often displaced from the enhancer and promoter regions by chromatin remodeling complexes to allow access of a variety of factors to DNA. During 1990s, evidence for chromatin remodeling and cellular memory became available in yeast (*Saccharomyces cerevisiae*), fruitfly (*Drosophila melanogaster*), and mammals, through genetic and biochemical studies.However, later towards the end of the last century and in the early years of he present century, chromatin remodeling has been studied and factors associated with this phenomenon in plant systems like *Arabidopsis*.

Activities catalyzed by chromatin remodeling: Chromatin-remodeling factors can catalyze the following activities: (i) mobilization and repositioning of nucleosomes, (ii) transfer of a histone octamer from a nucleosome to a separate DNA template, (iii) the facilitated access of nucleases (enzymes) to nucleosomal DNA, (iv) creation of di-nucleosome-like structures from mono-nucleosomes, (v) generation of superhelical torsion in DNA, (vi) disruption of histone-DNA contacts; and (vii) assembly and disassembly of nucleosomes.

Components of chromatin remodeling complexes: Three major strategies are used for chromatin remodeling. Each strategy makes use of a different set of proteins, described as components of chromatin remodeling complexes. Following are the three classes of chromosome remodeling complexes/components: (i) ATP-dependent chromatin remodeling complex, which makes use of ATP for chromatin modification through mere histone-DNA interactions. (ii) Histone modifying enzymes, which are used for post-translational modifications of histones (mainly acetylation and methylation); these modifications create signals that define the so-called 'histone code'. (iii) Variants of the histones (H2A, H2B, H3, H4 and H), which are synthesized and incorporated into nucleosome subunits (in place of normal histones), bringing about chromatin remodeling. More recently, the extreme use of H3 histone variants also led to the formulation of 'H3 barcode hypothesis' (Hake and Allis, 2006).

ATP-dependent chromatin remodeling machines/ complexes: ATP-dependent :chromatin remodeling complexes (CRCs); also called chromosome remodeling machines (CRM), consist of ATPase polypeptide subunits and also non-ATPase subunits. Although ATPases play an major important role in chromatin remodeling, non-ATPase components also have a role to play. The ATPases can be quite diverse and not all ATPases are important for chromosome remodeling (see below). These ATPases are classified into three superfamilies, each superfamily having several families. One of these families belongs to a specific class of ATPases, described as 'Snf2-like family of ATPases', which are relevant to chromosome remodeling and therefore will be discussed in some detail.

(*a*) *ATPases as subunits of ATP- dependent complexes:* Members of SNF2-like family of ATPases are classified in several subfamilies, depending upon which protein motifs outside the ATPase region they have. Atleast seven subfamilies are known, but only four subfamilies are important, which include the following: (i) Swi/Snf2 subunit of the SWI/SNF complex; (ii) ISWI (ISWI, hSNF2H, hSNF2L, yISW1, yISW2; h stands for human, and y stands for yeast), (iii) CHD1 (CHD1, Mi-2 /CHD3, Mi-2 /CJD4, Hrp1, Hrp3), (iv) INO80 (CSB, Rad 26, ERCC6).

(*b*) *Non-ATPase subunits of ATP- dependent complexes:* The ATP dependent chromatin remodeling complexes also have non-ATPase subunits, which may have the following functions: (i) enhance or regulate motor activity of ATPase subunits; (ii) mediate other specialized functions that are not related with chromatin remodeling– these may be recruited to promoters via interactions with sequence specific transcription factors (TFs). Rad54 is also an example, in which SNF2-like ATPase is programmed by another polypeptide. Rad54 and Rad51 (related with bacterial RecA protein) catalyze homologous strand pairing.

Chromosome remodeling through histone modifications: The 'histone code': In eukaryotes the fundamental unit of chromatin is the nucleosome, which is a protein octamer/DNA complex composed of 200 bp wrapped around a histone octamer that consists of two molecules each of four core histones, H2A, H2B, H3 and H4. One molecule of linker histone H1 is also associated with each nucleosome. Crystal structure of the core particle of this nucleosome unit consisting of 146 bp of DNA wrapped around histone octamer was determined in late 1990s at 2.8 A resolution (Luger *et al*., 1997).More recently, crystal structure of a tetranucleosome was also determined at 9 Å resolution to understand the manner of higher-order folding of the nucleosome sub-units (Schalch *et al*., 2005).

It was also shown in several recent studies that amino-terminal tails of histone proteins are targets for a series of post-translational modifications (PTMs), including acetylation, phosphorylation, and methylation. These modifications regulate chromatin structure and gene expression (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Turner, 2002). Multiple histone modifications in various combinations are thought to

form a 'histone code', since these modifications extend the information capacity of the associated DNA (Table 1)(Strahl and Allis, 2000). For example, histone H3 and H4 acetylation is consistently associated with transcriptionally active euchromatin, while methylation can be associated with either active or inactive chromatin depending on the residue involved in methylation. For instance, methylation at H3K4, H3K36 and H3K79 are hallmarks for active transcription, whereas methylation at H3K9, H3K27 and H4K20 are correlated with transcriptionally inert heterochromatin (Fischle *et al.,* 2003; Lachner *et al*., 2003; Margueron *et al*., 2005; Peters and Schubeler, 2005). Further, the lysines in histone N-terminal tails can be mono-methylated (me1), di-methylated (me2) or tri-methylated (me3), and each methylation state may have unique biological functions, increasing the potential complexity of the histone code (Dutnall, 2003).

Table 1. Histone modification combinations and their effect on transcription

Nature of histone modification	Effect on transcription
Histone acetylation	Transcription activation
Histone methylation	
H3K4, H3K36 and H3K79	Transcription activation
H3K9, H3K27 and H4K20	Transcription silencing

Table 2. Various combinations of histone methylations and their effect on state of chromatin condensation

Degree of methylation	State of chromatin
H3K9me3, H3K27me1 and H4K20me3	Highly condensed heterochromatin
H3K9me1, H3K9me2 and H3K27me3	Lightly condensed heterochromatin

It has been shown that methylation of H3K9, H3K27 and H4K20 is involved in hetero-chromatinization, but the degree of methylation of each of these lysine residues determines the degree of heterochromatinization (Table 2) (Peters *et al*., 2003; Schotta *et al*., 2004). For instance, H3K9me3, H3K27me1 and H4K20me3 mark the most deeply stained regions while H3K9me1, H3K9me2 and H3K27me3 mark the less condensed heterochromatin (Peters *et al*., 2003; Plath *et al*., 2003; Rice *et al*., 2003; Silva *et al*., 2003; Schotta *et al*., 2004; Okamoto *et al*., 2004). Several reports indicate that the mono- and di-methylated forms of H3K9 and H3K27 are enriched in heterochromatin (Jackson *et al*., 2004; Lindroth *et al*., 2004; Mathieu *et al*., 2005; Naumann *et al*., 2005), although degree of methylation also depends on the genome size (Houben *et al*., 2003). For instance, unlike in animals, in *Arabidopsis*, H3K27me3 is associated with euchromatin and H3K9me3 is extremely rare (Lindroth *et al*., 2004; Mathieu *et al*., 2005; Naumann *et al*., 2005). There is also evidence that H4K20 methylation is also present in *Arabidopsis* (Naumann *et al*., 2005; NG *et al*., 2006). It is not clear, however, whether there is a direct relationship between heterochromatin and histone methylation. However, in plants with large genomes including maize and barley and wheat, very little is known about histone methylation (Houben *et al*., 2003), which makes up the bulk of the angiosperms (Arumuganthan and Earle, 1991).

In a recent study, quantitative distribution of mono-, di-, and trimethylation at H3K9 and H3K27 was examined in maize on whole genome level using oachytene chromosomes. The data reveal that three marks (H3K9me1, H3K27me1, and H3K27me2) correlate with DAPI (DNA) staining, but that only H3K27me2 is specifically enriched in condensed areas. It was also observed that H3K9me2 is not abundant in heterochromatin, but is instead enriched between chromomeres along with H3K4me2. The study also reported that centromeres contain H3K9me2 and H3K9me3; that H3K27me3 occurs at several brightly focused euchromatic domains, and that H4K20 methylation is rare or absent.

The Histone Code Hypothesis states that chromatin-DNA interactions are guided by combinations of histone modifications. For example, phosphorylation of serine residues 10 and 28 on H3 is a marker for chromosomal condensation; similarly, phosphorylation of serine residue 10 and acetylation of residue 14 on H3 is a tell-tale sign of transcription.

Chromosome remodeling through histone variants: 'H3 barcode hypothesis': In addition to post-transcriptional modifications (PTM) of histones, a number of variants are known for histone proteins that are found in the core particle of the nucleosome (Henikoff *et al*., 2004). Perhaps H4 is the only exception, which does not seem to have any variant, but H3 and H2A are the two major classes of histones, which exhibit higher variation relative to H2B histone. Each histone variants differs from its corresponding normal histone in only a few amino acid residues (for a review, see Pusarla and Bhargava, 2005), and each histone variant plays an important role in chromatin remodeling. In particular, the histone variants for H3 offer the best example to illustrate the role of histone variants in chromatin remodeling, so that a H3 barcode hypothesis has also been formulated recently to describe the role of histone H3 variants (Hake and Allis, 2006).

The histone variants are generally synthesized directly from genes encoding them, but may also result

due to post-transcriptional modification of conventional histones. The genes encoding histone variants can be broadly classified into replication dependent (RI), replication independent (RI) and tissue-specific (TS), so that at least some of these variants occur in a tissue specific or developmental stage-specific manner. These histone variants replace the normal histones, and can be incorporated into the chromatin at any time during the cell cycle, although the conventional nucleosomes are produced and assembled into nucleosomes only during the S-phase of the cell cycle.

Whole genome sequencing : genomics and epigenomics

In the year 2000, with the publication of the whole genome sequence of the crucifer weed, thale cress (*Arabidopsis thaliana*), plant cytogenetics entered into a new era of research, the era of plant genomics. Consequently in the early years of the present century, whole genome sequences of rice and a draft sequence of the genome of poplar (*Populus trichocarpa*) became available (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). Sequencing of several other plant genomes is also in progress (as listed at NCBI site) According to some, the first wave of plant genome sequencing is over, and we are now entering a new era in plant genomics research. In this new era, genomes of many model species with small genomes or those of species of economic importance will be sequenced. Also the available sequences will be subjected to annotation (assigning functions to these sequences), and the choice of new genomes to be sequenced will be made on several criteria, including phylogeny (Jackson *et al*., 2006). The genomes of crops like maize and wheat will also be subjected to identification of gene-rich regions (GRRs), which will then be taken up for sequencing. There are also new technologies that will change the way we approach future genome sequencing projects.

As discussed earlier in this article, DNA methylation, nucleosome remodeling (including histone modification and histone variants), and noncoding RNAs can organize chromatin into accessible ('euchromatic') and inaccessible ('heterochromatic') sub-domains. This extends the information potential of the genetic code, and one genome can generate many 'epigenomes' in time and space, during the life-span of an organism. The implications of epigenetic research are far reaching, so that efforts are being made to study the epigenomes in a variety of eukaryotes including some plant systems. In a recent study, it was shown that these epigenetic modifications are not as conserved as was once thought. Further, very little is known about histone methylation in large genome plants (Houben *et al*., 2003), which make up the bulk of the angiosperms (Arumuganthan and Earle, 1991).

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