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REVIEW PAPER

# Tidying-up the plant nuclear space: domains, functions, and dynamics

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## Abstract

**Understanding how the packaging of chromatin in the nucleus is regulated and organized to guide complex cellular and developmental programmes, as well as responses to environmental cues is a major question in biology. Technological advances have allowed remarkable progress within this field over the last years. However, we still know very little about how the 3D genome organization within the cell nucleus contributes to the regulation of gene expression. The nuclear space is compartmentalized in several domains such as the nucleolus, chromocentres, telomeres, protein bodies, and the nuclear periphery without the presence of a membrane around these domains. The role of these domains and their possible impact on nuclear activities is currently under intense investigation. In this review, we discuss new data from research in plants that clarify functional links between the organization of different nuclear domains and plant genome function with an emphasis on the potential of this organization for gene regulation.**

**Keywords:** 3D Chromatin organization, chromocentres, gene expression, liquid–liquid phase separation (LLPS), nuclear domains, nuclear bodies, nucleolus, nuclear periphery, telomeres, topologically associated domains (TADs).

Abbreviations: CB, Cajal body or coiled body; CENH3, centromere-specific histone H3 variant; CRWN, CROWDED NUCLEI; FISH, fluorescence *in situ* hybridization; GISH, genomic *in situ* hybridization; HP1, Heterochromatin Protein 1; IDR, intrinsically disordered region; LAD, lamina-associated domain; LLPS, liquid–liquid phase separation; NAD, nucleolar-associated domain; NB, nuclear body; NE, nuclear envelope; NMCP, nuclear matrix constituent protein; NOR, nucleolus organizing region; NP, nuclear periphery; NPC, nuclear pore complex; NUP1/136, NUCLEOPORIN1/136; PB, photobody; Pc, Polycomb; PcB, Polycomb body; PcG, Polycomb-group; PRC, Polycomb Repressive Complex; PLAD, plant lamina-associated domain; PWO1, DOMAIN INTERACTOR OF POLYCOMBS1; TAD, topologically associated domain; TrF, transcription factory; SpS, splicing speckle; TERRA, TElomeric Repeat-containing RNA; TRB, TELOMERE REPEAT BINDING; VRN1, VERNALIZATION 1.

## Introduction

In eukaryotic cells, the genetic information is encoded by DNA, which can be several metres long, that needs to be packaged to fit into the cell nucleus. However, the function of the nucleus goes much further than just being a simple packaging entity. The three-dimensional (3D) organization of the interphase nucleus remained unknown for a long time and only with substantial improvements in microscope resolution and *in situ* staining techniques was it possible to visualize distinct chromatin domains and chromosome territories in the interphase nucleus (Fig. 1) (Cremer and Cremer, 2001, 2010). Historically, insights into the position and organization of chromatin domains and chromosomes within the plant cell nucleus come from visual approaches, such as fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) (reviewed in Santos *et al.*, 2015). In plants, the observation of chromosome territorial organization was not straightforward mainly due to the complexity of plant genomes with a high amount of dispersed repetitive sequences and the insufficient signal intensity of short unique sequences which hampered the understanding of plant territorial chromatin organization. Pioneer studies involving the ingenious implementation of GISH in interspecific and intergeneric hybrids (e.g. wheat/rye translocation or addition lines) made possible the visualization of chromosome territories in plant cell nuclei (Fig. 1) (Schwarzacher *et al.*, 1992; Aragon-Alcaide *et al.*, 1997; Bass *et al.*, 2000). These studies were based on labelling introgressed chromatin and only later was a 'true chromosome painting' done in *Arabidopsis thaliana* (Arabidopsis) and its close relative *A. lyrata* by using chromosome-specific probes (Fransz *et al.*, 2002). More recently, the use of synthetic oligonucleotide libraries enabled whole-chromosome oligo-FISH paints of maize chromosomes to be obtained (Albert *et al.*, 2019).

The higher order organization level of chromatin into functional nuclear domains has the potential to deeply affect gene regulation (reviewed in Adriaens *et al.*, 2018; Groves *et al.*, 2018). Some nuclear domains such as the nucleolus and large-scale heterochromatin foci (chromocentres) are well recognized by phase contrast microscopy due to differential DNA

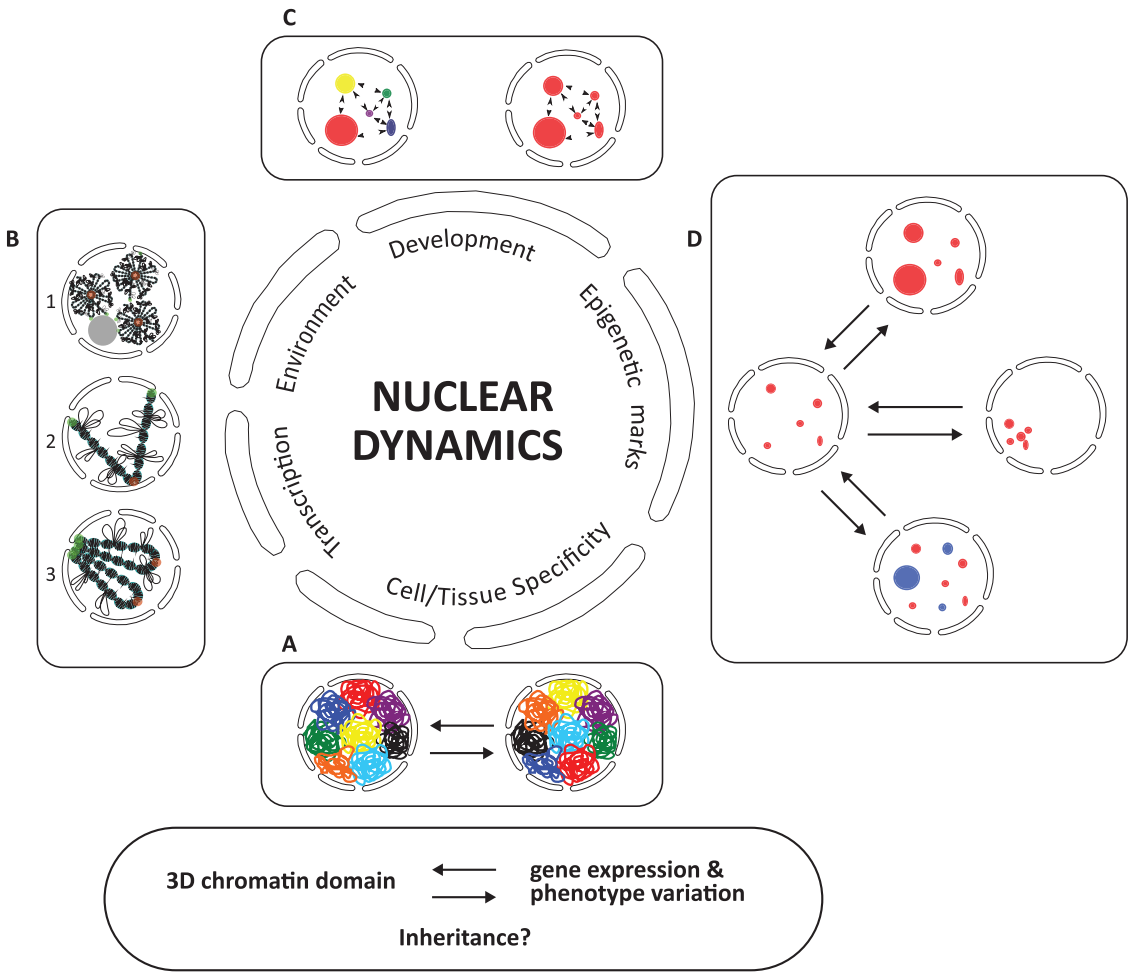


**Fig. 1.** 3D Nuclear organization and dynamics. Schematic representation of chromatin plasticity subjacent to transcriptional requirements, environmental responses, cell and tissue specificities, developmental stages, and epigenetic marks. (A) Flexible organization of chromosome territories. (B) Flexible positioning and organization of genes, chromocentres (red dots), and telomeres (green dots) inherent to distinct models of chromosome configurations: rosette-like configuration (1); Rabl configuration (2); and bouquet configuration (3). (C) Possible crosstalk between different nuclear domains may occur as part of nuclear dynamics (reviewed in Kumaran *et al.*, 2008). (D) Number, size, formation/disappearance, and organization of nuclear domains may change in response to different stimuli.

concentration or protein density. Other domains, such as nuclear bodies (NBs) enriched in regulatory proteins [e.g. Cajal bodies (CBs) or Polycomb bodies (PcBs)], can only be detected by immunostaining with domain-specific antibodies. In addition, the nuclear envelope (NE) and the underlying lamina have their own space and organization within the 3D nuclear structure. All nuclear subdomains have in common the lack of a membrane structure confining their space, yet each NB forms a discrete nuclear compartment exerting a specific function. Other nuclear structures, such as the telomeres, despite not being bona fide subdomains, also have a demonstrated special organization and function within the nucleus.

Furthermore, innovative approaches based on large-scale chromosomal capture approaches, in combination with deep profiling and high-throughput sequencing, make it feasible to annotate topologically associated domains (TADs) within the chromatin and even to identify TADs from a more specific perspective, namely nucleolar-associated domains (NADs) and lamina-associated domains (LADs) (Dixon *et al.*, 2012; Feng *et al.*, 2014; reviewed in Nicodemi and Pombo, 2014; Ciabrelli and Cavalli, 2015; Pontvianne and Grob, 2020). Moreover, chromatin domains often show specific epigenetic modification patterns and transcriptional states, suggesting that the higher level of chromatin regulation does entail a functional role. It remains a challenge to understand the mechanisms assisting chromatin organization and the extent of chromatin flexibility in response to specific transcriptional requirements or environmental stress conditions. The exposure to stress factors can trigger changes in large-scale genome organization including rearrangements of chromatin structure and spatial nuclear organization (reviewed in Probst and Mittelsten Scheid, 2015; Santos *et al.*, 2017) as predicted by McClintock (1984). Still much of the evidence has been gained from studies in metazoans and much less attention has been given to the details of nuclear organization in plants. While there are several organizing principles partially conserved between plants and animals, recent studies have started to provide details regarding specificities of plant nuclear organization. For instance, plants show several exclusive chromatin-associated characteristics, including plant-specific histone variants (reviewed in Zambrano-Mila *et al.*, 2019), novel DNA- and histone-modifying enzymes, plant-specific histone modifications (reviewed in Feng and Jacobsen, 2011), as well as a plant-specific lamina-like network at the nuclear periphery (Poulet *et al.*, 2017*b*). These specificities of chromatin organization and epigenetics make plants a very interesting model to study the plasticity of nuclear dynamics.

Our aim here is to give an overview of the different nuclear domains in plants, as well as to provide some insights into how they may be organized without the requirement of a membrane to compartmentalize them from surrounding domains and how they may impact gene expression.

## Nuclear bodies

Originally detected as cytological structures, NBs are membrane-less functional compartments interspersed in the nucleoplasm, that are now recognized to participate in the spatiotemporal control of various specialized nuclear processes (i.e. transcription, RNA processing, DNA replication, DNA repair, protein degradation, and signal transduction) (reviewed in Del Prete *et al.*, 2014; Guo and Fang, 2014; Staněk and Fox, 2017; Shah *et al.*, 2018). Various names are commonly used for NBs, such as foci, speckles, or paraspeckles—these terms are usually not clearly defined and are based on original cytological observations before functional elements were identified, and sometimes may refer to the size or organization of the observed pattern. Most of them are dynamic structures depending on physiological, developmental, or stress conditions (Fig. 1) (Boudonck *et al.*, 1999; reviewed in Reddy *et al.*, 2012; Scarpin *et al.*, 2013; Kim *et al.*, 2019). Beyond their diversity, a common feature of NBs is their role in the formation of specific microenvironments, characterized by supramolecular assembly of proteins, in some cases accompanied by RNA molecules. The local concentration of NB components contributes to the molecular crowding-increased binding rate and low diffusion rate, which favour biochemical reactions (reviewed in Zhu and Brangwynne, 2015). Thus, NBs are centres for enzymatic reactions but also sequestration, storage, modification, recycling, or degradation of proteins. Some of them play a hub role in scaffolding and recruiting genomic regions with similar regulation, thus participating in the 3D genome organization. The nucleolus, as the largest NB, is discussed in the following section of this review.

PcBs, first described in animal cells, are marked by local accumulation of Polycomb-group (PcG) proteins. PcBs concentrate distant transcriptionally inactive PcG-targeted genomic regions that form intra- or interchromosomal interactions also detectable by chromatin conformation capture techniques (reviewed in Matheson and Elderkin, 2018; Loubiere *et al.*, 2019). Unlike in animals, PcG-targeted regions are more dispersed throughout the plant genome (reviewed in Del Prete *et al.*, 2015). Nevertheless, genomic regions targeted by the Arabidopsis PcG protein LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) tend to be organized locally in clusters along the chromosomes (Molitor *et al.*, 2016). In the nuclear space, LHP1 and several Arabidopsis PcG proteins form foci when transiently overexpressed in heterologous systems [LHP1 (Gaudin *et al.*, 2001), CURLY LEAF (CLF) (Hohenstatt *et al.*, 2018), VERNALIZATION 2 (VRN2) (Gendall *et al.*, 2001), and EMBRYONIC FLOWER 1 (EMF1) (Calonje *et al.*, 2008)]. Such foci were also observed in complemented conditions for LHP1 (Kotake *et al.*, 2003) whose pattern is dependent on cell differentiation (Libault *et al.*, 2005), similarly to animal PcGs (Ren *et al.*, 2008; Kundu *et al.*, 2017). A non-uniform nuclear distribution is adopted by the protein PWWP-DOMAIN INTERACTOR OF POLYCOMBS1 (PWO1) under conditions of native promoter-driven expression in Arabidopsis (Mikulski *et al.*, 2019). Finally, a physical clustering of repressed alleles of the Polycomb Repressive Complex (PRC) 2 target *FLOWERING LOCUS C* (*FLC*) was reported in Arabidopsis interphase nuclei, which relies on PHD–PRC2 components (Rosa *et al.*, 2013). These data show that clustering of PcG proteins and their genomic targets also occurs in plants, implying that PcBs are likely to be common

features of animal and plant nuclei. The functional implications of PcBs and compact domains of PcG-targeted genes in PcG repression or other processes remain debated in animals (reviewed in Matheson and Elderkin, 2018), and further investigations are required in plants. MORC (Microrchidia) family ATPase-enriched NBs appear to be another type of Arabidopsis NB associated with silencing, implicated in repression of DNA-methylated pericentromeric genes (Moissiard *et al.*, 2012) or unmethylated pathogen-responsive genes (Harris *et al.*, 2016).

Components of the transcription machinery in animals were originally described as concentrated aggregates within the nucleus and were named transcription factories (TrFs) (Buckley and Lis, 2014). A dispersed and reticulated distribution of RNA polymerase II was reported in Arabidopsis (Schubert and Weisshart, 2015). TrF definition may evolve with microscopy and live imaging development (Buckley and Lis, 2014). TrFs were recently proposed in wheat (Concia *et al.*, 2020).

Cajal bodies (CBs, or coiled bodies) are dynamic substructures, which are found in animal as well as plant cells (Boudonck *et al.*, 1998, 1999; reviewed in Gall, 2000). CBs are enriched for splicing components such as small nuclear ribonucleoproteins (snRNPs) and are usually associated with the conserved coilin (Collier *et al.*, 2006; reviewed in Machyna *et al.*, 2015), both being structural scaffolds for CB formation (reviewed in Bassett, 2012; Staněk and Fox, 2017; Ohtani, 2017). CBs are involved in maturation of spliceosome snRNPs, snRNA chemical modifications, and RNP export complex assembly. Recent data support a role for CBs in the topological organization of spliceosomal snRNA and histone genes in inter- and intrachromosomal gene clusters and in their transcriptional regulation ( Sawyer *et al.*, 2016; Wang *et al.*, 2016). In addition, CBs contribute to telomere maintenance, ribosome biogenesis, or stress responses via poly(ADP ribose) polymerase interactions (Kotova *et al.*, 2009; reviewed in Boulon *et al.*, 2010; Love *et al.*, 2017). Plant CBs have additional functions in siRNA and miRNA processing (Pontes and Pikaard, 2008; Scarpin *et al.*, 2013; Shaw *et al.*, 2014). In plants, not all snRNP-containing bodies resembling CBs in size and shape are associated with the presence of coilin and it remains debated whether these should be classified as CBs (Pontes and Pikaard, 2008; Pontes *et al.*, 2013; Love *et al.*, 2017). Among them are splicing speckles (SpSs) and nuclear dicing bodies (D-bodies). SpSs are general sites of storage and assembly of splicing regulators in regions of active transcription, and are conserved in animals and plants. In addition to splicing factors, they also concentrate RNA polymerase II subunits, transcription elongation and polyadenylation factors, and chromatin proteins, suggesting broader functions (reviewed in Galganski *et al.*, 2017). Located in interchromatin regions, SpSs are dynamic and mobile depending on transcriptional activity, cell differentiation, or metabolic state (Docquier *et al.*, 2004; Kim *et al.*, 2019; for reviews see Reddy *et al.*, 2012; Galganski *et al.*, 2017). D-bodies are plant-specific NBs that are centres of pri-miRNA processing and contain microprocessor complex components including DICER-LIKE 1 or HYPONASTIC LEAVES 1 (Fujioka *et al.*, 2007; Fang and Spector, 2007; reviewed in Dolata *et al.*, 2018).

NBs can also form upon environmental or developmental stimuli. In plant nuclei, DNA damage and repair proteins form distinct foci upon DNA damage that are dynamic (Friesner *et al.*, 2005). The RAD54-marked foci, for instance, tend to locate at the nuclear periphery during DNA repair (Hirakawa and Matsunaga, 2019). Recently, antiviral immunity-related NBs were also found in plants, such as NBs formed with the WW-domain protein AtWWP1, which sequester viral nucleoprotein complexes in the nucleus and prevent their nucleoplasmic trafficking (Calil *et al.*, 2018). The plant-specific photobodies (PBs) are enriched in photoreceptors, light signalling, and proteasome degradation components, transcription regulators, or splicing factors that are involved in light-induced photoreceptor sequestration, photomorphogenesis inhibitor protein degradation, and light-responsive transcript processing (Bauer *et al.*, 2004; Galvão *et al.*, 2012; Kaiserli *et al.*, 2015; Xin *et al.*, 2017, 2019). PB assembly and dynamics correlate with plant growth and developmental processes in response to light signals (reviewed in Chen and Chory, 2011; van Buskirk *et al.*, 2012; Klose *et al.*, 2015). PB biogenesis depends on light signalling component interactions which can individually nucleate *de novo* formation of the PBs (Liu *et al.*, 2014). Recently, several novel factors required for the formation of PHY-B-containing PBs have been identified (Qiu *et al.*, 2017; H. Huang *et al.*, 2019; Yoo *et al.*, 2019). Phytohormone-induced NBs were reported, but remain poorly characterized (Ng *et al.*, 2004; Riera *et al.*, 2006).

Despite significant progress, many questions remain to be addressed regarding plant NB structure and functions, which may reveal originalities, connected for instance to the different subcellular sequestration of processes conserved in plants and animals, such as siRNA/miRNA processing or nonsense-mediated decay (reviewed in Pontes and Pikaard, 2008). Specific features of plant NBs such as higher plasticity and/or redundancy of NB components are also hinted at by the absence of phenotype effects upon depletion of coilin (Collier *et al.*, 2006) despite drastic effects of its depletion in animals (Walker *et al.*, 2009). With the development of high-resolution microscopy tools as well as biochemical approaches, the composition, classification, and substructure of plant and animal NBs are expected to evolve with expansion of the NB repertoire and redefinitions of NBs in terms of detailed structural or functional qualities. For instance, besides the internal nucleolar organization, subcompartmentalization has been reported for smaller NBs such as paraspeckles and speckles (Mintz and Spector, 2000; Hall *et al.*, 2006; West *et al.*, 2016; Fei *et al.*, 2017). This highlights a higher level of structural complexity of the cell nucleus that may account for the multitude of functions associated with certain NB types and opens up further questions regarding NB substructure dynamics. Improvement of live imaging and tracking techniques is expected to shed new light on NB distribution, mobility, and function. Recently, long-range, directional, actin-independent motion of speckles within chromatin-depleted channels highlighted a novel nuclear trafficking mechanism (Kim *et al.*, 2019). A novel technique allowing identification of NB-associated genomic regions (Baudement *et al.*, 2018) can help in addressing the interplay between the NB structure and 3D genome organization.

How NBs are assembled is a challenging interdisciplinary question addressed by cellular and structural biologists, biophysicists, and modellers. Assembly models are currently proposed. The first model is based on a sequential and ordered assembly of the NB components anchored to major scaffolding NB proteins or RNA molecules. The second model favours stochastic processes as contributing to the random assembly of different components in a self-organizing manner, which can start with any NB component (reviewed in Matera *et al.*, 2009; Mao *et al.*, 2011). However, the observed non-random organizations suggest more complex rules and question the physicochemical forces driving NB assembly.

## Chromocentres

In a chromosome, the centromeres together with the nearby chromatin regions can form heterochromatin structures that remain condensed during interphase (Heitz, 1931). In Arabidopsis, the term chromocentres refers to centromeric and pericentromeric regions forming heterochromatic domains during interphase (Fransz *et al.*, 2002). In other species, such as *Drosophila*, the term chromocentres originally and more formally refers to the congregation of pericentromeric heterochromatic regions from different chromosomes forming a small number of chromocentres (Jagannathan *et al.*, 2019). In maize, heterochomatic knobs are distal, satellite tandem repeats, not centromeric, and fit the 'staining' definition of chromocentres, but are not called chromocentres because they are not aggregations of centric heterochromatin (reviewed in Gent *et al.*, 2017). In general, centromeres are enriched in satellite repeats and retrotransposons (Nagaki *et al.*, 2003; Tek *et al.*, 2010; reviewed in Malik and Henikoff, 2009).

Cytologically, DNA staining intensity by DAPI has been used to visualize chromocentres which are generally round shaped, highly condensed, and heavily stained prominent structures (Fransz *et al.*, 2002; Tek *et al.*, 2011). Usually the boundaries of actual centromeres and pericentromeric regions within the chromocentres are not clearly defined, although in well-studied plants such as Arabidopsis this distinction has already been made (reviewed in Simon *et al.*, 2015). Specifically, in Arabidopsis, within the whole array of 180 bp centromeric DNA repeats, only a limited portion is recognized by immunolabelling with HTR12 protein, a homologue of the centromere-specific histone H3 variant (CENH3) (Shibata and Murata, 2004).

Although the DNA composition of chromocentres is broadly known, their function remains elusive (reviewed in Simon *et al.*, 2015; Jagannathan and Yamashita, 2017). More recently, new models of possible functions have proposed that chromocentre formation could be involved in the maintenance of the eukaryotic genome (reviewed in Jagannathan and Yamashita, 2017). Nevertheless, because of their tractability, chromocentres have largely been used as targets to study genome structure and organization. In Arabidopsis, the chromocentres tend to be preferentially located at the nuclear periphery (Fransz *et al.*, 2002; Pecinka *et al.*, 2004). It is from the chromocentres that chromatin loops (0.2–2 Mb in length) emanate, giving rise to a rosette-like interphase chromosome configuration (Fransz *et al.*, 2002) (Fig. 1). Contrastingly, in larger genomes such as wheat, chromocentres were not described, and centromeres were visualized by FISH as being polarized on one side of the nucleus to establish a special organization known as the Rabl configuration of interphase chromosomes (Santos and Shaw, 2004) (Fig. 1). These studies illustrate that centromere positioning in mitotic anaphase results in establishing the polarized Rabl nuclear arrangement, thereby affording centromeres more opportunities for interaction than would occur if chromosomes were randomly distributed. The morphological variability of chromocentres present in different Arabidopsis ecotypes has also been used to determine the genetic factors governing chromocentre formation and maintenance, as it was possible to determine several genetic loci affecting chromocentre structure. These results indicate the involvement of complex genetic mechanisms in chromatin organization (Snoek *et al.*, 2017).

Chromocentres exhibit epigenetically distinct chromatin features. The most obvious property of chromocentres is their heavy DNA methylation, as evidenced by immunolabelling with 5-methylcytosine antibodies (Fransz *et al.*, 2002). Also H3K9me2 levels were increased at Arabidopsis chromocentres (Zhang *et al.*, 2008). On the other hand, histone marks related to transcriptionally active chromatin, such as the acetylation of histone H4 (H4K5ac and H4K8ac), were not detected at chromocentres (Fransz *et al.*, 2002), reinforcing the idea of transcriptional inactivity of chromocentres in Arabidopsis. Indeed, chromocentre DNA sequences are transcriptionally repressed, forming silenced domains (Soppe *et al.*, 2002), and transcription of transposable elements in the chromocentres is reduced during *de novo* chromatin formation (Benoit *et al.*, 2019).

Environmental factors are known to affect the chromatin structure (reviewed in Probst and Mittelsten Scheid, 2015), and chromocentres have in fact been used as target sites in the nuclei to track the effect of environmental stresses. Imposed heat stress on Arabidopsis caused decondensation of the chromocentre structure with an increase in transcriptional activation of repetitive elements but without any change in DNA methylation (Pecinka *et al.*, 2010). HEAT-INTOLERANT 4 (HIT4) orchestrates heat-mediated chromocentre decondensation and subsequent activation of centromeric sequences (Wang *et al.*, 2013). As a negative regulator of gene expression under various stress conditions, STRESS RESPONSE SUPPRESSOR1 (STRS1), a DEAD-box RNA helicase involved in RNA metabolism, is located at the chromocentres but leaves the chromocentres in response to salt stress, possibly contributing to gene silencing via an interacting partner protein (Khan *et al.*, 2014). The environmental-mediated changes in chromocentric structure are therefore a very good indicator of the dynamism of this nuclear structure as well as of the chromocentre-associated proteins and of the transcriptional regulation of chromocentre-localized sequences. This dynamic response in chromocentre organization has also been shown during development. For instance, in Arabidopsis, the non-random arrangement of chromocentres in the diploid cell and also in the triploid endosperm implicates specific interactions of parental chromosomes for the developmental-mediated epigenetic mechanisms

during seed development (Baroux *et al.*, 2017). An increased number of chromocentres is correlated with dosage of maternal chromosomes, indicating the requirement for the separation of maternal chromosomes (Baroux *et al.*, 2017). Clearly, defined features of chromocentres in Arabidopsis along with variable developmental stages and environmental stimuli could pave the way for refinement of genetic determinants affecting the chromatin organization and its effect in gene regulation. Formation of chromocentres during developmental stages requires highly regulated changes in nucleosome structure and histone post-translational modifications (Benoit *et al.*, 2019).

Satellite DNA sequences have been used to track the chromocentres in Arabidopsis, for example by FISH and zinc finger DNA recognition coupled to green fluorescent protein (GFP) (Lindhout *et al.*, 2007). The development of tracking methods for the chromocentres using fluorescent transcription activator-like effectors (TALEs) (Fujimoto *et al.*, 2016) or fluorescent versions of the CRISPR/Cas [clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein] system (Anton *et al.*, 2014) in live tissues while retaining the morphology will provide novel insights in the analysis of chromatin dynamics during variable environmental and developmental conditions. These, together with other tools, such as GFP-tagged CENH3 (De Storme *et al.*, 2016), will hopefully broaden the current understanding of chromocentre properties and functions in a variety of cells and tissues.

## The nucleolus

Our knowledge on this nuclear compartment goes back to the 18th century, when it was first observed and reported by Felice Fontana, who noticed its occurrence in the slime of an eel (reviewed in Pederson, 2011). In 1839, Gabriel Gustav Valentin described this structure as a 'nucleus within the nucleus' and named it 'nucleolus'. In the 1930s, the link between the nucleolus and ribosome biogenesis was established by other leading scientists. In 1931, the cytogeneticist Heitz described the presence of a secondary constriction on some chromosomes, different from the centromere, which Barbara McClintock correlated with the nucleolus in corn (Heitz, 1931; McClintock, 1934). Barbara McClintock designated the secondary constrictions as 'NORs' for 'nucleolus organizer regions', without knowing the content of these genomic regions. The direct link between NORs, ribosome biogenesis, and the nucleolus was then clearly established in the 1960s (Brown and Gurdon, 1964; Ritossa and Spiegelman, 1965; Warner and Soeiro, 1967). The structure of the nucleolus, which is the consequence of ribosome biogenesis, is divided into three different subcompartments: the fibrillar compartment (FC), surrounded by the dense fibrillar component (DFC), and the granular component (GC). These subcompartments correspond to different phases of the ribosome biogenesis process, starting from the transcription of rRNA precursor by RNA polymerase I at the FC/DFC boundary, to the formation of the pre-ribosome complexes in both DFC and then GC compartments (reviewed in Stępiński, 2014). Hundreds of factors and steps are required to generate mature ribosome subunits, and current knowledge has been recently reviewed in Sáez-Vásquez and Delseny (2019). Although formation of the nucleolus is a direct consequence of ribosome biogenesis, additional functions have been linked to the nucleolus and include cell growth regulation, stress response, cell ageing, ribonucleoprotein complex formation, RNA degradation, and genome organization (reviewed in Boisvert *et al.*, 2007; Boulon *et al.*, 2010).

In plant cells, a first proteomic analysis identified >200 nucleolar proteins, mainly implicated in ribosome biogenesis, as well as in RNA metabolism (Pendle *et al.*, 2005). More recent reports almost doubled this number and revealed an additional link with the proteasome (Palm *et al.*, 2016; Montacié *et al.*, 2017). At the genomic level, although the presence of rRNA genes was clearly established, additional works revealed that both active and inactive rRNA genes co-exist in every cell (reviewed in Grummt and Pikaard, 2003). However, only active rRNA genes are present within the nucleolus, as demonstrated by the analysis of purified nucleoli of Arabidopsis (Pontvianne *et al.*, 2013). Expressed rDNA copies associate with active chromatin marks: cytosines are poorly methylated in every context and histones are mainly methylated at Lys4 and Lys36 of the histone 3 (H3K4me3 and H3K36me3, respectively). Conversely, silent copies remain excluded from the interior of the nucleolus and contain constitutive heterochromatin marks such as H3K9me2 and H3K27me1 (Lawrence *et al.*, 2004; Pontvianne *et al.*, 2012, 2013).

In 2010, large chromatin domains, other than active NORs, were shown to associate with the nucleolus in mammalian cells (Németh *et al.*, 2010; Van Koningsbruggen *et al.*, 2010) and were named nucleolus-associated chromatin domains (NADs). NADs were also identified in Arabidopsis after isolation of nucleoli by fluorescent-assisted nucleoli sorting (FANoS) (Pontvianne *et al.*, 2016*a*; Carpentier *et al.*, 2018). As in mammalian cells, NADs in plants are primarily gene-poor regions enriched in repetitive sequences (reviewed in Picart-Picolo *et al.*, 2019; Pontvianne and Grob, 2020). In Arabidopsis, excluding rRNA genes, NADs represent 4% of the genome. At the chromosomal scale, most of the NADs are found on the short arm and at the subtelomeric regions of chromosome 4 (Pontvianne *et al.*, 2016*b*). These locations correspond to genomic regions flanking active rRNA genes located in the NOR on chromosome 4, as well as the telomeres that cluster at the nucleolar periphery in Arabidopsis, as will be further discussed (Fransz *et al.*, 2002; Chandrasekhara *et al.*, 2016) (Fig. 2). Little overlap can be found between NADs and genomic regions present at the nuclear periphery (i.e. LADs; see below) (reviewed in Picart-Picolo *et al.*, 2019; Pontvianne and Liu, 2020; Pontvianne and Grob, 2020). Interestingly, in the Arabidopsis *nucleolin 1* mutant, where both rRNA gene expression and nucleolar structure are altered, additional genomic regions that juxtapose the NOR2 on chromosome 2 now associate with the nucleolus. In addition, telomere nucleolar clustering is affected and telomeres are shorter (Pontvianne *et al.*, 2007, 2016*b*; reviewed in Picart and Pontvianne, 2017).

Around 900 genes localizing in the NADs or NAD genes have been identified in Arabidopsis leaf cells. Most of them are poorly expressed genes, and only pseudogenes and tRNA

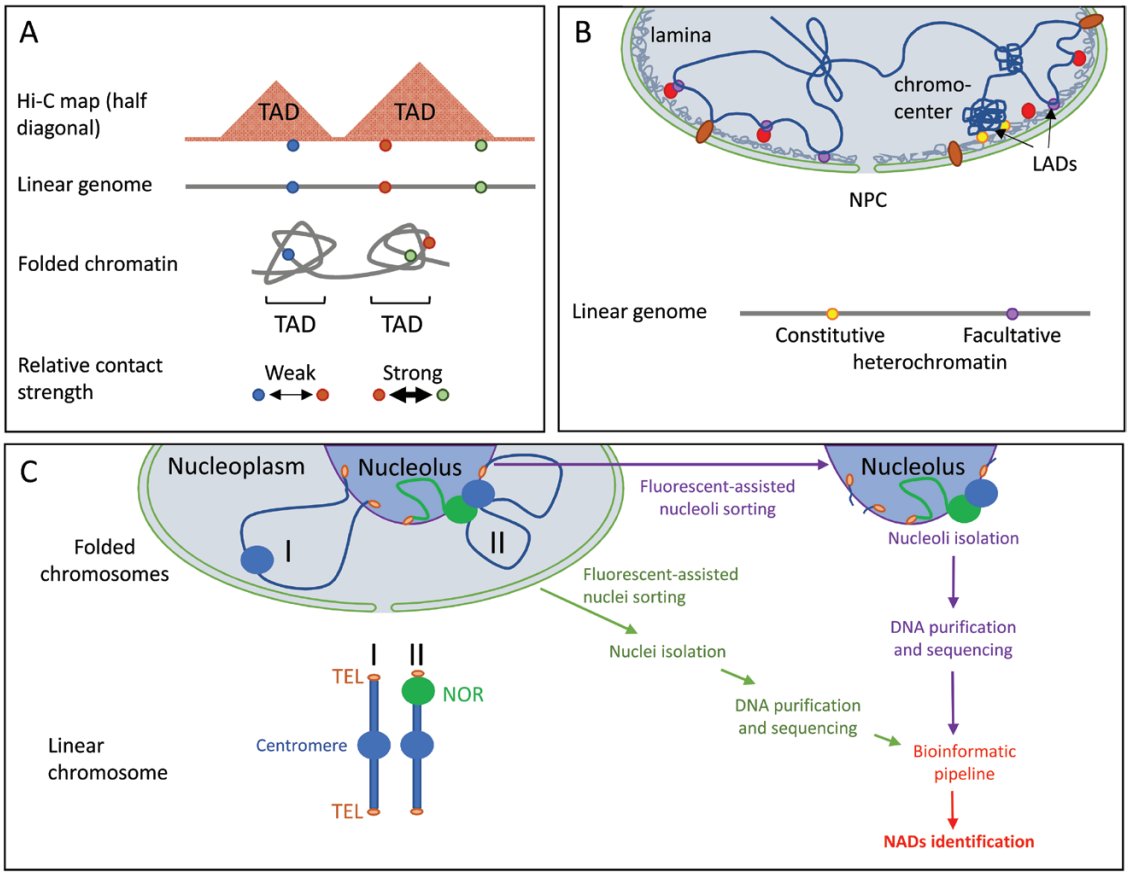


**Fig. 2.** Schematic illustration of TADs, NADs, and LADs. (A) Self-organized chromatin domains and their corresponding TAD patterns on a Hi-C map. In this sketch, three evenly distributed genomic loci are distributed in two TADs; the two located in the same TAD show stronger chromatin contact (shorter physical distance) than loci in different TADs. (B) Self-organized LADs, at either H3K9me2-marked constitutive heterochromatin (mainly at chromocentres) or H3K27me3-marked facultative heterochromatin. Association of chromatin at LADs is either directly with structural lamina components (grey mesh) or with lamina-interacting proteins (red and brown circles). (C) Self-organized chromatin domains associating with the nucleolus. In this sketch, two chromosomes are presented: one without an NOR (I) and one with an NOR (II). Chromosome (I) mainly associates with the nucleolus at its subtelomeric regions, while the NOR-bearing chromosome (II) shows stronger nucleolar association. For NAD identification, nuclei and nucleoli are isolated by fluorescent-assisted cell sorting. Nuclear and nucleolar DNA are then purified and sequenced to perform NAD identification.

genes have been shown to be enriched in NADs (Pontvianne *et al.*, 2016*b*). It is important to note that RNA polymerase II is absent from the nucleolus (Schubert and Weisshart, 2015). The nucleolus could therefore be considered as a sequestering area in order to maintain certain genes silent. In mammals, actively transcribed regions are excluded from the nucleolar compartment, supporting the idea that the positioning close to the nucleolus is mainly linked to repressive states (Quinodoz *et al.*, 2018). In human cells, acidosis or heat shock stress provokes the retention of particular nucleoplasmic proteins within the nucleolus (Audas *et al.*, 2012; Jacob *et al.*, 2013). This phenomenon, known as stress-induced sequestration in the nucleolus, depends on production of long non-coding RNA from the intergenic regions of rRNA genes (reviewed in Audas and Lee, 2016). Whether the nucleolus could also sequester particular genes to regulate their expression remains an open question. However, this hypothesis is supported by the recent discovery of two types of NADs in mammalian cells: type I, which present heterochromatin features; and type II, enriched in developmentally regulated genes (Vertii *et al.*, 2019). Type I and II NADs could be considered as constitutive and facultative NADs, respectively. Are developmentally regulated genes still released from the nucleolar area when expressed? If this is the case, the factors responsible of the nucleolus association of NAD genes are still unknown, although some nucleolar proteins were already found to tether centromeric regions at the nucleolar periphery (Padeken *et al.*, 2013).

In addition to rRNA transcription and ribosome biogenesis, the nucleolus should be considered as a platform tethering genomic regions enriched in repressive chromatin marks. In Arabidopsis, rRNA genes derived from NOR-bearing chromosome 4 associate with the nucleolus and are actively transcribed, while the rRNA genes derived from NOR-bearing chromosome 2 are silent and excluded from the nucleolus. However, in mutants in which both chromosomal NORs are expressed, NADs also become enriched in chromosome 2 genomic regions. These results suggest that the NOR locations on the chromosome and its expression levels seem to be an important aspect of the composition of NADs (reviewed in Picart-Picolo *et al.*, 2019; Pontvianne and Grob, 2020). Identification of the NADs in different cell types and in stress conditions should help in understanding how dynamic the NAD composition is and the importance of the nucleolus function in gene regulation, but a first analysis of NADs under

heat stress did not reveal significant differences compared with the control plants (Picart-Picolo *et al.*, 2020).

## The nuclear periphery

The nuclear periphery (NP) which surrounds the nucleoplasm consists of the nuclear envelope (NE), a double membrane layer with a continuum to the endoplasmatic reticulum (ER), nuclear pore complexes (NPCs), and the membrane adjoining the NPCs. The NP ensures the regulated transfer of mRNAs from the nucleus to the cytoplasm and of proteins in the reciprocal direction, and has essential roles in gene regulation and genome organization in providing attachment sites for genomic regions and chromatin. While the cell biology of the NP and the associated chromatin has been extensively reviewed (e.g. Meier *et al.*, 2017), we will here only highlight recent advances and particularly address the role of the NP in gene regulation.

The inner nuclear membrane can be associated with a protein mesh, the nuclear lamina, and interacts with transmembrane components that are part of the linker of nucleoskeleton and cytoskeleton (LINC) complex spanning the NE. The nuclear lamina in animals consists of lamins, type-5 intermediate filament proteins, and lamin-associated proteins. While some of the LINC complex members are conserved in plants, strictly defined lamins and lamin-associated genes are not present in plant genomes (Poulet *et al.*, 2017*b*). However, putative lamin analogues were initially identified in *Daucus carota* as the nuclear matrix constituent protein (NMCP) family (Masuda *et al.*, 1997). Subsequent analysis uncovered orthologues in various plant species, including Arabidopsis whose genome contains four orthologues of *DcNMCP1*, the *CROWDED NUCLEI* (*CRWN1–CRWN4*), and maize with two orthologues called *NMCP/CRWN homolog* (*NCH1* and *2*) genes (Dittmer *et al.*, 2007; Sakamoto and Takagi, 2013; Gumber *et al.*, 2019). Further lamina components were revealed by proteomic analysis of the nuclear lamina, which also identified CRWN1 and CRWN4 (Sakamoto and Takagi, 2013). Another plant-specific component of the lamina, lacking a transmembrane domain, is *KAKU4*, which interacts with CRWN1 and CRWN4 and is only present in angiosperm genomes (Goto *et al.*, 2014; Poulet *et al.*, 2017*b*). Importantly, lack of functional *CRWN1* and *KAKU4* (and other components of the NE and NP) in Arabidopsis results in nuclear morphology changes such as reduced nuclear size and increased circularity, but does not lead to obvious growth defects (Dittmer *et al.*, 2007; Sakamoto and Takagi, 2013; Wang *et al.*, 2013; Goto *et al.*, 2014). Only higher order *crwn* mutant combinations with *crwn1* result in severe dwarfism and cell death or, when all four *CRWN* genes are lacking, in lethality (Wang *et al.*, 2013). In particular, the cell death phenotype is to a large extent explained by an ectopic expression of defence genes and induction of the phytohormone salicylic acid (SA) (Choi *et al.*, 2019). Consistently, *crwn1 crwn2* double mutants are more resistant to infection by bacterial pathogens, in an SA-dependent manner (Guo *et al.*, 2017; Choi *et al.*, 2019). CRWN proteins possibly serve as direct transcriptional repressors of immunity-related genes, as CRWN1 interacts with the transcription factor NAC WITH TRANSMEMBRANE MOTIF1-LIKE9 (NTL9) which is involved in plant immunity (Guo *et al.*, 2017). Overall, lack of functional *CRWN* genes (and a disruption of nuclear lamina components in general) results in de-regulation of many genes which are involved in stress and defence responses, but also include other Gene Ontology (GO) terms (Langen *et al.*, 2014; Guo *et al.*, 2017; Mikulski *et al.*, 2019).

By microscopy analysis, it has been long known that most of the heterochromatin-containing Arabidopsis chromocentres are associated with the NE (Fransz *et al.*, 2002; Poulet *et al.*, 2017*a*). In *crwn4* mutants, chromocentres are dispersed, while the number of chromocentres is reduced in *crwn1 crwn2* double mutants (Dittmer *et al.*, 2007; Sakamoto and Takagi, 2013; Wang *et al.*, 2013). Hi-C analysis of *crwn1* and *crwn4* revealed reduced spatial separation of different chromatin compartments and therefore increased chromatin interactions between different chromatin compartments and interchromosomal interactions compared with wild-type nuclei. This indicates a reduced organization of chromatin in the *crwn1* and *crwn4* mutants and possibly higher interchromosomal compaction which may be partially explained by the decreased nuclear size (Grob *et al.*, 2014; Hu *et al.*, 2019). However, there is no overall misexpression of transposable or repetitive elements detected in any of the *crwn* single or multiple mutants (Wang *et al.*, 2013; Choi *et al.*, 2019). While the genes that are ectopically expressed in *crwn* mutants do not carry marks of constitutive heterochromatin, they are enriched in a specific chromatin state which harbours marks both of facultative heterochromatin (the PcG mark H3K27me3) and of active chromatin (H2A.Z and H3K4me3) (Sequeira-Mendes *et al.*, 2014; Choi *et al.*, 2019). This bivalent state may poise genes for fast activation upon differentiation signals or stress exposure. Indeed, two recent studies identified the genomic regions associated with the nuclear lamina, based on the association of DNA with NUCLEOPORIN1/136 and CRWN1 (Bi *et al.*, 2017; Hu *et al.*, 2019). These plant lamina-associated domains (PLADs) were enriched with repressive chromatin marks, such as H3K9me1/H3K27me1 for constitutive heterochromatin and H3K27me3 for facultative heterochromatin, and contained low expressed genes and non-accessible chromatin, suggesting that PLADs are mainly transcriptionally silent regions (Fig. 2) (reviewed in Pontvianne and Grob, 2020).

A link between PcG and the nuclear lamina may be mediated by PWO1, which interacts with both PRC2 components and CRWN1 (Hohenstatt *et al.*, 2018; Mikulski *et al.*, 2019). Mutations in *PWO1* lead to a reduction in nuclear size and misexpression of a set of genes which are also misregulated in *crwn1 crwn2* mutants (Mikulski *et al.*, 2019). Thus, PWO1 may tether PRC2 to certain genomic regions at the NP by interacting with CRWN1 or recruit specific PRC2 target genes to the NE. A direct link between chromatin/genomic regions and the NP may also be provided by the DNA-binding factor AtbZIP18 which interacts with the NE-associated protein1 (NEAP1) which is part of the LINC complex (Pawar *et al.*, 2016).

The NPC has also been extensively studied in plants, including a detailed proteomic analysis of the NPC, revealing conserved and

plant-specific factors (Tamura *et al.*, 2010). NPC members have various roles in development and disease, and have been extensively reviewed (see, for example, Meier *et al.*, 2017). However, direct links to chromatin regulation have largely not been identified, except for NUCLEOPORIN1/136 (NUP1/136) which shows similar chromatin contacts to CRWN1 (Bi *et al.*, 2017; Hu *et al.*, 2019). NUP1/136 is a likely functional analogue of metazoan Nup153 which is part of the NPC basket and mediates interactions with the lamina (Smythe *et al.*, 2000). Similar to plant lamina mutants, loss of functional NUP1/136 results in smaller nuclei (Tamura and Hara-Nishimura, 2011). Further analysis is required to disentangle roles of NPC components in chromatin regulation or protein/mRNA shuffling between the nucleoplasm and the cytoplasm.

Despite recent advances, we still need to assess the dynamic association of genomic regions with the NP in response to developmental and environmental cues and reveal the functional role of the NP in gene regulation. A first analysis uncovered that light triggers a rapid repositioning of several Arabidopsis light-regulated genes from the nuclear interior to the NP when they were transcriptionally activated (Feng *et al.*, 2014). Whether this a general feature of induced genes and whether repositioning is functionally relevant needs to be determined.

## Telomeres

Telomeres are repetitive elements assembled into nucleoprotein protective caps located at the ends of linear chromosomes. They safeguard chromosomal ends against cellular exonucleases and gross chromosomal reorganization arising from the action of DNA repair machineries (Weaver, 1998; Nakamura *et al.*, 2002). While telomeres do not fit the typical definition of an NB *per se*, they are discussed here given their role in 3D chromosome organization in plants and regulation of gene expression (Fig. 1).

In plants with large genomes such as wheat, rye, or barley, interphase telomeres are polarized on one side of the nucleus in Rabl configuration (reviewed in Santos *et al.*, 2015). In some other species, such as Arabidopsis and sorghum, telomeres cluster around the nucleolus (Dong *et al.*, 1998; Fransz *et al.*, 2002) as a part of NADs (Pontvianne *et al.*, 2016*b*), introduced above. Specific telomere conformation is established during meiosis, when a telomere bouquet is seen (Bass *et al.*, 2000; Colas *et al.*, 2008). Interestingly, the observation of bouquet formation belongs to one of the earliest descriptions (Digby, 1919), even before telomeres themselves were discovered by Herman Muller and Barbara McClintock in the 1930s. They noticed an ambiguous behaviour of the terminally located DNA in irradiated cells and described the chromosome healing phenomenon (McClintock, 1941; Melek and Shippen, 1996). Later, the end replication problem was uncovered (Olovnikov, 1971), revealing that telomere erosion occurs in every S phase and, if not counteracted, limits the cellular life span (Hayflick, 1982). That further confirmed that chromosomal ends are essential functional elements and led to the most recent definition of telomeres as difficult to replicate sequences and fragile sites (reviewed in Ozer and Hickson, 2018).

Although telomeres were first discovered in plants nearly a hundred years ago, what guides their clustering, chromatin organization, or protein composition is still not clear. It is partially because plant telomeres are quite heterogeneous, thus the general model cannot be easily pictured. Their sizes range from the very short telomeres (~3 kb) in Arabidopsis to 200 kb in tobacco (Kovarik *et al.*, 1996), Moreover, plants show phylogenetic divergence at the sequence level, as the common plant telomere motif (TTTAGGG)*n* (Richards and Ausubel, 1988) is not present in all plant species (Moyzis *et al.*, 1988).

Telomerase is the most important complex interacting with telomeres, but a large number of other factors modulate telomere homeostasis. Telomere components are the dsDNA-binding factors TELOMERE REPEAT BINDING (TRB) proteins, members of the Single-Myb-Histone protein family (Marian *et al.*, 2003; Kuchar and Fajkus, 2004). The TRB proteins form speckles preferentially in the nucleolus (Dvorackova *et al.*, 2010; Schrumpfová *et al.*, 2014; Dreissig *et al.*, 2017) where they interact with factors important for telomerase biogenesis (Schorova *et al.*, 2019). The functions of the AtTRB proteins, however, are not exclusively telomeric, suggesting a link to the genome-wide chromatin remodelling via the PRC2 complex (Zhou *et al.*, 2016, 2018). Therefore, it is possible that one function of AtTRB on telomeres is to recruit other chromatin factors to incorporate H3K27me3.

The telomeric epigenetic pattern depends neither on telomere sequence composition nor on telomere lengths, and shows large variability (Adamusova *et al.*, 2020). In the case of Arabidopsis, telomeric histones are predominantly labelled by H3K9me2 and H3K27me1, with lower, but detectable enrichment of H3K4me2/me3 and H3K27me3 (Vrbsky *et al.*, 2010; Majerova *et al.*, 2014; Adamusova *et al.*, 2020). Due to the retention of H3K27me1 and H3K4me3, the Arabidopsis telomeric chromatin state is considered intermediate or bivalent (Sequeira-Mendes *et al.*, 2014; Liu *et al.*, 2016). Whether H3K27me1 and H3K4me3 co-exist at the neighbouring nucleosomes as described for other regions with bivalent chromatin (Sequeira-Mendes *et al.*, 2014) is not yet clear. Arabidopsis telomeres are also enriched with the H3.3 histone variant (Vaquero-Sedas *et al.*, 2012), which could stand in support of the view that its telomeres are not fully heterochromatic (Vaquero-Sedas and Vega-Palas, 2013). Consistently, the so-called telomere position effect (TPE), mediating silencing of telomere-adjacent genes, was not detected in Arabidopsis (Gottschling *et al.*, 1990; Aparicio *et al.*, 1991; Vrbsky *et al.*, 2010). New insights into the effects of telomeres on gene regulation could be found by using the Hi-C approach. For instance, the existence of a long-distance TPE (~10 Mb) was shown in mammals where it occurs naturally and affects the global gene expression in a telomere length-dependent manner (Kim *et al.*, 2016; Kim and Shay, 2018). Telomeres can also modulate gene transcription via long non-coding RNAs called 'TElomeric Repeat-containing RNAs (TERRAs) (Azzalin *et al.*, 2007; Schoeftner and Blasco, 2008; Luke and Lingner, 2009; Chu *et al.*, 2017). In mammals, TERRA helps to establish telomeric heterochromatin via binding to shelterin components (Deng *et al.*, 2009), by recruitment of PRC2 (Montero *et al.*, 2018), or by competing for binding sites with the histone chaperone

ALPHA THALASSEMIA-MENTAL RETARDATION X-LINKED (ATRX) not only at telomeres, but genome wide (Chu *et al.*, 2017). ATRX is known to deposit a transcription-coupled histone variant H3.3 to heterochromatic sites as well as telomeres, where the function of H3.3 is not fully understood (McKittrick *et al.*, 2004; Lewis *et al.*, 2010).

The impact of plant telomeres on long-distance interactions, the telomere gene silencing in other plant species, or participation of telomeres in formation of NBs also represent open questions.

Progressive approaches including CRISPR-based telomere labelling (Dreissig *et al.*, 2017), single RNA detection (Duncan *et al.*, 2016), Hi-C, or super-resolution microscopy have a great potential to provide new insight into the these open questions in plant telomere biology and help to understand how telomeres and telomerase modulate the chromatin in 3D nuclear space.

## Topologically associated domains

The term 'topologically associated domains' (TADs) describes chromatin regions that appear as 'self-organized' structures, formation of which is associated with chromatin compartmentalization, chromatin looping, and chromatin insulation (reviewed in Pontvianne and Grob, 2020). The invention of the Hi-C technique and its derivatives enabled researchers to investigate genome-wide chromatin organization patterns with resolution as high as 1 kb (Lieberman-Aiden *et al.*, 2009). As the most important finding made with Hi-C, TADs were originally reported in mammalian cells (Dixon *et al.*, 2012) and since then different Hi-C maps have been created, which are essentially a numeric matrix describing relative chromatin interaction frequencies between any two genomic regions. Upon visualizing a Hi-C map with colours with different intensities that correspond to chromatin interaction values, one can observe TADs as distinct squares lined up along the diagonal, in which each TAD labels a genomic region showing stronger *cis* contacts inside this TAD than interaction across its boundaries. It should be noted that nowadays TAD calling is still a challenging task on a genomic scale; rather variable results can be obtained with different algorithms.

In a 3D prospective, a TAD on a 2D Hi-C map can be considered as a self-organized chromatin domain that is relatively insulated from its neighbouring chromatin regions (Fig. 2). Such an interpretation is supported by high-resolution microscopic studies demonstrating spatial isolation of TADs (Bintu *et al.*, 2018; Szabo *et al.*, 2018; Mateo *et al.*, 2019), although these investigated TADs were only a small fraction of those identified across the genome. In animals, TAD formation has been shown to be mainly contributed by chromatin insulators [e.g. protein complexes containing CCCTC-binding factor (CTCF) and cohesin], as well as chromatin states that reflect local epigenetic marks and transcriptional activities (reviewed recently by Szabo *et al.*, 2019; Zheng and Xie, 2019; Pontvianne and Grob, 2020). Therefore, how TADs are demarcated is dependent on both the genome sequence and chromatin activities/marks, where the latter can vary from one cell type to another. The space constraint of chromatin contact patterns in TADs creates chromatin interaction specificity; in some cases, the demarcation of TADs appears to be critical for gene expression regulation and cell differentiation, such as limb development (Andrey *et al.*, 2013; Lupianez *et al.*, 2015) and oncogene activation (Flavahan *et al.*, 2016; Hnisz *et al.*, 2016; Dixon *et al.*, 2018). However, it is not always true that TADs play dominant roles in determining gene expression. For instance, a recent study on several structural variations in the *Drosophila* genome causing changes in chromatin topology (including TADs) showed that they are not predictive of changes in gene expression. These results suggest that the expression of a gene can endure alternative TAD patterns as long as it maintains the same enhancer–promoter contact profile (Ghavi-Helm *et al.*, 2019).

Over the past few years, Hi-C analyses have been conducted on many plant species showing a diversity of TAD patterns in distinct plant genomes (reviewed in Dogan and Liu, 2018; Sotelo-Silveira *et al.*, 2018; Pontvianne and Grob, 2020). First, unlike animals, not all plant species display extensive TAD patterns throughout their genomes. In particular, two closely related species in the *Brassicaceae* family do not even have TADs at their chromosome arms. Secondly, for those plants showing TAD patterns, none of them has TADs featured with the presence of chromatin loops that connect TAD borders, which is a prominent characteristic of many animal TADs. Thirdly, distinct from animals TADs, plant TADs do not show pattern conservation between syntenic regions in different species, and genes residing in the same plant TAD lack co-expression (Dong *et al.*, 2017; Zhou *et al.*, 2018). Nevertheless, similar gene transcription and epigenetic profiles at plant and animal TAD borders indicate that it is a feature of these studied eukaryotes to have active and open chromatin preferentially associated with these chromatin regions (reviewed in Dogan and Liu, 2018; Sotelo-Silveira *et al.*, 2018; Pontvianne and Grob, 2020). This notion is further supported by comparative Hi-C studies. For instance, in a recent study comparing TAD patterns among different plant tissues, Dong *et al.* (2019) showed that tissue-specific TAD borders in rice and maize often overlap with gene up-regulation. Another example comes from comparisons of TAD patterns in diploid and subgenomes of tetraploid (formed via polyploidization) cotton genomes, which shows that conserved TAD boundary regions tend to have a higher level of chromatin accessibility and the euchromatin histone mark H3K4me3 than do non-conserved regions (Wang *et al.*, 2018). Accordingly, this study reveals that non-conserved TAD borders have a higher probability of displaying differential gene expression. At present, studies comparing TAD structures in normally growing plants and those in plants responding to biotic and/or abiotic stimuli are extremely limited, and the extent to which TAD formation regulates plant growth and development is unclear.

Mechanisms underlying plant TAD formation are unknown at the moment. CTCF-mediated TAD formation in mammals happens along with the formation of chromatin loops that link TAD boundary regions (Sanborn *et al.*, 2015; Fudenberg *et al.*, 2016). This mechanism, which results in strong chromatin insulation at TAD borders, seems to be missing in plant genomes, as plants do not have *CTCF*-like genes. However, the

strong correlation between active local gene expression and TAD borders suggests that plant TADs are shaped by transcriptional regulation. Recent motif analyses of rice TAD border regions revealed enrichment of sequences recognized by TCP (TEOSINTE BRANCHED 1, CYCLOIDEA, PCF1) and bZIP (basic leucine zipper) proteins, which belong to two large plant transcription factor families (Dogan and Liu, 2018). Further investigation of their potential roles in chromatin insulation would be helpful for plant scientists to better understand plant TAD formation. Additional potential plant TAD formation mechanisms have been discussed recently. Based on surveying TAD patterns from different plants, Stam *et al.* (2019) hypothesized that TAD formation, as well as TAD identification, are feasible in plant genomes bearing large-sized and dispersed repeat-containing chromatin regions. In other words, plant TADs largely reflect the spatial separation of repressive chromatin domains from their flanking chromatin. This idea can be tested by checking chromatin interaction patterns of TAD-containing genomic regions after inserting them into the Arabidopsis genome. Technically, the transformation-competent artificial chromosome vector (TAC) system enables the insertion of large genomic DNA (50–100 kb) into host plants (Liu *et al.*, 1999, 2002), which is sufficient to harbour most TADs identified in the rice genome (Liu *et al.*, 2017). In summary, the very limited knowledge of plant TAD function and formation calls for more efforts to be made to better understand 3D plant genomes.

## Nuclear subdomains are formed by intrinsically disordered domain proteins via liquid–liquid phase separation

Considering the information discussed in the previous sections, the question arises of how the nuclear domains are assembled without the formation of a surrounding membrane, which is necessary in organelles to maintain a local concentration of cellular components and to separate different metabolic activities. The answer lies in the liquid-like behaviour of biomolecular structures that undergo phase transition. The basic principle is that above a critical concentration, proteins aggregate and form a network of interactions with other molecules, resulting in dense liquid droplets. The first evidence of such a mechanism came from studies of segregating RNA and protein-rich P granules in *Caenorhabditis elegans* (Brangwynne *et al.*, 2009). Their posterior localization in the nematode embryo involves condensation of its macromolecular components. During the past few years, an increasing number of studies in different organisms have provided evidence that the physicochemical forces underlie the segregation of biological macromolecules into droplets, leading to the assembly of membraneless compartments in animals (Li *et al.*, 2012; Feric *et al.*, 2016; Langdon and Gladfelter, 2018; Maharana *et al.*, 2018; Sabari *et al.*, 2018; Shin *et al.*, 2018; Boeynaems *et al.*, 2019; Falk *et al.*, 2019; Ukmar-Godec *et al.*, 2019 Wang *et al.*, 2019) and in plants (Fang *et al.*, 2019; Zhang *et al.*, 2019; Zhou *et al.*, 2019).

Liquid–liquid phase separation (LLPS) or liquid phase condensation is the de-mixing of a homogeneous solution of proteins into two liquid phases where one is enriched for the protein (reviewed in Alberti, 2017; Strom and Brangwynne, 2019). Multiple droplets with similar content can form a compartment. The value of the critical concentration at which phase transition occurs depends on several factors, including intrinsic molecular properties of the protein domains and the nature and intensity of the interaction between the macromolecules. For example, a high RNA–protein ratio and a high number of interacting molecular domains (multivalency) reduce the critical concentration and hence induce LLPS (Li *et al.*, 2012; Maharana *et al.*, 2018). In this context, post-translational modifications, such as phosphorylation or methylation, which can alter protein interactions, play an important role in phase separation, as is further discussed.

Several chromatin proteins have so-called intrinsically disordered regions (IDRs) providing the biomolecules with an intrinsic capacity for LLPS required to form nuclear bodies involving chromatin. The extensively studied non-histone chromatin protein family, Heterochromatin Protein 1 (HP1), first described in *Drosophila* (James and Elgin, 1986), is a prominent component of heterochromatin. The human HP1α can phase separate and induce compaction of associated DNA (Larson *et al.*, 2017). The formation of phase-separated droplets was promoted by phosphorylation of HP1α and by DNA binding, confirming the importance of post-translational modifications in LLPS. De-mixing into droplets has also been demonstrated for the *Drosophila* HP1a protein and forms the driving force behind the assemblage of heterochromatin domains (Strom *et al.*, 2017). Arabidopsis contains a functional equivalent of HP1, agenet domain-containing protein (ADCP), which binds to methylated H3K9 and localizes to chromocentres. ADCP can also undergo liquid–liquid de-mixing and forms DNA-rich droplets upon methylated H3K9 recognition (Zhang *et al.*, 2018; Zhao *et al.*, 2019). These data provide evidence that animal HP1 and plant ADCP mediate the formation of constitutive heterochromatic chromocentres via phase separation.

The formation of PcBs in mammalian nuclei is established by Chromobox 2 (CBX2) via LLPS. CBX2 is the homologue of chromodomain-containing Polycomb (Pc) protein of *Drosophila* and a component of PRC1. CBX2 undergoes phase separation, thereby compacting PcG-associated chromatin (Tatavosian *et al.*, 2019). Phosphorylation of serines in CBX2 is an important step in the phase separation event. The low-complexity disordered region in CBX2 appears important for both LLPS and chromatin compaction, clearly indicating the link between the two processes (Plys *et al.*, 2019). Although plant LHP1 has the chromodomain and the chromoshadow domain (like HP1), it does not bind to methylated H3K9. Instead, LHP1 binds to H3K27me3 and maintains repression of PRC2 target genes (Turck *et al.*, 2007; Zhang *et al.*, 2007; Exner *et al.*, 2009), being considered the plant equivalent of Pc/CBX2 in PRC1. In addition, and as we have previously commented, LHP1 forms nuclear speckles (Libault *et al.*, 2005). Both nuclear distribution in speckles and H3K27me3 interaction are dependent on the presence of the chromodomain (Exner *et al.*, 2009). Whether LHP1 is involved in LLPS activity and responsible for PcB formation is very likely but needs to be determined. In this context, VERNALIZATION

1 (VRN1) might be an interesting factor. The plant-specific VRN1 mediates vernalization and binds DNA via its two B3 domains in a sequence-non-specific manner to repress gene targets such as *FLC* (Levy *et al.*, 2002). VRN1 is considered a member of PRC1 in plants although it is not a core component (Y. Huang *et al.*, 2019; reviewed in Holec and Berger, 2012; Kim and Sung, 2014). VRN1 has been demonstrated to undergo LLPS when it interacts with DNA giving rise to nuclear speckles. Both B3 domains and the IDR located in between them are essential for the phase separation event (Zhou *et al.*, 2019).

Another protein family involved in heterochromatin-linked LLPS are MORC ATPases that catalyse changes in chromatin structure in plants and animals (Koch *et al.*, 2017). In mammals, MORC proteins dimerize via their ATPase domain, which enables MORC to associate with chromatin and form NBs via LLPS (Zhang *et al.*, 2019. Its CW-type zinc finger domain can inhibit binding to DNA, forming an inactive state of MORC. The inactive state is released when CW interacts with H3K4me3 (Zhang, 2019). In Arabidopsis, AtMORCs are concentrated in discrete NBs at the boundary of chromocentres, while AtMORC4 and AtMORC7 also appear more diffuse in the nucleoplasm (Harris *et al.*, 2016). However, AtMORCs do not have a CW domain (Langen *et al.*, 2014), which makes it unclear how they are directed to chromatin.

The nucleolus, the most prominent NB (see above), is a clear example of a nuclear compartment formed by LLPS. Fibrillarin (FIB1) in the dense fibrillar component and nucleophosmin (NPM1) in the granular component of the nucleolus can phase separate *in vitro* into droplets as the nucleolar compartments do (Feric *et al.*, 2016; Yao *et al.*, 2019). In addition, both NPM1 and FIB1 require the presence of rRNA to phase separate into droplets, indicating the importance of protein–RNA interactions in compartment formation. The disordered regions in FIB1 and NPM1 are necessary for the phase separation process, while the RNA-interacting domains are needed to segregate into the proper nucleolar subcompartment (Yao *et al.*, 2019). Moreover, the latter promotes the sorting of correctly folded but not unfolded pre-rRNA into the DFC, pointing at a delicate interaction between multivalent FIB1 and rDNA transcripts. The tripartite organization of the nucleolus can be disturbed when aberrant, intrinsically disordered proteins interfere with the nucleolar phase separation. For example, when aberrant arginine-enriched dipeptide domains of the C9Orf72 protein interact with NPM1, the GC droplets dissolve. This phenomenon is observed in the human disease amyotrophic lateral sclerosis (ALS) (White *et al.*, 2019).

The list of studies on LLPS-mediated formation of NBs is still growing, indicating that phase transitions are key mechanisms for nuclear organization. For example, phosphatidylinositols, in particular phosphorylated PIP2, can play an important role in nuclear compartment formation since they have been detected in the nucleoplasm, nuclear speckles, and nucleolus. They interact with >300 nuclear proteins (Lewis *et al.*, 2011), including RNA polymerase II, RNA polymerase I, and FIB1, and associate with NORs during mitosis in human cells (Yildirim *et al.*, 2013; Sobol *et al.*, 2018). They form 40–100 nm sized nuclear lipid islets (NLIs), which have been observed in many organisms, including animals, plants, and yeast (Sztacho *et al.*, 2019). A direct interaction between phosphatidylinositols and chromatin is found for phosphatidyl 5-phosphate, which facilitates the binding of UHRF1, a ubiquitin-like PHD and RING finger domain protein, to methylated histone H3K9, thereby regulating epigenetic states (Gelato *et al.*, 2014).

Considering that the histone code is based on the post-translational modification of histone tails, it is likely that histone modifications play an important role in the organization of chromatin via LLPS. Indeed, in a phase separation experiment with reconstituted chromatin and physiological salt, nucleosomes with histone tails clearly showed LLPS, whereas 'tail-less' nucleosomes did not (Gibson *et al.*, 2019). Moreover, linker histone H1 and nucleosome linker length promote phase separation, while histone acetylation antagonizes chromatin phase separation. In addition, the interaction between chromodomain proteins and methylated histone H3K9 leads to condensed droplets in a tube, indicating that LLPS is a major driving force behind heterochromatin formation (Wang *et al.*, 2019).

LLPS has also been associated with the formation of euchromatin compartments. In mammalian cells, the co-activators Mediator (Med1) and Bromodomain-containing protein 4 (BRD4) are enriched at clusters of enhancers and transcription machinery components, so-called super enhancers (Pott and Lieb, 2015; Sabari *et al.*, 2018). It is proposed that Med1 and BRD4 form phase-separated condensates via their IDRs which enable compartmentalization with enhancers and transcription factors (Sabari *et al.*, 2018).

In conclusion, the LLPS studies point to a universal biological mechanism that explains the segregation of nuclear proteins into concentrated, small areas and the formation of chromosomal compartments with a specific epigenetic signature. The discovery of membraneless compartments by LLPS enables us to understand the dynamics of individual RNAs inside the nucleus (Pitchiaya *et al.*, 2019). The intrinsic molecular properties and a critical concentration of the associating macromolecules determine the unique identity of NBs, while specific epigenetic features along the chromosomes contribute to local compartment formation within chromatin. In fact, almost every topological change in the nucleoplasm is subject to LLPS forces.

## Conclusion

Technological advances in the analysis of chromatin organization, together with the application of next-generation sequencing (NGS) to decipher in more depth the genetic and epigenetic information of an increased number of plant species, have allowed the existence of several compartments in the 3D nucleus to be highlighted and are helping to uncover connections between regulatory domains of the 3D genome. Nevertheless, how spatial and structural organization of the nucleus influences its function is far from being understood. Recent discoveries in this area have indeed proven to be key in depicting the complexity of gene

expression regulation within the reduced nuclear space as we have discussed. However, caution needs to be present in attributing functions to 3D nuclear organization. The intrinsic capacity of chromatin to be plastic allied to adjustments of chromatin organization and nuclear domain arrangements in relation to plant species, specific transcriptional requirements, developmental stages, cell and tissue type, and epigenetic and stress factors can aid in allowing fast changes in gene expression and thus to induce quicker responses to specific requirements (Fig. 1).

LLPS is increasingly emerging as an important player in 3D genomics. Understanding the formation and organization of highly conserved and plant-specific nuclear domains in response to external and internal cues will contribute to our understanding of the regulation of these processes and plant phenotypes but also, most probably, to the discovery of future molecular tools. On the other hand, this complex nuclear dynamism illustrates well the difficulty in finding universal rules for making direct connections between 3D nuclear organization and predictable gene expression. Furthermore, chromatin-mediated regulation of genome accessibility may also be involved in molecular memories of responses given to different types of stimuli, but very little is known about perpetuating chromatin states and even less about inheritable gene expression patterns between generations as a way to ensure more prompt responses to challenges (Fig. 1) (reviewed in Mozgová *et al*., 2019). Increasing the amount of knowledge on the connections between interphase nuclear domains and the regulation of gene expression may enable identification of novel chromatin-based markers, ideally stable and associated with predictable impacts on plant traits, which in future plant breeding programmes can aid in improving crop growth under suboptimal environmental conditions.

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