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REVIEW PAPER

The matrix revolutions: towards the decoding of the plant chromatin three-dimensional reality

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Abstract

In recent years, we have witnessed a significant increase in studies addressing the three-dimensional (3D) chromatin organization of the plant nucleus. Important advances in chromatin conformation capture (3C)-derived and related techniques have allowed the exploration of the nuclear topology of plants with large and complex genomes, including various crops. In addition, the increase in their resolution has permitted the depiction of chromatin compartmentalization and interactions at the gene scale. These studies have revealed the highly complex mechanisms governing plant nuclear architecture and the remarkable knowledge gaps in this field. Here we discuss the state-of-the-art in plant chromosome architecture, including our knowledge of the hierarchical organization of the genome in 3D space and regarding other nuclear components. Furthermore, we highlight the existence in plants of topologically associated domain (TAD)-like structures that display striking differences from their mammalian counterparts, proposing the concept of ICONS—intergenic condensed spacers. Similarly, we explore recent advances in the study of chromatin loops and R-loops, and their implication in the regulation of gene activity. Finally, we address the impact that polyploidization has had on the chromatin topology of modern crops, and how this is related to phenomena such as subgenome dominance and biased gene retention in these organisms.

Keywords: Chromatin conformation, chromatin loops, chromosome configuration, ICONS, ploidy, R-loops, TADs, transcription factories.

Introduction

The spatial distribution of the eukaryotic genome is constrained by the nuclear envelope, a lipidic double membrane separating the nucleus from the cytoplasm. Due to the considerably large size of the genome compared with other cellular components, it is tightly packed and wrapped around histone proteins, forming a complex known as chromatin. Besides containing the genetic information for the vast majority of cellular processes, the nucleus serves as an integrator of environmental and developmental signals. Accordingly, the organization of the genome within the nucleus must

permit the accessibility and expression of a pertinent set of genes in response to specific stimuli in order to allow the cell to maintain its correct function, while preserving a high degree of compaction.

Understanding how the spatial organization of chromatin influences gene activity has become a relevant area of study in the biological sciences. The recent development of high-resolution -omic and microscopy-based techniques has contributed to unprecedented advances in the animal and plant fields, leading to the identification of diverse levels of organization of the genome (Doğan and Liu, 2018). While the general patterns governing genome organization appear to be conserved between the eukaryotic taxa, including the existence of chromosome territories, as well as A/B compartments (i.e. two separated compartments corresponding to active and inactive chromatin, respectively), some fundamental differences have also been observed between and within kingdoms. We previously addressed the generalities and specificities of the plant nuclear architecture, as well as the different elements that have been associated with its regulation (Rodriguez-Granados *et al.*, 2016). However, due to the rapid advances in the field and the increasing availability of data for an exponential number of plant species, we consider it both pertinent and relevant to discuss its state-of-the-art, questions, and challenges.

Distinct levels of organization govern the plant nucleus

Chromosome conformation

When cells are in interphase, each chromosome occupies a limited and exclusive subdomain in the nucleus, a phenomenon commonly referred to as chromosome territories (Fig. 1) (Rabl, 1885; Boveri, 1909; Cremer and Cremer, 2010). In plants, the first evidence of the existence of chromosome territories emerged through the application of FISH (fluorescence *in situ* hybridization) in *Arabidopsis thaliana*, the model organism for flowering plants (Lysak *et al.*, 2002). Even when the existence of chromosome territories is conserved within eukaryotes, it has been observed that chromosomes tend to acquire specific conformations during interphase in diverse species. In general, these have been classified into different categories in plants, including the Rabl (in honor of the anatomist Carl Rabl) and rosette conformations. The Rabl configuration consists of

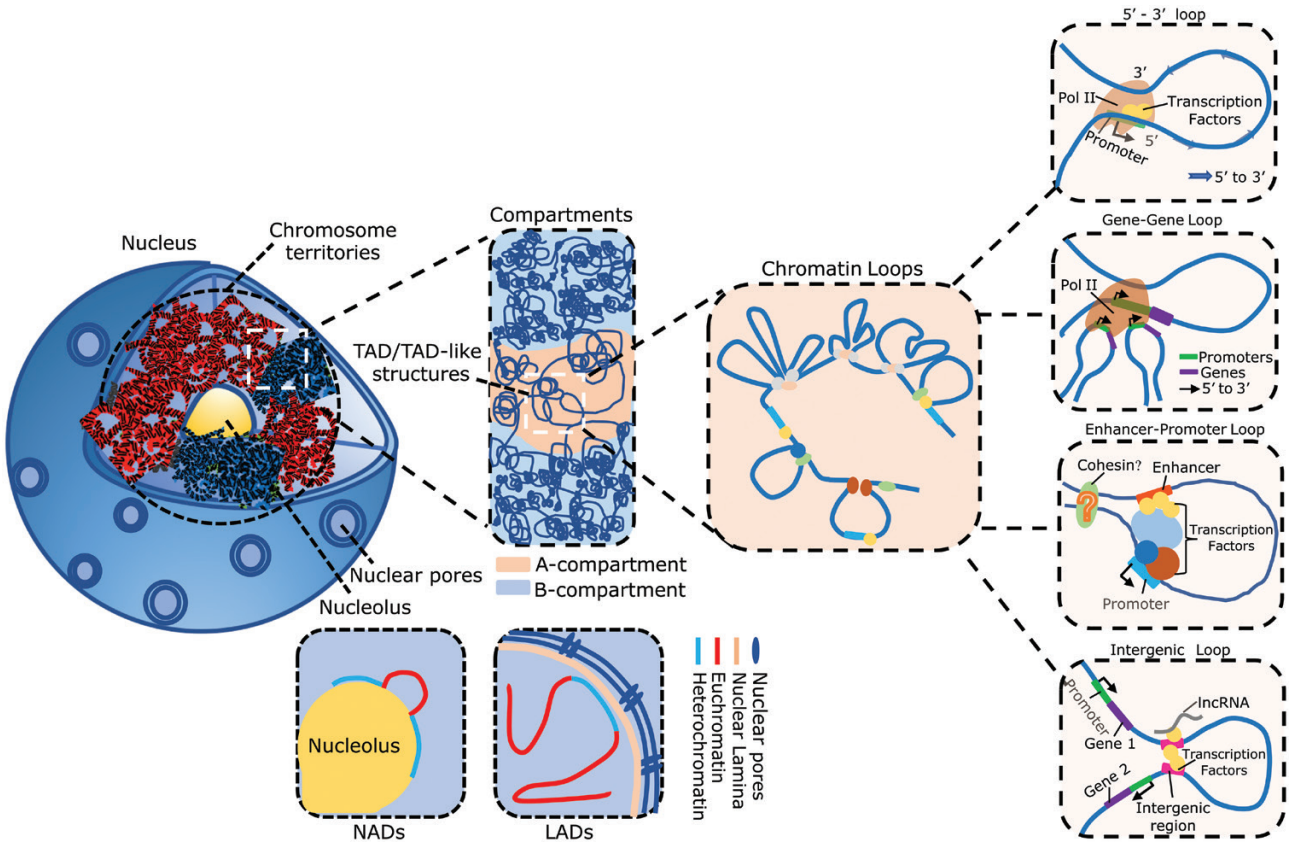

Fig. 1. Hierarchical organization of the plant chromatin in the nucleus. Chromosomes occupy specific territories in the plant nucleus. Chromosomes territories are further organized into A and B compartments, representing predominantly euchromatic and heterochromatic regions, respectively. NADs are chromatin regions interacting with the nucleolus, while LADs are associated with the lamina of the nuclear envelope. TADs/TAD-like structures are compartments where the regions inside these structures interact with each other with a higher frequency than with the surrounding regions. Chromatin loops are lower scale chromatin interactions that allow the establishment of regulatory networks between distant elements through their physical proximity. Chromatin loops can be established between different regions of the same gene (5'–3'/gene looping), between different co-regulated genes, between an enhancer and a promoter, and between non-coding genomic regions.

chromosomes folded on the centromeres, leading to the polarized separation of these structures and the telomeres (Fig. 2A) (Cremer *et al.*, 1982). This configuration has been observed in a high diversity of organisms, including animals such as the fruit fly and the salamander (Rabl, 1885; Hochstrasser *et al.*, 1986), budding and fission yeast (Jin *et al.*, 2000; Goto *et al.*, 2001), and plants with large genomes such as barley, oats, rye, and wheat (Fig. 2A) (Abranches *et al.*, 1998; Dong and Jiang, 1998; Mascher *et al.*, 2017). It has been proposed that the Rabl configuration plays an important role in the correct functioning of the genome in these organisms. For instance, in fission yeast, centromere clustering during interphase contributes to the capture of kinetochores during mitosis, and their de-clustering has been associated with defects in chromosome segregation and chromosome loss during cell division (Hou *et al.*, 2012). More recently, it has been shown through biophysical approaches that this configuration physically limits the topological entanglement of chromosomes produced by chromatin condensation in budding yeast (Pouokam *et al.*, 2019), a phenomenon that may be conserved in the plant species that present this chromosome configuration.

In 1998, Dong and Jiang observed that, in contrast to what was accepted at the time, the Rabl conformation was not universal among all plant species. Based on an *in situ* hybridization method, the authors concluded that maize, rice, and sorghum nuclei display a non-Rabl configuration when in interphase, characterized by disperse centromeres and telomeres (Dong and Jiang, 1998). A recent study that analyzed the three-dimensional (3D) conformation of rice through Hi-C [a chromatin conformation capture (3C)-based genome architecture assay] confirmed the non-Rabl conformation of its nuclei (Liu *et al.*, 2017); however, a previous *in situ* hybridization study showed that chromosomes from xylem vessel precursor cells display a Rabl conformation in this species (Prieto *et al.*, 2004). The existence of different chromosome configurations within the same organism suggests that these might be specific to diverse cell types. However, until recently, the 3C-based techniques to study chromosome configuration have required a considerable amount of tissue, hindering the characterization of specific cell types. In plants, this obstacle is even larger, as no specific cell type *in vitro* cultures can be established, and the only means by which specific cells can be obtained is through their

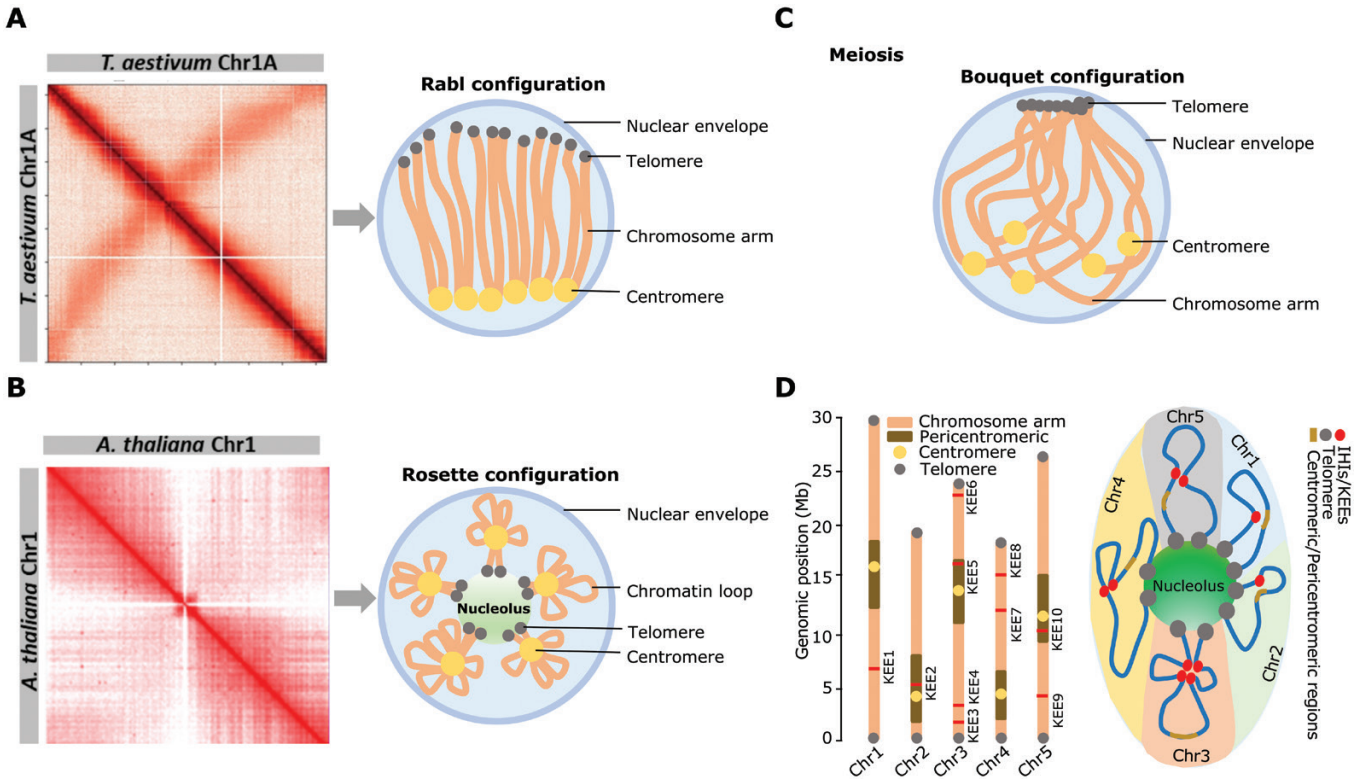

Fig. 2. Chromatin configurations in the plant nucleus. (A) Hi-C contact matrix of the wheat chromosome 1A. The intense interaction signal in the anti-diagonal represents frequent interactions between the chromosome arms, evidence of the Rabl configuration acquired by interphase chromosomes in this organism. The Gene Expression Omnibus (GEO) accession number for the data set used for generating this figure is GSE133885 and corresponds to the study by Concia *et al.* (2020). (B) Hi-C contact matrix of the *A. thaliana* chromosome 1. *A. thaliana* chromosomes are known to be organized into chromocenters, composed of centromeric and pericentromeric chromatin, from where euchromatic loops emanate. This is known as the rosette conformation. The GEO accession number for the data sets used for generating this figure is GSE76571 and corresponds to the study by Veluchamy *et al.* (2016). (C) The bouquet conformation is a transient chromatin configuration observed in meitotic cells in various organisms, including plants. In this, telomeres of all chromosomes co-localize on a specific site of the nuclear periphery, whilst the rest of the chromatin remains dispersed on the nuclear space. (D) In *A. thaliana*, telomeres tend to be associated with the nucleolus, and each chromosome bears at least one IHI/KEE, heterochromatic patches located inside euchromatic regions. IHIs/KEEs tend to strongly interact with each other, contributing to the formation of the KNOT, a 3D nuclear structure that has been proposed to be involved in the protection of the genome against DNA invasive elements.

purification via tissue dissection or cell sorting. Consequently, to date, most of the available 3C-based data in plants represent the average of a cell bulk from a specific organ (leaves, roots, flowers, etc.), or whole plantlets, which most probably eclipses the conformational differences presented by nuclei from different cell types.

The emergence of single-cell resolution in 3C-derived (and other) technologies will allow in the near future the generation of high-resolution maps of a variety of cell types in plants, permitting us to assess the link between chromatin organization and cell identity. These techniques have already started to be applied, revealing the existence of subtle but intriguing differences between distinct cell types in the same organism. In rice, for instance, single-cell 3C and Hi-C genome-wide approaches were performed in four different cell types: egg cells, sperm cells, mesophyll cells, and unicellular zygote (Zhou *et al.*, 2019). Comparative analyses showed that even though chromosome territories are conserved between these, some topological structures might be specific to certain cell types, as is the case of the compact silent centre (CSC). The CSC, in which the chromatin fiber is folded in and back out of the center of the nuclear space, is observed in egg and mesophyll cells as well as unicellular zygotes, but not in sperm cells. Additionally, the CSC appears to be reorganized after fertilization, thus suggesting that it may play a role in the regulation of zygotic genome activation (ZGA) (Zhou *et al.*, 2019); however, the precise functional impact of this phenomenon remains to be elucidated. In agreement with this study, through an *in situ* Hi-C analysis, Dong and collaborators found that in rice, foxtail millet, and maize, global chromatin architectures are conserved between different cell types. Nonetheless, at a local level, chromatin compartments were found to display tissue-specific characteristics, which the authors associated with the differential gene expression of each cell type (Dong *et al.*, 2020). Altogether, these studies suggest that the general principles governing 3D chromatin topology are constant—to a large extent—for each plant species, and flexible in response to developmental processes—to another degree.

Regarding its nuclear architecture, *A. thaliana* differs significantly from most plants so far described (Fig. 2B) (Pontvianne and Grob, 2020). The term rosette conformation has been used to describe the spatial distribution of its chromosomes, as (peri-)centromeric heterochromatin forms condensed chromocenters (CCs) from which euchromatic loops emanate (Fransz *et al.*, 2002) (Fig. 3A). CCs present a tendency to cluster so that in general 8–10 CCs co-localize close to the nuclear periphery (Fransz *et al.*, 2002; Fang and Spector, 2005; Poulet *et al.*, 2017). Traditionally, this conformation has been attributed to the particularly small genome of this plant (Liu and Weigel, 2015), especially compared with various staple monocots, whose genomes have expanded throughout their evolutionary history (Santos and Shaw, 2004; Leitch *et al.*, 2010). However, the *A. thaliana* genome size is comparable with that of *Drosophila melanogaster* and sorghum, neither of which present this chromosome conformation (Muller *et al.*, 2019). Furthermore, yeasts, which have even smaller genomes than *A. thaliana*, present a Rabl configuration (Muller *et al.*, 2019), indicating that genome size *per se* is not a determinant of the spatial organization of the chromosomes in the nucleus. On the other hand, the particular genome organization of this plant has also been attributed to its low abundance of repetitive sequences and transposable elements (TEs), as well as their concentration in pericentromeric regions (Tiang *et al.*, 2012; Lee and Kim, 2014; Rowley *et al.*, 2017); however, this remains a hypothesis and needs to be experimentally assessed.

Despite its significant particularities in chromosome conformation, *A. thaliana* has been a useful model for studying nuclear architecture in the context of developmental processes, as well as biotic and abiotic stress-responsive pathways. Consequently, it has been observed that CC number and organization in this organism are determined by a plethora of factors, including the genetic background, ploidy level, cell type, as well as loss-of-function mutations for chromatin modifiers (Probst and Mittelsten Scheid, 2015). Developmental phase transitions have been shown to significantly alter CC compaction. For instance, a previous study assessed heterochromatin condensation in different developmental phase transitions of the seed, going from embryo development to seed germination. Heterochromatin condensation—determined by the DAPI signal intensity at CCs—was found to increase during seed maturation, and decondensation is observed soon after imbibition and germination (van Zanten *et al.*, 2011). During photomorphogenesis, light induces expansion of the nuclei and condensation of heterochromatin in cotyledons. This contrasts with the chromatin architecture observed in etiolated seedlings, in which pericentromeric domains and other heavily methylated heterochromatic regions remain decompacted, a condition regulated by the two morphogenesis repressors *DE-ETIOLATED 1* (*DET1*) and *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*COP1*) (Bourbousse *et al.*, 2012). Floral transition has also been related to changes in chromatin organization. This phase accounts for an evident decondensation of pericentromeric repeats and gene-rich regions in mesophyll cells prior to bolting and a gradual recovery of chromatin compaction ~4 d after floral bud appearance (Tessadori *et al.*, 2007). Similarly, prolonged heat stress and pathogen attack have both been reported to induce CC deconsensation in *A. thaliana* leaf cells (Pavet *et al.*, 2006; Pecinka *et al.*, 2010). These changes in the spatial organization of chromatin at high order levels have been associated with the transcriptional reprogramming occurring during cell differentiation and stress responses (Mathieu *et al.*, 2003; Bourbousse *et al.*, 2015); however, to date, establishing their functional consequences and transcriptomic impact in a genome-wide fashion remains a challenge.

A third chromatin configuration, the so-called bouquet conformation, is characterized by telomere clustering on the nuclear periphery while the remaining chromatin is distributed in the nucleoplasm (Fig. 2C). This conformation has been described in various plant species, including rice and *A. thaliana* (Zhang *et al.*, 2017; Hurel *et al.*, 2018); however, the bouquet appears to be a universal and transient feature of meiotic cells in plants but also in yeast, the flat worm *Dendrocoelum lacteum*, the salamander, and humans (Zickler and Kleckner, 2016).

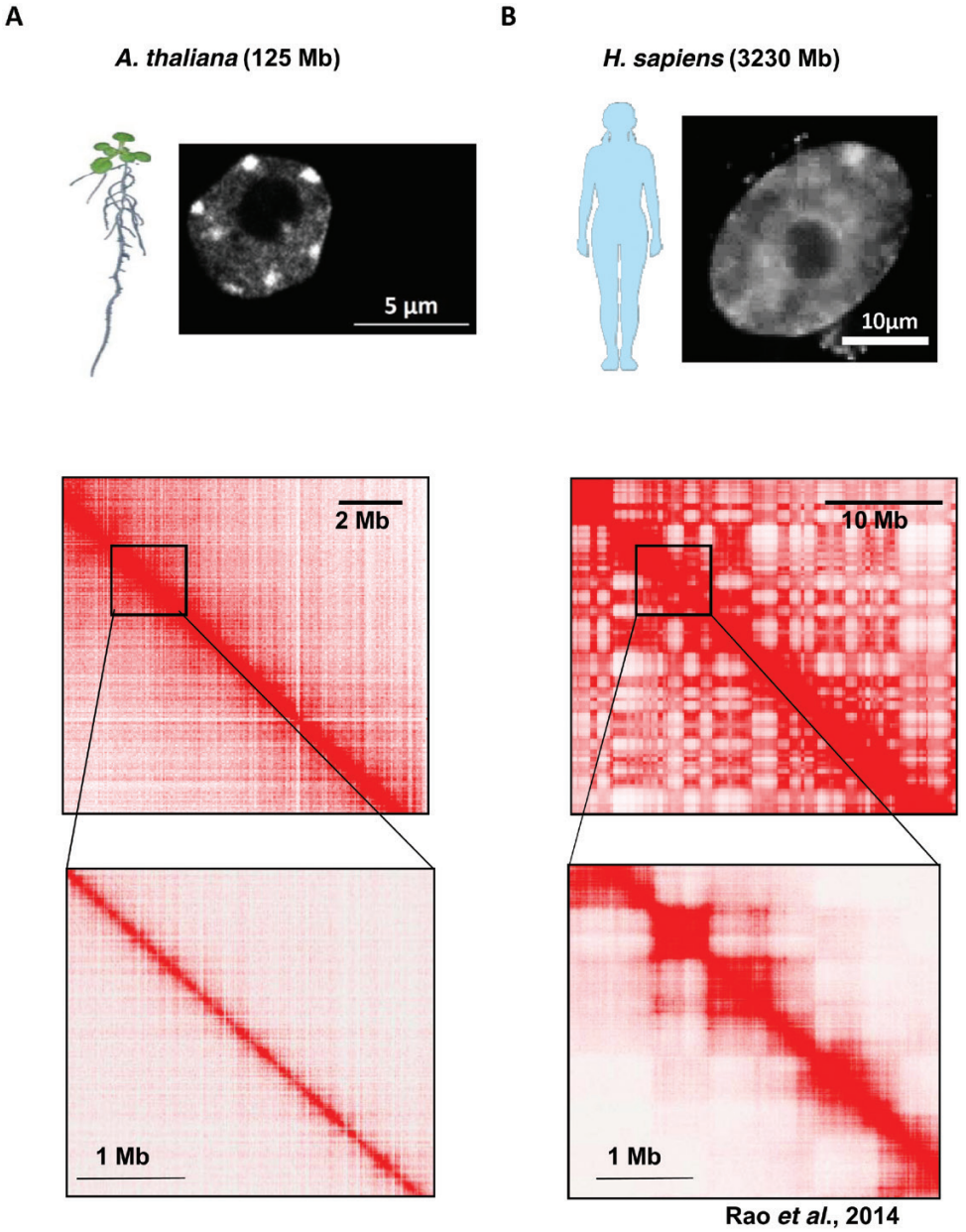

Fig. 3. Significant differences between the *A. thaliana* and the metazoan nuclear topology. (A) *A. thaliana* presents highly condensed chromocenters, visualized as bright defined foci in a DAPI-stained nucleus. The lack of TADs in the Arabidopsis genome is evidenced in the absence of high interaction squares along the diagonal on its Hi-C contact matrix. The Gene Expression Omnibus (GEO) accession number for the data sets used for generating this figure is GSE76571 and corresponds to the study by Veluchamy *et al.* (2016). (B) Even when chromocenters have been reported in human nuclei (Jagannathan *et al.*, 2018), these structures are not clearly visible through DAPI staining. The bright foci in the top right represents the inactive X chromosome of a diploid human fibroblast. The human genome presents a plethora of TADs, evidenced as high interaction squares along the diagonal on its Hi-C contact matrix. The GEO accession number for the data sets used for generating this figure is GSE63525 and corresponds to data from the study by Rao *et al.* (2014). This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

A/B compartments

There is evidence of a further spatial chromatin compartmentalization inside chromosome territories in eukaryotes. Chromatin regions can be subdivided into A or B compartments (Fig. 1), enriched with active and repressed genomic regions, respectively (Lieberman-Aiden *et al.*, 2009). This classification can be performed through a principal component analysis (PCA) on Hi-C data, based on an eingenvector analysis of the genome contact matrix (Fortin and Hansen, 2015). Functionally, this signifies that regions displaying similar epigenomic states interact with each other with a higher frequency than with regions displaying a different state (Lieberman-Aiden *et al.*, 2009; Grob, 2019); therefore, in PCA analyses of Hi-C data of *A. thaliana* and other plant species, A compartments highly overlap with chromosome arms, while B compartments coincide with heterochromatic pericentromeric regions (Grob *et al.*, 2014; Dong *et al.*, 2017; Liu *et al.*, 2017; Wang *et al.*, 2018).

The KNOT

There are several inter- and intrachromosomal interactions that further shape the plant genome (Rodriguez-Granados *et al.*, 2016). For instance, an outstanding characteristic of the *A. thaliana* genome is the presence of the KNOT. Both Hi-C and FISH approaches have shown that this 3D structure is formed by the interaction of 10 KEEs (KNOT engaged elements) or IHIs (interactive heterochromatic islands) (Fig. 2D) (Feng *et al.*, 2014; Grob *et al.*, 2014). KEEs/IHIs are present in all the chromosomes and are characterized by patches of heterochromatic histone modifications (H3K9me2) and transposons within a euchromatic region (Grob *et al.*, 2014). KEE-like structures have been recently identified in the *Brassica* spp. and rice genomes, sharing similar characteristics to those in *A. thaliana*; apart from their heterochromatic features, rice KEEs also present an enrichment in *VANDAL6* and *ATLANTYS3* TEs (Grob *et al.*, 2014; Dong *et al.*, 2018; Grob, 2019; Xie *et al.*, 2019). It was previously thought that the heterochromatic nature of KEEs contributed to the formation of the KNOT in *A. thaliana*; however, mutations in the machinery involved in the deposition of DNA methylation (*ddm1* and *met1*) do not disrupt the interaction between these elements (Feng *et al.*, 2014), suggesting that their heterochromatic features are not a requirement for the KNOT establishment. Besides, until recently, the function of the KNOT remained obscure, since it could not be correlated to a particular transcriptional regulation; nonetheless, Grob and Grossniklaus (2019) contributed with evidence of a role for this structure in the silencing of invasive DNA elements. In their study, they evaluated the 3D genome architecture of eight independent *A. thaliana* lines from the SALK collection, carrying the same transgene at different genomic positions along chromosome 1 and harboring the kanamycin resistance gene *NPTII*. 4C experiments revealed that transgenic plants display novel interactions between transgenes [i.e. TRANSGENE INSERTION SITE (TIS)] and KEEs (especially KEE6) as well as pericentromeric regions. Additionally, these ectopic contacts not only led to changes in the TIS organization but also to changes in transgene activity, as TIS–KNOT interaction intensity negatively correlated with *NPTII* expression and, therefore, viability in selective media. These results indicated that the KNOT participates in the repression of invasive DNAs in *A. thaliana*—a process they named KNOT-linked silencing (KLS). KLS was found to be independent from the epigenetic environment of TIS (i.e. heterochromatin and euchromatin) and surprisingly does not require DNA methylation or the activity of small RNAs. Therefore, it appears to be independent from DNA methylation-mediated transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS), the two canonical mechanisms of gene silencing in plants (Vaucheret and Fagard, 2001). However, KLS appears to behave as a paramutation, since silenced T-DNAs were shown to induce *de novo* silencing of active transgenes *in trans*. The molecular mechanisms underlying the KNOT establishment and KLS remain to be described. For this, it would be pertinent to identify the proteins participating in the recruitment of KEEs, as well as the epigenomic profiles of these elements and their target transgenes during KLS; however, this remains extremely challenging due to the technical limitations for the screening of KLS phenotypes at a large scale (Grob and Grossniklaus, 2019).

Chromatin organization in the nuclear space

Even though 3C-derived techniques have permitted assessment of the interaction frequencies between different genomic regions, these methods do not allow evaluation of the position of chromatin relative to other nuclear components, including the envelope and the nucleolus. Hence, cytological and other molecular approaches have been used to investigate these aspects, contributing with evidence of the profound effect that this positioning has on the transcriptional regulation of specific genomic regions (Kubben *et al.*, 2010; Shevelyov and Ulianov, 2019). Initial studies in *A. thaliana* nuclei allowed detection of a preferential association of CCs with the nuclear periphery and of the telomeres with the nucleolus (Armstrong *et al.*, 2001; Fransz *et al.*, 2002) (Fig. 2B). Recent molecular approaches—including isolation of nucleoli through fluorescence-activated cell sorting (FACS) followed by sequencing of interacting DNA—have confirmed these observations and allowed the identification of the nucleolus as an important structure for ribosome biogenesis and telomere maintenance (Pontvianne *et al.*, 2016; Picart-Picolo *et al.*, 2019). NADs [nucleolus-associated domains] have been detected in both plants and animals (Fig. 1). These structures include NORs [nucleolus organizer regions), consisting on actively transcribed rRNA genes arranged in tandem. In *A. thaliana*, NORs are located in chromosomes 2 and 4 (Haberer *et al.*, 1996), hence, these two chromosomes associate frequently around the nucleolus (Pecinka *et al.*, 2004). On the other hand, NADs are also enriched in inactive protein-coding genes and TEs (Pontvianne *et al.*, 2016; Matheson *et al.*, 2017; Kalinina *et al.*, 2018), suggesting that the nucleolus is associated with both the activation and repression of transcription; however, the molecular mechanisms and the proteins participating in the recruitment and regulation of these sequences in plants remain to be described. Remarkably, Picart-Picolo and collaborators showed that in plants exposed to heat stress, NADs remained unchanged when compared with non-stressed plants. This result is in contrast to previous findings showing that heat stress affects the global organization of the genome in this organism, and the fact that nucleolar morphology itself is also affected by this stimulus (Pecinka *et al.*, 2010; Picart-Picolo *et al.*, 2020). However, NAD stability has been demonstrated in animal cells during senescence, despite the massive nuclear organization occurring during this process (Dillinger *et al.*, 2017), suggesting that NADs are highly stable domains among eukaryotic nuclei.

The nuclear periphery has also been shown to play a key role in the regulation of the 3D organization of the nucleus in plants and other eukaryotes, as well as in transcription. In animals, peripheral chromosomal regions were first detected for their association with lamin proteins, components of the nuclear lamina in these organisms. Lamin-associated domains (LADs) are characterized for being transcriptionally repressed,

with gene-poor and A/T-rich genomic regions (Fig. 1) (Van Steensel and Belmont, 2017). Studies in humans have shown that LADs create a repressive environment that overall contribute to gene repression; however, it has also been observed that the sensitivity level to LAD repression varies between promoters and is determined by the complex interplay between the chromatin features of a specific LAD and the promoter sequence (Leemans *et al.*, 2019). Furthermore, the loss of chromatin tethering to the nuclear lamina has been associated with cell aging, senescence, and lamin-associated diseases in animals, as well as autoimmunity and cell death in plants, highlighting the crucial role that the chromatin interaction with the nuclear envelope plays in cell homeostasis (Choi *et al.*, 2019; Shevelyov and Ulianov, 2019).

In contrast to metazoans, lamin homologs are absent in plants, a phenomenon that has hindered the identification of genomic regions anchored to the nuclear periphery in these organisms. Ingeniously, the *A. thaliana* nucleoporin NUP1 was the first protein used for the identification of genomic regions interacting with the nuclear envelope (Bi *et al.*, 2017). Through a restriction enzyme-mediated ChIP, Bi and collaborators determined that genomic regions associated with the nuclear envelope in *A. thaliana* are pericentromeric regions enriched in heterochromatic histone modifications (H3K9me2), silenced protein-coding genes, and TEs, differing from those in animals. However, considering that NUP1 is a nuclear pore protein, it remains unclear whether these interactions may be specific for this protein or for the whole nuclear periphery. Remarkably, the authors also found that the identified chromatin organization at the nuclear periphery displays similar patterns among different tissues (Bi *et al.*, 2017). These results are coherent with previous observations in wheat interphase nuclei, where the chemical perturbation of DNA methylation and histone acetylation [with azacytidine (5-AC) and trichostatin A (TSA), respectively] does not seem to alter telomere and centromere organization towards opposite nuclear poles, suggesting that the general processes governing these interactions are independent of developmental programs and epigenomic states (Santos *et al.*, 2002).

More recently, the putative plant lamin-like proteins CROWDED NUCLEI 1 (CRWN1) and CRWN4 were shown to mediate the tethering of chromatin at the nuclear envelope (Hu *et al.*, 2019). Loss-of-function mutants for these proteins displayed a loss in differential probe positioning patterns in a chromosome painting assay, CRWN1 being the major contributor to chromatin binding to the nuclear periphery. Interestingly, *crwn1* and *crwn4* mutations were found to perturb chromatin compartmentalization, as chromatin interactions between different A/B compartments from the same or different chromosomes were increased in both mutants compared with the wild type, thus indicating a reduction in chromatin organization. These structural changes are accompanied by profound modifications of gene expression, as observed in *crwn* double mutants (Choi *et al.*, 2019). Altogether, these results showed that perinuclear chromatin anchoring by CRWN1 and 4 contributes to nuclear organization in *A. thaliana*, suggesting that they represent functional analogs of animal lamins in this organism (Hu *et al.*, 2019). Interestingly, a study by Guo and collaborators demonstrated that upon infection by virulent bacterial pathogens, CRWN1 proteins are degraded in a salicylic acid (SA)-dependent fashion (Guo *et al.*, 2017). Even though the effect of CRWN1 degradation on chromatin tethering to the nuclear lamina has not been assessed, it can be predicted that upon SA signaling, the reduction on CRWN1 levels leads to chromatin conformational changes associated with stress responses; however, this remains to be experimentally addressed (Hu *et al.*, 2019). Additionally, Polycomb repressive complex (PRC)-mediated regulation might be related to these peripheral chromatin domains (Mikulski *et al.*, 2019). It has been reported that PRC-associated components such as PWO1 physically interact with CRWN1 and 2, controlling the expression of a common subset of PRC2 targets. These results provide evidence of the functional relevance of chromatin association with the nuclear periphery and its relationship to repressive chromatin environments (Mikulski *et al.*, 2019).

In contrast to the abundant information existing for animals, there are significant advances to be made in the field for plants, since studies of this nature have only been performed in *A. thaliana*. Due to the reviewed high diversity in plant 3D chromatin configuration and the particularities of *A. thaliana*, it remains indispensable to explore the mechanisms governing chromatin–nucleolus and chromatin–periphery interactions in other plant species. This will allow us, for instance, to evaluate the extent to which these interactions influence developmental processes, responses to environmental stimuli, or traits of agricultural interest.

TADs/TAD-like structures

From all the chromatin interactions described since the introduction of 3C-based techniques, TADs (topologically associated domains) remain the best characterized, as they are visually recognizable as high interaction squares along the diagonal in Hi-C matrices at a relatively low resolution (Fig. 3B) (Maass *et al.*, 2019). They are defined as 3D structures where the chromatin regions included within a TAD interact with each other *in cis* with a higher frequency than with chromatin outside it (Fig. 1). The first Hi-C analyses performed in plants were done in *A. thaliana*, revealing the absence of these structures in this organism (Fig. 3A). Rapidly, the scientific community started speculating that these structure were absent from the plant kingdom (Grob, 2017); however, the application of 3C-derived techniques to other plant species has revealed that TAD-like structures are widely distributed within both monocots and dicots, as well as in the animal kingdom (Fig. 3B) (Dixon *et al.*, 2012; Liu *et al.*, 2017; Dong *et al.*, 2017; Wang *et al.*, 2018). Furthermore, the generation of high-resolution Hi-C maps for *A. thaliana* revealed that even when the existence of TADs is not obvious in this organism, its genome harbors >1000 TAD boundary-like and TAD interior-like regions (Wang *et al.*, 2015), suggesting that after all plant nuclei follow overall similar principles of chromatin packaging to their metazoan counterparts.

In the context of chromatin organization, one of the most striking differences between plants and metazoans is the absence of genes encoding CCCTC-binding factors (CTCFs) in plant genomes. CTCFs are known to participate in TAD establishment in animals through the formation of CTCF–cohesin complexes that stall TAD borders, a role that has been supported by experimentally editing CTCF motifs and by depleting these proteins (de Wit *et al.*, 2015; Guo *et al.*, 2015; Nora *et al.*, 2017). On the other hand, cohesins do exist in plants; however, whether there are other structural proteins carrying out the function of CTCFs in these organisms remains to be determined. Remarkably, TAD boundaries in rice were found to be enriched with motifs recognized by the plant-specific TCP transcription factor (TF) family and bZIP proteins, suggesting that these may contribute to TAD formation in rice (Liu *et al.*, 2017; Doğan and Liu, 2018); however, this has not been experimentally addressed to date.

In animals, TADs are not completely abolished in the absence of CTCFs and cohesins, suggesting that some of these structures are independent from these proteins. In fact, these CTCF- and cohesin-independent TADs correlate with the compartmentalization of chromatin into transcriptionally active and repressed compartments, suggesting that the transcriptional status of a genomic region contributes to TAD formation (Nora *et al.*, 2017; Rao *et al.*, 2017; Schwarzer *et al.*, 2017; Doğan and Liu, 2018). Interestingly, when comparing *Drosophila* and *A. thaliana* transcriptional densities, researchers observed that even if these organisms have a similar genome size, transcriptional density is relatively uniform in *A. thaliana* chromosome arms, while *Drosophila*'s genome presents large non-transcribed regions. Remarkably, a strong correlation between regions with a high transcription density and the positioning of TAD borders has been detected in various organisms, including *Drosophila* (Rowley and Corces, 2016). Based on this, it has been proposed that the lack of TADs *sensu stricto* in *A. thaliana* is a result of the homogeneous transcriptional rates along its genome (Rowley *et al.*, 2017). Alternatively, this phenomenon has also been attributed to the small genome size of this plant, since TADs have been reported in plants with medium and large genomes (including rice, millet, sorghum, tomato, cotton, and corn), while *Arabidopsis lyrata*, with a small genome of ~230 Mb, does not present these structures either. However, *A. lyrata* is a close relative of *A. thaliana*; hence, TAD absence may well be a characteristic of the Arabidopsis genus. In order to assess the contribution of genome size to TAD establishment, it would be appropriate to assess the 3D genome conformation of small genome plants unrelated to *A. thaliana* such as plants from the *Genlisea* genus, which possess some of the smallest known angiosperm genomes (~61 Mbp) (Fleischmann *et al.*, 2014).

Transcription states have been shown to be an important predictor of Hi-C contact maps in various eukaryotic species, including *Drosophila* and *A. thaliana* (Rowley *et al.*, 2017). However, a recent study has demonstrated that the loss of TAD isolation does not necessarily lead to changes in gene expression (Despang *et al.*, 2019). Despang and collaborators have shown that mice transgenic lines lacking CTCF sites located not only at the boundaries but also inside the two TADs of the *Sox9–Kcnj2* locus, displayed a reduced isolation and, therefore, fusion of these domains. Surprisingly, this phenomenon did not have significant effects on developmental gene regulation or developmental phenotypes, indicating that enhancer–promoter contacts occur for these loci even after TAD loss (Despang *et al.*, 2019). To date, studies assessing the contribution of particular TADs to the transcriptional regulation of specific loci remain scarce; for this reason, it is premature to hypothesize whether this phenomenon is generalized or specific to certain TADs. The detailed depiction of the molecular mechanisms governing TAD establishment will certainly allow us to deepen our understanding of their regulatory nature in the nucleus.

In a recent study where we described the 3D genome conformation of hexaploid wheat, we provided evidence of folding domains in this organism that differ from canonical TADs (Concia *et al.*, 2020). While animal TADs are isolated units where genes inside the same TAD are co-regulated (Dixon *et al.*, 2012, 2016), ICONS—for intergenic condensed spacers—are characterized by being deprived of genes and enriched in TEs in their interior. Consequently, in wheat, active genes and euchromatic marks are enriched on the borders of these domains, representing a radical difference from TADs. Previous studies depicting chromatin topology in other plant species have described a similar phenomenon without placing emphasis on it. For instance, it has been observed that protein-coding genes and euchromatic marks display a significantly higher density outside TADs than inside them in rice (Liu *et al.*, 2017; Dong *et al.*, 2018), and TAD boundaries were also found to display an enrichment in actively transcribed genes in cotton and *Brassica* species (Wang *et al.*, 2018; Xie *et al.*, 2019). Similarly, genes in maize are organized in clusters or gene islands, which are separated by blocks enriched in retrotransposons. Chromatin loops have been shown to emanate from between different gene islands, whereas gene-poor regions remained highly condensed (Dong *et al.*, 2017), facts that are in line with the earlier replication of gene islands compared with gene-poor regions (Bass *et al.*, 2015). In sum, these results may suggest that ICONS are widely distributed among plants, which present folding domains reminiscent of mammalian TADs, but harboring mostly TEs and non-coding sequences in their interior, and therefore possibly fulfilling a different function. As TADs, ICONs seem to have a regulatory function, since genes located at ICON boundaries were found to frequently form loops between them. Hence, we hypothesized that, while TADs bring together genes inside them for their co-regulation, ICONs contribute to the compaction of heterochromatin to allow co-regulated genes to interact, modulating their accessibility to the transcriptional machinery. This hypothesis is supported by the fact that the genes involved in these loops tend to be enriched in the same epigenetic marks, display similar expression levels, and behave similarly when differentially expressed in different organs. This mechanism of co-regulation may be particularly useful for increasing the efficiency of nuclear metabolism in highly complex genomes (Misteli, 2007).

Transcription factories contribute to the transcriptional regulation of wheat (and other plant species?)

The concept of a transcription factory has gained popularity in molecular biology to refer to the specialized nuclear sites where transcription occurs. These foci have been detected in mammalian genomes, and their existence has been proposed to contribute to transcription efficiency by augmenting the spatial and temporal availability of the transcriptional machinery, and to facilitate the co-regulation of genes with similar expression profiles (Cook, 1999; Weipoltshammer and Schöfer, 2016). Observations in *A. thaliana* nuclei allowed the determination that in this organism RNA polymerase II (RNA Pol II) molecules are dispersed along the euchromatic regions of the nucleus (Schubert and Weisshart, 2015) which, together with the lack of intergenic loops between actively transcribed genes in this organism (Liu *et al.*, 2016), challenged the existence of transcription factories in plants. However, recently, we proposed the existence of these structures in wheat, based on the detection of RNA Pol II-associated intra- and interchromosomal interactions involving numerous co-regulated genes (Fig. 4) (Concia *et al.*, 2020). Such spatial organization of transcription may represent an advantage for the regulation of a large genome such as that of wheat, facilitating the positioning of transcriptional regulatory factors at specific focal points and their simultaneous targeting of different genomic regions. Furthermore, it is possible that ICONS contribute to the establishment of these structures in this organism and, reciprocally, the absence of TAD-like structures in *A. thaliana* could be linked to the reported lack of transcription factories. The fact that co-regulated highly expressed genes physically interact through chromatin loops in other plants such as maize and rice, and that some of these are associated with Pol II, could indicate the existence of transcription factories in these organisms (Li *et al.*, 2019; Peng *et al.*, 2019; Zhao *et al.*, 2019). Nonetheless, the distribution of these structures in diverse plant taxa remains to be determined, as does their association with genome size or transcriptional regulation.

Chromatin loops influence the local regulation of gene activity in plants

Chromatin loops represent long- and short-range interactions at the gene body level (Pontvianne and Liu, 2020; Gagliardi and Manavella, 2020). They have been reported throughout the eukaryotic taxa and comprise the physical interaction between genes and their regulatory elements (regardless of their genomic distance, even between different chromosomes) (Fig. 1). It has been determined that loop formation significantly contributes to the regulation of gene expression in plants and other organisms through the establishment of regulatory networks based on physical proximity. We previously proposed a regulatory element (RE)-based classification of chromatin loops in plants (Rodriguez-Granados *et al.*, 2016). Consequently, chromatin loops can be classified into gene loops, involving loops that bring together the 5' and 3' termini of the same gene, enhancer–promoter loops, occurring between a gene and its distant enhancer, and intergenic chromatin loops, comprising intergenic regions (Fig. 1) (Rodriguez-Granados *et al.*, 2016). The recent improvements in the resolution of ChIA-PET (chromatin interaction analysis by paired-end tag sequencing) and 3C-derived methods, in sequencing capacity, as well as in the power of bioinformatic analyses, has allowed the detection of thousands of chromatin loops in *A. thaliana* and other organisms with complex genomes at a local scale and high resolution (Liu *et al.*, 2016; Dong *et al.*, 2018; Li *et al.*, 2019; Peng *et al.*, 2019; Zhao *et al.*, 2019); however, to date, only a few of these structures and their regulatory mechanisms have been explored in detail. Chromatin loops remain elusive to depict, in part because of the laborious and intricate methods required for their study, as well as their instability in response to specific environmental signals and developmental processes such as vernalization and cell division (Crevillén *et al.*, 2013; Cao *et al.*, 2014; Jégu *et al.*, 2015). However, in the last years, we have seen a substantial increase in studies in the field and foresee their number to continue growing, which will allow us to increase our understanding of the transcriptional regulation of genes associated with phenotypical traits

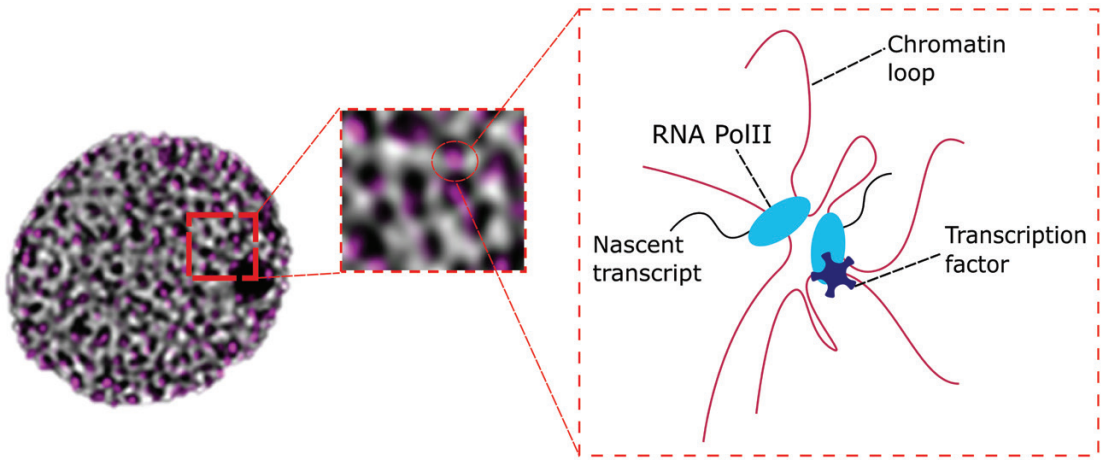

Fig. 4. Transcription factories in wheat nuclei. DAPI staining and an immunostaining (αPol II ab, pink) in a wheat interphase nucleus. RNA Pol II distribution is restricted to specific foci in the wheat nucleus, suggesting the existence of transcription factories in this organism. Transcription factories may represent a strategy for the spatial transcriptional regulation of large and highly complex genomes, bringing together co-regulated genes.

of agricultural importance, such as flowering time, yield, stress tolerance, and disease resistance. Probably one of the best described examples of gene loops in plants corresponds to the vernalization-sensitive gene loop formed between the 3' and 5' ends of the *FLC* locus, encoding a MADS domain TF involved in flowering repression in *A. thaliana* (Michaels and Amasino, 1999; Sheldon *et al.*, 2000; Seo *et al.*, 2009; Crevillén *et al.*, 2012). As an early event during vernalization, this loop is disrupted after cold exposure, which coincides with the accumulation of repressive histone modifications on the *FLC* locus and its down-regulation. Interestingly, the disruption of this loop also correlates with the accumulation of the *COOLAIR* antisense transcript, suggesting that this disruption might play a role in exposing *COOLAIR* promoter elements, therefore favoring its transcription and setting a conformational switch to an epigenetic repressed state of *FLC* (Csorba *et al.*, 2014).

Soon after the discovery of the gene loop at the *FLC* locus, another study provided for the first time an inventory of chromatin loops in a plant species through the integration of Hi-C and ChIP-seq in *A. thaliana*. Liu and collaborators observed that H3K27me3, a repressive histone mark, is commonly enriched in the promoters of interacting genes, a fact that is in agreement with other studies that have associated this particular histone modification with the regulation of chromatin topology in various organisms (Cheutin and Cavalli, 2014; Liu *et al.*, 2016; Veluchamy *et al.*, 2016; El-Sharnouby *et al.*, 2017; McLaughlin *et al.*, 2019). Furthermore, this study revealed that in *A. thaliana*, actively transcribed genes do not tend to associate in intergenic loops, self-loops being far more common in this organism (Liu *et al.*, 2016). On the other hand, loops involving repressed genes or those genes which are expressed at a low level were found to display particular epigenomic characteristics, including the presence of the histone variant H3.3 in their flanking regions, as well as the absence of repressive marks such as heterochromatic histone modifications and DNA methylation (Liu *et al.*, 2016).

A similar approach was implemented in rice by Dong and collaborators, who found that euchromatic loops (located in A compartments) were found to be significantly enriched in H3K27me3, as had been previously reported in *A. thaliana* (Liu *et al.*, 2016; Dong *et al.*, 2018). Furthermore, and similar to *A. thaliana*, several genes were found to self-loop and this phenomenon associated with the active expression of neighboring genes, indicating that this may be a conserved mechanism between plants (Liu *et al.*, 2016; Dong *et al.*, 2018). More recently, the ChIA-PET method was implemented for the study of the chromatin loops occurring in the rice genome at a significantly higher resolution (Zhao *et al.*, 2019). In this study, the authors reported that in this plant the majority of chromatin loops occur intrachromosomally, connecting co-regulated actively transcribed genes (Zhao *et al.*, 2019). This result was surprising and in contrast to the observations made in *A. thaliana*, where expressed genes do not physically associate (Liu *et al.*, 2016). Besides, in rice, the genes associated with active chromatin loops were found to be more conserved in different accessions than genes that do not participate in these structures, suggesting the existence of a selection pressure on these loci. On the other hand, through the study of H3K9me2-marked heterochromatin, the researchers were able to determine that a significant proportion of regions marked with this heterochromatic modification act as interaction anchors in the rice genome, displaying high TE density and stability. Interestingly, in this study, the effect of genetic variation on chromatin topology was assessed. For this, they compared two different rice varieties—Minghui 63 (MH63) and Zhenshan 97 (ZS97)—finding that functional genetic variations are linked to differential H3K4me3-associated 3D genome conformation and its impact on gene expression (Zhao *et al.*, 2019). This result suggests that differences in chromatin topology, and specifically chromatin loops, contribute to the existing phenotypic variation between varieties of this plant, and most probably, other plant species.

Two independent studies have applied the ChIA-PET technique for the generation of high resolution chromatin interaction maps in maize, combining it with ChIP-seq experiments to identify REs through their enrichment in specific chromatin features (Li *et al.*, 2019; Peng *et al.*, 2019). On the one hand, the generated maps revealed the existence of frequent intrachromosomal interactions involving REs, and that genes involved in the same loop tend to be co-expressed, similar to the observations in rice (Zhao *et al.*, 2019). Furthermore, by integrating chromatin interactions with distal elements and promoter-proximal regions with genome-wide association study (GWAS) data, the authors were able to identify a significant number of REs involved in chromatin interactions overlapping with QTLs (quantitative trait loci) associated with metabolic and agronomic traits. These results highlighted the potential effect of the identified distal elements on the expression of their target genes through chromatin interactions, contributing to the phenotypic variation associated with QTLs (Peng *et al.*, 2019). On the other hand, while the study by Li and collaborators corroborated some of the previous results by showing a correlation between chromatin interactions and gene expression, it also contributed with additional results regarding tissue-specific chromatin interactions (Li *et al.*, 2019). Interestingly, the authors found that constitutive genes often form stable self-loops with distant regulatory regions, ensuring their expression. In contrast, tissue-specific genes were found to be dynamic regarding the combination of regulatory elements with which they interacted, allowing them to reach appropriate temporal and spatial expression levels. Finally, this study allowed validation of putative enhancers that had been previously described in maize through genetic methods, including those of *TB1*, *ZmRAP2.7*, *UB3*, and *BX1*, which contribute to agronomic traits (Li *et al.*, 2019). Therefore, the implementation of ChIA-PET and 3C-derived methods could be used for the *de novo* discovery of REs involved in the regulation of complex phenotypical traits.

In parallel to the genome-wide identification of chromatin loops, some research groups have recently performed studies for the detailed characterization of specific previously described—as well as unknown—chromatin loops. For instance, Guo *et al.* (2018) contributed to the understanding of the molecular mechanisms governing the expression of *WUSCHEL* (*WUS*), a well-studied *A. thaliana* gene involved in development. *WUS* is an homeobox gene that has been exhaustively characterized

for its central role in the regulation of meristem determinacy and maintenance. This locus is both temporally and spatially regulated by various regulatory pathways including the direct transcriptional repression by AGAMOUS and KNUCKLES TFs, as well as the indirect pathway, mediated by PRCs (Yanofsky *et al.*, 1990; Lenhard *et al.*, 2001; Sun *et al.*, 2009; Liu *et al.*, 2011; Cao *et al.*, 2015; Sun and Ito, 2015). Although the indirect pathway has been well characterized, the direct mechanism remained to be understood in detail. Therefore, recently, Guo *et al.* (2018) evidenced the role of chromatin architecture as an additional regulatory layer governing *WUS* expression. Through the integration of 3C and ChIP approaches, the authors showed that the direct repression of *WUS* by AG involves the formation of a chromatin loop between the *WUS* 5'-transcription start site (TSS) and a sequence in its 3'-untranslated region (UTR). Furthermore, they found that the establishment of this loop is promoted by AG and that this structure has a repressive effect on *WUS* transcription, highlighting that the local 3D conformation of this gene is a determinant factor for its fine-tuning (Guo *et al.*, 2018).

Another recent study described the existence of an enhancer–promoter loop mediating hormone signaling in *A. thaliana*. Wang *et al.* (2019) demonstrated that jasmonate (JA) signaling regulates the chromatin looping between JA-enhancers and MYC2 targets in a MED25-dependent manner. MYC2 is known as the master regulator of the JA-mediated immunity pathway in *A. thaliana*, mediating physiological responses against diverse biotic and abiotic stresses (Dombrecht *et al.*, 2007; Kazan and Manners, 2013). The activity of this TF depends on its physical interaction with MED25, a subunit of the Mediator transcriptional co-activator complex, since MED25 recruits Pol II to MYC2 targets and positively regulates the H3K9 acetylation of these loci for their induction (Çevik *et al.*, 2012; Chen *et al.*, 2012; An *et al.*, 2017). In their study, Wang and collaborators showed that upon JA signaling, the identified enhancers interact with MYC2 targets (including the *MYC2* locus) through chromatin loops dependent on MYC2 and MED25. Surprisingly, the study also found that *ME2*, one of the regulatory elements forming loops with *MYC2*, has an inverse effect on the expression of this locus in response to short- and long-term MeJA treatment, as evidenced by the lower and higher sensitivity of the *me2* mutants to these treatments, respectively (Wang *et al.*, 2019). While it is well known that REs come into physical proximity with their targets through chromatin loops, the mechanisms by which the first regulate the second remain obscure. This phenomenon is especially true in plants, where studies on REs are significantly more recent and limited than those in animals (Weber *et al.*, 2016). Genome-wide studies have revealed the existence of thousands of uncharacterized chromatin loops in various eukaryotic genomes, which may indicate that the regulation of gene activity through these structures is not an exception but a particularly common process. Therefore, understanding the mechanisms behind the effect of a regulatory element on the transcription of a particular locus, or the establishment of a DNA loop between them, is crucial for depicting the regulatory role of the non-coding genome and lower scale chromatin interactions in responses to environmental signals.

R-loops represent a poorly characterized regulatory nuclear mechanism

In recent years, R-loops—triple-stranded nucleic acid structures composed of a DNA–RNA duplex and a displaced single DNA strand—have gained increasing attention in plants, since they have been shown to be involved in the regulation of various molecular and physiological processes, such as splicing, genome stability, flowering time, auxin signaling, and root development (Sun *et al.*, 2013; Ariel *et al.*, 2014, 2020; Conn *et al.*, 2017; Shafiq *et al.*, 2017; Yang *et al.*, 2017).

Like various other studies, the first genome-wide R-loop characterization for a plant was carried out in *A. thaliana*, through the implementation of ssDRIP-seq (ssDNA ligation-based library construction from DNA:RNA hybrid immunoprecipitation, followed by sequencing) (Xu *et al.*, 2017). In this study, Xu and collaborators were able to determine that these structures are highly prevalent in the *A. thaliana* genome, which presents >47 000 R-loop peaks. R-loops were shown to be enriched in specific genomic regions including promoters, TSSs, and gene bodies of protein-coding genes, as well as TEs and annotated long non-coding RNA (lncRNA) regions, centromeres, and telomeres. Interestingly, in *A. thaliana*, these structures were found to be frequently formed in both the sense and antisense orientation relative to transcription, a radical difference from human cells, where sense R-loops are far more common than antisense loops (Sanz *et al.*, 2016). By integrating ssDRIP-seq with ChIP-seq and bisulfite sequencing data, *A. thaliana* R-loops were found to be negatively correlated to GC DNA hypermethylation—a phenomenon that has been previously reported in mammals (Ginno *et al.*, 2012)—and associated with genomic regions decorated with CHG and CHH methylation and the repressive histone modifications H3K27me1 and H3K9me2 (Xu *et al.*, 2017).

A more recent study performed the genome-wide identification of R-loops in rice, addressing their relationship to gene expression and epigenomic features of chromatin (Fang *et al.*, 2019). Similar to *A. thaliana*, in rice there is not a direct and simple link between R-loop formation and transcription. In fact, the position of the R-loop within the gene structure appears to be determinant for its effect on gene expression: on the one hand, antisense and sense/antisense R-loops were found to be enriched around the TSS and positively correlated with transcription of overlapping genes. In contrast, sense-only R-loops are spread over the coding regions of genes, and inversely correlated to the expression of these loci. Furthermore, R-loops were found to be associated with genomic regions depleted of CG DNA methylation, indicating that this is a conserved characteristic among different eukaryotic lineages, including mammals, eudicots, and monocots (Ginno *et al.*, 2012; Xu *et al.*, 2017). Finally, the chemical (zebularin and trichostatin A) and genetic perturbation of the levels of DNA methylation and diverse histone modifications, including H3K9me2/3, H3K4me3, and H3ac, were shown to have a profound effect on R-loop formation in a genome-wide fashion, highlighting the complex regulatory network interlinking these structures with other epigenomic components (Fang *et al.*, 2019).

Similar to chromatin loops, some research groups have focused on depicting the regulatory nature of specific R-loops in diverse plants species (Sun *et al.*, 2013). For instance, widely studied *A. thaliana* genes have recently been shown to be regulated via these structures. A study by Conn and collaborators explored the regulation of *SEPALLATA3* (*SEP3*), encoding a MADS-box gene implicated in the development of all floral organs (Kaufmann *et al.*, 2009). They found that a circular RNA (circRNA) derived from exon 6 of *SEP3* regulates the abundance of an alternative splicing variant of the *SEP3* mRNA (lacking exon 6) through the establishment of an R-loop *in cis*. The formation of this R-loop was found to induce transcriptional pausing and the recruitment of splicing factors, leading to alternative splicing of its cognate transcript and its associated homeotic transformations, including reduced stamen and increased petal number (Conn *et al.*, 2017). Another example corresponds to the R-loop formed at the terminator region of the *FLC* locus, since the stabilization of this structure has been shown to influence *COOLAIR* and, indirectly, *FLC* expression (Sun *et al.*, 2013). This study added another layer involved in the regulation of flowering, thus making *FLC* a remarkable example of the complex gene expression regulation mechanisms in plants, with chromatin architecture playing an important role. In addition, a classic plant chromatin loop, involving the *PID* (*PINOID*) and *APOLO* (*AUXIN-REGULATED PROMOTER LOOP*) loci from *A. thaliana*, has been associated with downstream R-loops with a regulatory function (Ariel *et al.*, 2020). *PID* expression has been previously described to be regulated via a loop encompassing the promoter region of *PID* and *APOLO*, encoding an lncRNA molecule (Ariel *et al.*, 2014). In response to auxin signaling, the repressive loop is opened, allowing the transcription of both loci. The accumulation of *APOLO* transcripts is associated with the re-establishment of the *PID–APOLO* loop via RNA-directed DNA methylation (RdDM) and the recruitment of PRCs, involved in the deposition and maintenance of H3K27me3 (Ariel *et al.*, 2014). In a recent study, Ariel and collaborators demonstrated that the *APOLO* transcript can act *in trans* on various auxin-responsive distant loci recognized by short sequence complementarity. Upon their recognition, *APOLO* binds its targets, forming R-loops, a process that recruits the PRC1 protein LHP1 and modulates the 3D chromatin topology for the regulation of transcription (Ariel *et al.*, 2020).

Another equally recent study highlighted a direct link between R-loop formation and the establishment of chromatin loops in maize (Liu *et al.*, 2020). Centromeric retrotransposons from kind 1 (CRM1), which, together with CRM2, constitute the majority of centromeric sequences in maize, were found to encode different linear ncRNAs and back-spliced circRNAs able to bind to maize centromeres through R-loops. Furthermore, these R-loops were found to be associated with the formation of chromatin loops in centromeres, and the perturbation of circRNA levels through RNAi to alter chromatin looping in the CRM1 regions, indicating a direct link between circRNAs, R-loops, and chromatin looping. Finally, through protoplast transformations, the researchers assessed the presence of retrotransposon back-splicing in different crop plants, confirming the conservation of this phenomenon between numerous crops species, including oat, rice, wheat, sorghum, and soybean (Liu *et al.*, 2020). This result may suggest the existence of centromeric R-loops and chromatin loops mediated by these circRNAs in several plant species; however, this remains to be experimentally addressed, and their function elucidated.

Overall, these findings suggest the potential role of R-loops in gene silencing, transcription, and genome organization in plants, while highlighting the obvious knowledge gaps in the field. These include understanding the molecular mechanisms and proteins involved in the establishment of these loops, as well as the factors determining their activating or repressive effect on transcription. In yeast, R-loops have been previously associated with histone 3 phosphorylation and chromatin condensation at centromeric and pericentromeric regions (Castellano-Pozo *et al.*, 2013), suggesting that these structures have an active role in the 3D organization of the genome in various eukaryotic lineages.

Ploidy level alters genome topology

The largest known genomes can be found within the flowering plants. Several processes have contributed to genome expansion in these organisms, including the accumulation of TEs and regulatory sequences as well as polyploidization (Bennetzen *et al.*, 2005). Polyploidization can confer several evolutionary advantages, including heterosis and gene redundancy (Comai, 2005). This phenomenon can be evidenced in the higher adaptability, tolerance to abiotic stress, and pathogen resistance of various modern polyploid crop plants compared with their ancestors with a lower ploidy level (Alix *et al.*, 2017). Polyploidization increases the complexity of transcriptional regulation, a phenomenon that is predicted to be a consequence, at least partially, of changes in chromatin topology.

To date, few studies have assessed the effect of polyploidization on the nuclear architecture of plants, demonstrating that the number of genome copies has a remarkable effect on the configuration of chromatin in the nucleus. In an initial study, Wang *et al.* (2018) generated and compared 3D genome maps for two cultivated tetraploid cotton accessions and two extant diploid progenitors. In this study, they evidenced that polyploidization leads to A-to-B and B-to-A compartment switching of some genomic regions in the tetraploids compared with the diploids. Furthermore, when analyzing TAD (ICON?) conservation among species, it became evident that ~84% of diploid TAD-like structures were conserved in tetraploids, indicating a high degree of conservation of these structures after polyploidization; however, when integrating these results with transcriptomic data, it became evident that genes located in non-conserved TAD boundaries have a higher probability of displaying differential expression, suggesting that changes in their abundance and localization during polyploidization are associated with the establishment of new regulatory levels. In contrast, only 48.5% and 55.7% of regions predicted as putative enhancers in diploid species were conserved in tetraploids, and the remaining non-conserved REs were predicted to control the transcription of >10 000 genes on each progenitor. These results indicate the post-allopolyploidization

resetting of long-range RE–gene interactions for enormous amounts of genes. Finally, they found that inter-subgenomic interactions represented a high proportion (45.5%, 47.1%) of the interchromosomal interactions in both tetraploid species, providing evidence of their influence on the shaping of the nuclear topology. Additionally, within these inter-subgenomic interactions, the authors detected a number of interactions between homoeologous genes, which indicates that several of these are subjected to a common regulatory system for the coordination of their expression (Wang *et al.*, 2018).

More recently, another study assessed the effect of autopolyploidization (duplication of the same genome) on 3D genome topology by studying *A. thaliana* Columbia accession (Col-0) and its tetraploid (4×Col-0), experimentally generated through the use of colchicine (Zhang *et al.*, 2019). From this study, the authors concluded that autopolyploid *A. thaliana* displays reduced intrachromosomal and more interchromosomal interactions. Furthermore, even when diploid and tetraploid plants displayed similar interaction matrix patterns in a PCA, they presented certain regions with differences in their compaction state (12%), indicating again that genome duplication can induce A-to-B (and vice versa) switches of certain genomic regions. Such a transformation was correlated to changes in levels of histone modifications: the new so-called loose structure domains (LSDs, or A compartments) in the tetraploid displayed higher levels of H3K4me3 (an active histone modification) and lower H3K27me3 (a repressive histone modification), whilst the opposite was observed for the new compact structure domains (CSDs, or B compartments), indicating that switches in chromatin structure domains are associated with changes in histone modification profiles during autopolyploidization. As expected, these structure domain switches, as well as changes in chromatin interactions, were found to have an effect on the transcription of hundreds of genes, including the well-characterized floral repressor *FLC*, leading to the late-flowering phenotype of 4×Col-0 (Zhang *et al.*, 2019).

Another recent study addressed the 3D genome configuration of two related plants from the *Brassica* genus, which represents the group of crops most closely related to *A. thaliana* (Xie *et al.*, 2019). All species from this genus suffered lineage-specific whole-genome triplication (WGT) followed by diploidization, processes that have significantly reduced duplicated gene redundancy in this lineage (Lysak *et al.*, 2005; Town *et al.*, 2006; Liu *et al.*, 2014; Parkin *et al.*, 2014; Xie *et al.*, 2019). Several duplicated genes have been retained in this genus; however, there is evidence that this has not been random, since in these species one of the subgenomes has retained the majority of the ancestral copies of the duplicated genes, which, at the same time, display a higher expression level than their paralogs in the other two subgenomes. This phenomenon is known as subgenome dominance and has also been reported in other polyploid plants including maize and cotton (Bird *et al.*, 2018). Characterizing the nuclear topology of *B. oleracea* and *B. rapa*, Xie and collaborators observed that in both species, the dominant subgenome—the least fractioned—displays stronger physical interactions than the other two subgenomes, suggesting a link between subgenome dominance and 3D genome architecture. Furthermore, they showed that a significant proportion of the retained paralogs resulting from WGT co-localize in the nuclei of both species and display similar DNA methylation and histone modification profiles, several of these co-localization events being conserved between them. This phenomenon is similar to the observations made in tetraploid cotton, where homoeologous genes also co-localize, which suggests that keeping functionally linked genes in physical proximity may be advantageous for their co-regulation and dosage balance (Wang *et al.*, 2018). Furthermore, the physical proximity of genes in *Brassica* species was correlated to the biased gene retention occurring during polyploidization (Xie *et al.*, 2019), as was previously observed in rice (Zhao *et al.*, 2019), and may indicate that the spatial interaction between sequences contributes to their fixation in a population. This is supported by the finding that in animal genomes, co-localized homologs display a higher resistance to copy number variations than other genes (Xie *et al.*, 2016).

The phenomenon of subgenome dominance has also been observed in experimentally generated Arabidopsis hybrids (*A. thaliana* ×*A. lyrata*) (Zhu *et al.*, 2017). Through the integration of Hi-C, transcriptomic, DNA methylation, FISH, and ChIP-seq data, Zhu and collaborators determined that in such a hybrid, the *A. thaliana* genome displays an overall lower expression level compared with that of *A. lyrata*, a phenomenon that the authors associate with its higher compaction, indicating that the *A. lyrata* subgenome is dominant in this hybrid. Furthermore, they found that genes labeled with H3K27me3 were over-represented among *A. thaliana* genes differentially expressed in the hybrid, indicating a link between this histone mark and the transcriptional regulation of the *A. thaliana* genome in the hybrid. H3K27me3 has been associated in both plants and animals with the shaping of 3D genome structures (Feng *et al.*, 2014; Grob *et al.*, 2014; Wang *et al.*, 2015; Liu *et al.*, 2016; Veluchamy *et al.*, 2016), and it has been described that genes harboring this mark tend to co-localize (Bantignies *et al.*, 2011; Denholtz *et al.*, 2013; Schoenfelder *et al.*, 2015; Vieux-Rochas *et al.*, 2015; Wani *et al.*, 2016). For instance, in *Drosophila* spp. and mammalian cells, *Hox* homeotic genes establish clusters between them and with other H3K27me3-marked genes, structures that have been associated with their transcriptional repression (Denholtz *et al.*, 2013; Schoenfelder *et al.*, 2015; Vieux-Rochas *et al.*, 2015). Thus, the authors propose that the local conformational changes suffered by the *A. thaliana* subgenome in the hybrid contribute to both the chromatin compaction of this subgenome and the positioning of H3K27me3-marked genes in different regulatory compartments (Zhu *et al.*, 2017).

Even when subgenome dominance appears to be established from the first generation of hybridization, it has been observed that gene expression dominance of a specific subgenome tends to increase over successive generations; hence, this phenomenon tends to be stronger in naturally established allopolyploids than in recently experimentally generated ones (Edger *et al.*, 2017; Bird *et al.*, 2018). However, subgenome dominance does not seem to be a universal characteristic of allopolyploids, since in plants such as *B. napus* and allohexaploid wheat (AABBDD), global subgenome dominance is not detected (Chalhoub *et al.*,

2014; Pfeifer *et al.*, 2014; Harper *et al.*, 2016). Alternatively, in these plants, local regions favor the expression of specific homeologs over others (Bird *et al.*, 2018). Another particularity of modern wheat is that the A and B genomes interact with a higher frequency between them than with the D genome (Fig. 5A), indicating the existence of defined genome territories, and reflecting the evolutionary history of wheat domestication (Fig. 5B) (Concia *et al.*, 2020): the hybridization origin that gave rise to tetraploid *Tritichum turgidum* (AABB) occurred hundreds of thousand of years before the hybridization of this tetraploid ancestor with *Aegilops tauschii* (DD) giving origin to modern wheat. Therefore, it could be thought that the A and B genomes had established a degree of physical contact in *Tritichum turgidum* that was inherited after the introduction of the genome D through hybridization (Concia *et al.*, 2020). However, this remains to be proven, for example through the analysis of reconstructed hybrids.

Conclusion and future perspectives

The second half of the 20th century represented a crucial time for life sciences, as groundbreaking biological techniques were introduced, allowing—not without a large effort—the study, sequencing, and assemblage of the first genomes. Rapidly after genomes of model organisms became available, the scientific community undertook a quest for annotating them and deciphering the biological function of protein-coding genes, with the hope that studying the genetic code of the 'non-junk DNA' would provide an answer to the most relevant biological questions.

Even though understanding the biological function of specific proteins and protein complexes has allowed significant advances in all the fields of biology, it has also become clear that the genetic content itself is not the absolute determinant behind phenotypic traits in all organisms, including plants. It is in this way that in the 1990s various research teams around the world started studying the so-called at the time 'junk DNA', discovering that these sequences—which in some cases represent the vast majority of the eukaryotic genome—play crucial regulatory roles in gene activity. Therefore, in the last three decades, great efforts have been made towards exploring the non-genetic—or epigenetic—elements and mechanisms behind the phenotypic plasticity observed in living beings.

Until relatively recently, studying the organization of the genome of complex organisms was a challenging task due to the technical and financial limitations that it imposed; however, in the last years, we have witnessed a boom in the development of a diversity of techniques that allow these endeavors with increasing resolution and accessibility. For instance, chromatin organization was initially assessed through fluorescence microscopy, whereby specific labeling and co-localization of different chromatin substructures can be observed up to a resolution of ~200 nm (Schubert, 2017). Latterly, the emergence of 3C-based techniques, their application at the genomic scale, and integration with other -omic approaches allowed not only the identification of chromatin substructures that are not observable by conventional microscopy but also the definition of distinct and shared folding principles between different organisms. The development and refinement of techniques such as Capture-HiC (Mifsud *et al.*, 2015), ChIA-PET (Fullwood *et al.*, 2009), and HI-ChIP (Mumbach *et al.*, 2016) have allowed a better resolution of short- and long-range interactions for genomic regions or chromatin domains of interest and, therefore, the identification and characterization of smaller chromatin interacting units such as chromatin loops, transcription factories, and interactomes related to regulatory elements (Li *et al.*, 2019; Ricci *et al.*, 2019; Concia *et al.*, 2020). In addition, with the integration of robotics and microfluidics, 3D chromatin topology can be now analyzed for thousands of individual cells in a reasonable time, providing a strong statistical power to these methods, thereby opening up an era of single cell and cell type-specific studies (Boettiger and Murphy, 2020). Furthermore, the resolution limit imposed by conventional microscopy can now be overcome by a new generation of super-resolution techniques such as structured illumination microscopy (SIM), photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy

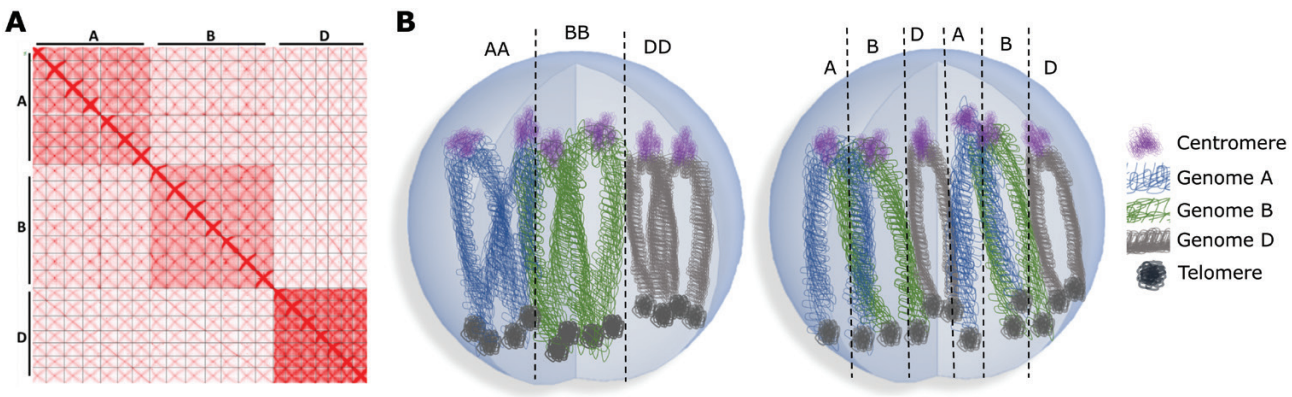

Fig. 5. Preferential subgenome interactions in hexaploid wheat. (A) Hi-C contact matrix of the hexaploid wheat genome. A and B subgenomes interact with a higher frequency between them than with the D subgenome, reflecting the evolutionary history of this staple crop. The Gene Expression Omnibus (GEO) accession number for the data set used for generating this figure is GSE133885 and corresponds to the study by Concia *et al.* (2020). (B) Schematic representation of the plausible predicted subgenome organization in the wheat nucleus. Each chromosome represents a copy of each subgenome.

(STORM), and stimulated emission depletion microscopy (STED), which allow an optical resolution of 50 nm or higher (Schubert, 2017). In the context of chromatin topology, super-resolution microscopy has been commonly used in mammals for visualizing chromatin architecture during carcinogenesis (Xu *et al.*, 2020), spatial distribution of chromatin modifications (Xu *et al.*, 2018), and chromosome regions involved in the formation of A and B compartments (Xie *et al.*, 2016; Nir *et al.*, 2018). These techniques have seldom been implemented in plant species, such as for elucidating the folding pattern at centromeres (Jankowska *et al.*, 2015; Ribeiro *et al.*, 2017) and telomeres (Schubert *et al.*, 2016), as well as for the localization of specific proteins such as RNA Pol II (Schubert, 2014; Schubert and Weisshart, 2015); nonetheless, they offer a surfeit of possibilities for the complementation of -omic-based studies in these organisms.

Due to the reviewed significant technical advances and the availability of an increasing number of sequenced and annotated plant genomes, our knowledge of chromatin organization and dynamics in plant nuclei is foreseen to rapidly expand. The scientific community has a variety of technical approaches to study chromatin topology with a resolution power that can be feasibly improved by the combination of several of these techniques and their adaptation for single-cell studies. On the one hand, 3C-based approaches can provide an even higher resolution when they are anchored on a specific genomic region or chromatin state of interest; on the other hand, super-resolution microscopy can equally complement these techniques by providing a nano-scale imaging of chromatin interacting units with a high resolution. This imaging resolution can be further enhanced by the combination of different super-resolution techniques and deep imaging techniques such as two-photon or multiphoton microscopy, allowing fast and dynamic imaging of nuclei (Komis *et al.*, 2015).

The application of 3C-derived and high-resolution imaging techniques in crop plants has significantly contributed to the understanding of the biology of these organisms. As herein reviewed, in the last years, we have found evidence that chromatin topology is involved in processes such as genome dominance and gene retention in species resulting from hybridization, as well as phenotypic traits associated with QTLs. Consequently, techniques for the study of chromatin conformation could be potentially implemented in research projects addressing topics as diverse as evolutionary questions or breeding programs. Depicting the genomic interactors of genes in specific tissues or in response to environmental signals—beside their molecular function—can be highly informative. Moreover, the implementation of state-of-the-art techniques such as CRISPR/Cas9 for the editing of interacting regions and regulatory elements, as well as techniques for studying chromatin interactions with RNA molecules can be of particular interest for understanding the molecular mechanisms regulating gene activity at a local level. We are confident that understanding the spatial organization of the genome in the nucleus, as well as its functional implications, will become a fundamental quest in the post-genomic era, as this will allow integration of our knowledge on the linear genome with molecular regulatory networks and phenotypic data.

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