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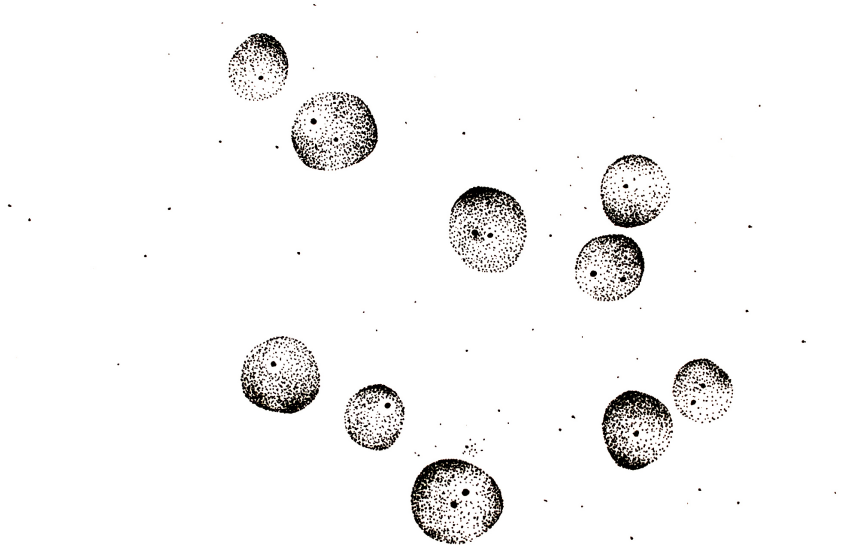
Characterization of chromatin mobility upon DNA damage in *Arabidopsis thaliana*

Romeo and Juliet: Act 5

Meschichi-Duriez Anis



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Abstract

Plant cells are subject to high levels of DNA damage from dependence on sunlight for energy and the associated exposure to biotic and abiotic stresses. Double-strand breaks (DSBs) are a particularly deleterious type of DNA damage, potentially leading to chromosome rearrangements or loss of entire chromosome arms. The presence of efficient and accurate repair mechanisms may be particularly important for sedentary organisms with late separation of the germline, such as plants. DSB repair is accomplished by two main pathways: nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ is achieved by stabilization and re-ligation of broken DNA ends, often with a loss or mutation of bases. HR is a more complex and conservative mechanism in which intact homologous regions are used as a template for repair. The molecular mechanisms that control DSB signaling and repair have been characterized extensively. Nonetheless, little is known about how the homology search happens in the crowded space of the cell nucleus. This thesis reveals the methodology to capture chromatin motion to investigate nuclear dynamics in different developmental and cellular contexts. Using live imaging approaches, we measured chromosome mobility by tracking the motion of specific loci using the *lacO*/LacI and ParB/*parS* tagging systems in *Arabidopsis thaliana*.

Our results have shown that chromatin mobility is affected by cell differentiation level, cell cycle phase, or genomic position, and that chromatin mobility increases when DNA damage is induced. Moreover, we observed an increase in chromatin mobility upon the induction of DNA damage, specifically at the S/G2 phases of the cell cycle. Importantly, this increase in mobility in S/G2 was lost on *sog1-1* mutant, a central transcription factor of the DNA damage response (DDR), indicating that repair mechanisms actively regulate chromatin mobility upon DNA damage. Studies have shown that HR is the predominant DSB repair pathway occurring during S/G2 phase. Therefore, we investigated the mobility of two GFP-tagged HR regulators, RAD51 and RAD54, corresponding to early and late HR. DSB sites show remarkably high mobility levels at the early HR stage. Subsequently, a drastic decrease in DSB mobility is observed, which seems to be associated with the relocation of DSBs to the nucleus periphery.

Altogether, our study suggests chromatin mobility as a non-negligible factor for DNA repair in plants, which may facilitate physical searching in the nuclear space thereby helping to locate a homologous template during homology-directed DNA repair.

Keywords: Arabidopsis, chromatin mobility, DNA damage, SOG1, RAD51, homologous recombination, cell cycle, chromatin dynamic, mean square displacement, DNA damage response

Karakterisering av rörlighet vid DNA-skada i *Arabidopsis thaliana*

Abstrakt

Växtceller utsätts för höga nivåer av DNA-skador från energin i solljus samt från exponering av biotiska och abiotiska påfrestningar. Dubbelsträngsbrott (DSB) är en särskilt skadlig typ av DNA-skada, som potentiellt kan leda till kromosom-rearrangemang eller förlust av hela kromosomarmar. Närvaron av effektiva och exakta reparationsmekanismer är då viktig för organismer med sen separation av könscellsceller, såsom växter. DSB-reparation åstadkoms genom två huvudvägar: icke-homolog ändfogning (NHEJ) av DNA strängen och homolog rekombination (HR). NHEJ uppnås genom stabilisering och förening av trasiga DNA-ändar, ofta med förlust eller mutation av baser som resultat. HR är en mer komplex och konservativ mekanism där intakta homologa regioner används som mall för reparation av skadan. Studier av de molekylära mekanismer som styr DSB-signalering och reparation är omfattande. Icke desto mindre är lite känt om hur homologisökningen sker i det trånga utrymmet i cellkärnan. I denna avhandling studeras metodiken för att fånga kromatin-rörelser för att kunna följa nukleär dynamik i olika utvecklings- och cellulära sammanhang. Med hjälp av levande avbildningsmetoder mätte vi alla kromosomer genom att spåra rörelsen hos specifika lokus med hjälp av lacO/LacI och ParB/parS-taggningssystemet.

Våra första resultat har visat att kromatin-rörligheten förändras beroende på differentieringsnivå, cellcykelfas eller genomisk position. Detta avslöjar vikten av att använda så kallade encells-metoder för denna typ av cellulära studier. Ett annat resultat var en generell ökning av kromatin-mobilitet vid induktion av DNA-skada. En ökning som var specifik under S/G2-faserna i cellcykeln. Denna ökning av rörlighet i S/G2 faserna förlorades när en *sog1-1* mutant analyserades. Denna mutation ligger i en central transkriptionsfaktor för DNA-skaderesponsen (DDR). Resultaten indikerar att reparationsmekanismer aktivt reglerar kromatin-mobilitet vid DNA-skada. Studier har tidigare visat att HR är den dominerande DSB-reparationsvägen som inträffar under S/G2-fasen. Därför undersökte vi rörligheten hos två GFP-märkta HR-regulatorer, RAD51 och RAD54, motsvarande tidig och sen HR. Platser för DSB uppvisade anmärkningsvärt höga mobilitetsnivåer i det tidiga HR-stadiet. Därefter observeras en drastisk minskning av DSB-rörligheten, vilket verkar vara associerat med förflyttning av DSB:er till kärnans periferi.

Sammantaget indikerar vår studie att kromatin-mobilitet är en icke försumbar faktor för DNA-reparation. Den skulle kunna fungera som en mekanism för att förbättra den fysiska sökningen i det nukleära utrymmet i cellkärnan för att lokalisera en homolog mall under homologiriktad DNA-reparation.

Keywords: Arabidopsis, chromatin mobility, DNA damage, SOG1, RAD51, homologous recombination, cell cycle, chromatin dynamic, mean square displacement, DNA damage response

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Meschichi A., Rosa S. (2021) Visualizing and Measuring Single Locus Dynamics in Arabidopsis thaliana. In: Sanchez-Serrano J.J., Salinas J. (eds) Arabidopsis Protocols. *Methods in Molecular Biology*, vol 2200.

II. Meschichi A*, Ingouff M*, Picart C, Mirouze M, Desset S, Gallardo F, Bystricky K, Picault N, Rosa S and Pontvianne F (2021) ANCHOR: A Technical Approach to Monitor Single-Copy Locus Localization in Planta. *Front. Plant Sci.* 12:677849. * Equal contribution

III. Meschichi A, Zhao L, Reeck S, Sicard A, White C, Da Ines O, Pontvianne F, Stefanie Rosa. The plant-specific DDR factor SOG1 increases chromatin mobility in response to DNA damage bioRxiv 2021.11.03.466744; (Submitted; *EMBO Reports*)

IV. Meschichi A., Kutashev K., Fonseca Cárdenas A., Zhao L., Algeret N., Sicard A., White C., Rosa S. Single-cell analysis of RAD51 transcription dynamic upon DNA damage (Manuscript in preparation)

Papers I-II are reproduced with the permission of the publishers.

The contribution of Meschichi-Duriez Anis to the papers included in this thesis was as follows:

I. Performed experimental design; Carried out experimental procedures; Analyzed data and discussed results; wrote the manuscript.

II. Performed experimental design; Carried out experimental procedures; Analyzed data and discussed results; wrote and commented the manuscript.

III. Performed experimental design; Carried out experimental procedures; Analyzed data and discussed results; wrote the manuscript.

IV. Performed experimental design; Carried out experimental procedures; Analyzed data and discussed results; wrote the manuscript.

Additional publications produced during the course of this doctoral thesis but not included are listed below:

I. Antoniou-Kourounioti R*., Meschichi A*., Reeck S*., Berry S., Menon G., Zhao Y., Fozard J., Holmes T., Wang H., Hartley M., Dean C*., Rosa S*., Howard M*. (2022) Integrating analog and digital modes of gene expression at Arabidopsis FLC (Submitted, eLife). * Equal contribution

List of figures

1. Introduction

DNA is constantly damaged by biotic or abiotic stresses and endogenous factors such as replication or transcription; therefore, the existence of repair mechanisms that maintain genome integrity is vital for all living organisms. Photo-autotrophic organisms such as plants need light for their metabolism and growth, making them more susceptible to genotoxic radiation such as UV. Furthermore, soil components such as aluminium or other heavy metals and chemicals can lead to DNA damage and compromise genome integrity. Because of their post-embryonic development, plants have to keep their genome integrity not to disrupt organs' development in later stages, such as germlines. Therefore, throughout evolution, plants had to develop mechanisms to resist those diverse genotoxic stresses and avoid the accumulation of DNA damage.

1.1 Exogenous and Endogenous sources of DNA damage

DNA damage can be caused by a variety of factors, including endogenous processes linked to intracellular activity, and exogenous stresses, such as abiotic (climate, soil composition, pollution) or biotic (organisms).

One primary endogenous source of DNA damage is metabolic activity such as free radical or reactive oxygen species (ROS) produced by the mitochondria and the chloroplasts (Sharma et al., 2012). The oxidative stress from ROS production generates genotoxic stress on the DNA (Poetsch, 2020). DNA-associated processes such as replication and transcription can lead to DNA damage when both machineries collide against each other (Zeman and Cimprich, 2014) or to an unrepaired damage site (Amiard et al., 2013). Exogenous stressors can also increase intracellular ROS production, including metals such as copper, cadmium, and mercury (Küpper and Andresen, 2016), heat (Zhao et al., 2018), drought (Lee et al., 2012), cold (Kawarazaki et al., 2013), and pathogens (Kim and Hwang, 2014; Yang et al., 2017). Within the exogenous factors, we can also find alkylating agents used in reverse genetic studies to induce random mutations in the genome (Natarajan, 2005; Till et al., 2003). Moreover, Ionizing Radiation like X-rays or gamma rays generated from radioactive pollution or space leads to DNA damage in plants (Manian et al., 2021; Pogribny et al., 2004). However, ultraviolet radiation from the sunlight is still the most abundant source of DNA damage (Britt, 1999, 2004)

DNA damage can be classified into four main categories: base mismatch, single-strand DNA breaks (SDB), DNA adducts, and double-strand breaks (DSB). Different genotoxic stresses will lead to specific DNA damage types. For example, UV radiation exposure produces cyclobutane pyrimidine dimers (CPD), which correspond to two cytosines or two thymidines interacting together. Other abiotic sources such as gamma radiation or some chemical agents can lead to the formation of more deleterious damages such as DSBs. Double strand breaks are lesions on both DNA strands, leading to pernicious consequences such as chromosomal rearrangement, partial chromosomal loss, and cell death *in fine*.

For DNA repair mechanisms to be initiated, it is essential that the DNA damage response pathway is activated (Hu et al., 2016; Yoshiyama et al., 2013a). The DDR pathway is closely conserved between eukaryotes except for some differences in the plant's kingdom. This pathway controls the cell cycle progression throughout some checkpoints to repair damage more efficiently (Campos and Clemente-Blanco, 2020).

1.2 DNA damage repair pathway

1.2.1 Sensors and mediators

The DNA damage response (DDR) pathway evolved to detect damage sites and transduce a signaling cascade to induce several mechanisms such as cell cycle arrest, DNA repair, or apoptosis (Figure 1).

In *Arabidopsis thaliana* (Arabidopsis), the two leading players of DDR sensing are Ataxia Telangiectasia Mutated (ATM) and ATM and Rad3-related (ATR), which belong to the phosphatidylinositol 3-kinase-like family and have homologs in animals. ATM is activated by the MRN complex (MRE11, RAD50, and NBS1) after DSB detection, and ATR is activated through the 9-1-1 complex (RAD9, RAD1, and HUS1), which recognizes replication stress and DNA single-stranded damages (SSD) (Amiard et al., 2010; Saldivar et al., 2017). Both kinases phosphorylate targets are involved in cell cycle arrest or DNA repair. In case of DSB induction, ATM will phosphorylate the histone variant H2AX through the MRN (Mre11-Rad50-Nbs1) complex. The phosphorylation of H2AX (γ-H2AX) is a crucial element in the DDR, which help to recruit and accumulate DNA damage repair proteins to the damaged site (Amiard et al., 2010; Burma et al., 2001). Therefore, antibodies against γ-H2AX are often used in cytology to visualize DSB sites. DNA damage sensors (MRN, 9-1-1) and most signal transducers (ATM/ATR) are conserved between animals and plants (Bundock and Hooykaas, 2002; Culligan et al., 2004, 2006; Gallego and White, 2001; Garcia et al., 2003; Heitzeberg et al., 2004). Other signal transducers such as CHK1 and CHK2 have not been identified in plants.

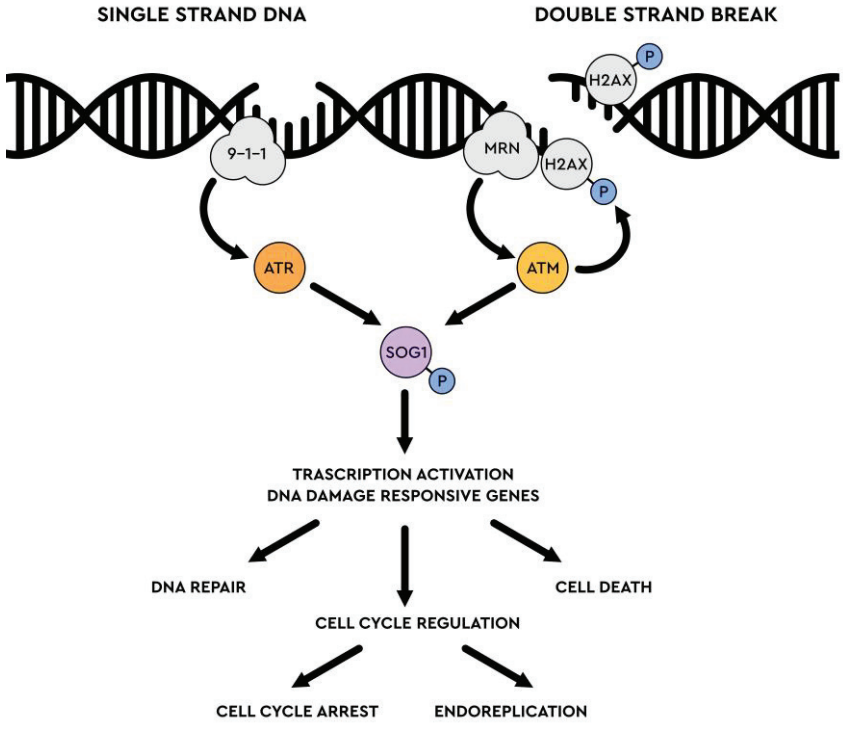

Figure 1: Schematic representation of the DNA damage Response pathway in plants. Single-stranded DNA is sensed by 9-1-1 complex which activates ATR, while double-strand breaks (DSBs) are detected through the MNR (MRE11 NBS1 RAD50) complex which activates ATM. Both kinases, ATM and ATR, phosphorylate the transcription factor SOG1, which will induce the expression of genes involved in cell cycle regulation, cell death control, and DNA repair.

1.2.2 SOG1 master regulator

Downstream to ATM/ATR, a transcription factor, called SUPPRESSOR OF GAMMA RESPONSE1 (SOG1), controls DNA damage repair pathways and cell cycle arrest. SOG1 was isolated from a genetic screen of suppressors of *xpf-2*, which is defective in DNA repair endonuclease activity (Preuss and Britt, 2003). To explore the expression pattern of *SOG1*, the promoter region and coding region of *SOG1* were fused with GUS reporter revealing specific expression to shoot and root apical meristems and in lateral root primordia (Yoshiyama et al., 2013b). *Sog1-1* mutant plants lacked responses to DNA damage that are typically triggered in wild-type plants, such as growth arrest (Yoshiyama et al., 2009). SOG1 was found to be part of the plant-specific NAC transcription factors (Yi et al., 2014; Yoshiyama et al., 2009) and is activated once hyperphosphorylated by ATM during DNA damage (Yoshiyama et al., 2013b). Phosphorylation of SOG1 is ATR independent but it seems that both act together to induce cell cycle arrest upon DNA damage (Yoshiyama et al., 2009). The phosphorylation of SOG1 by ATM, genetic interaction with ATR, and the transcription factor activity suggested SOG1 as the DDR master regulator, similarly to p53 in animals. However, the amino acid sequences of SOG1 and p53 share a low degree of homology, suggesting that independent DDR regulatory mechanisms evolved in both models (Yoshiyama et al., 2014). Further studies using ChIP-seq analysis have revealed genes targeted directly by SOG1, including genes responsible for cell cycle regulation, such as CDK inhibitors (CDKi) and DNA repair, with a preference for genes involved in homologous recombination (Ogita et al., 2018). Transcriptomic analysis has shown that SOG1 directly targets around 300 genes involved in DNA repair, cell cycle, and other networks such as plant

defense (Bourbousse et al., 2018). Suggesting SOG1 is crucial in coordinating DNA repair with the cell cycle.

1.2.3 Cell cycle regulation upon DNA damage

The defects in root growth inhibition upon irradiation in *sog1-1* mutant, its target genes, and transcriptomic analysis showed SOG1 as a critical player in cell cycle arrest via the checkpoint activation. The cell cycle progression can be stopped depending on the cell cycle phase where the damage is detected by four main checkpoints; G1/S, intra-S, G2/M, and M (Gentric et al., 2021) (Figure 2). Most of the checkpoint activation results from CDKi transcription induced by SOG1. Within the CDKis expressed by SOG1, we can find WEE1, which is involved in the cell cycle G1/S intra-S and G2/M during replicative stress, and SMR5 and SMR7, which induce endoreplication (Cools et al., 2011; De Schutter et al., 2007; Yi et al., 2014). Other genes involved in the cell cycle, such as CYCLINB1.1 (CYCB1:1), are expressed by SOG1 to regulate the cell cycle and act conjointly with RAD51 during homologous recombination (Schnittger and De Veylder, 2018). Transcriptomic analysis revealed that SOG1 upregulates cell cycle regulators' expression, while MYB3R transcription factors inhibit the expression of G2/M cell cycle genes (Bourbousse et al., 2018). Altogether, DNA damage response and the cell cycle progression are coordinated to activate the appropriate DNA repair pathway in the appropriate cell cycle phase.

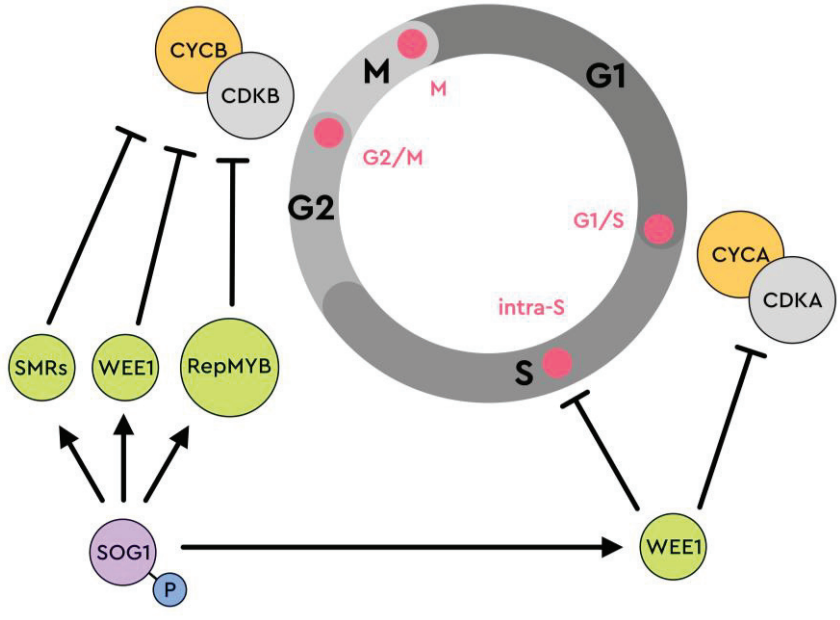

Figure 2: Cell cycle regulation in response to DNA damage in plants. Four canonical checkpoints in the cell cycle G1/S, intra-S, G2/M, M (indicated in red). Upon DNA damage, SOG1 regulates the G1/S, intra S and G2/M checkpoint by targeting the core cell cycle genes WEE1, SMRs (SMR5 and SMR 7) and by rep-MYB3Rs.

1.2.4 Two ways, error-prone or error-free?

Following the detection of DSBs and the cell cycle arrest, the appropriate DNA repair pathway will be activated. The repair pathway chosen is determined depending on when the DSB occurred during the cell cycle.

Double strand breaks can be repaired via two main pathways, non-homologous end joining (NHEJ), which is the junction of the two broken ends independently of sequence homology, or the homologous recombination (HR) which requires a homologous DNA sequence to serve as a template (Chang et al., 2017; Huertas, 2010). NHEJ is a short and straightforward pathway to repair DSBs but is error-prone because of the loss of genetic information or the introduction of mutations (Chang et al., 2017). Unlike NHEJ, HR is a precise and accurate mechanism to repair damaged locus by using DNA sequences homologous as templates (Huertas, 2010). HR is primarily active during the S/G2 phase, and NHEJ, although active throughout the cell cycle, is used mainly during G1 (Chang et al., 2017; Hustedt and Durocher, 2017; Johnson and Jasin, 2000). In Arabidopsis, NHEJ and HR pathways are highly conserved across eukaryotes. The initial step in NHEJ is binding Ku70/80 heterodimers, the recruitment of XRCC4 at the DNA ends, and LIG4 to finalize the ligation (Davis and Chen, 2013). Accordingly, *atKu80*, *atKu70*, *and atLIG4* have been identified in Arabidopsis, and the corresponding mutants show hypersensitivity to gamma radiation (Riha, 2002; West et al., 2002). In dicot plants, including Arabidopsis, NHEJ plays a significant role in the T-DNA insertion during transformation (Saika et al., 2014).

1.2.5 Homologous recombination error-free

Homologous recombination is a repair pathway that uses a homologous template without integrating mutations. This repair pathway plays an essential role in somatic cells and meiosis. Studies in animal cells show that HR is predominantly active in pluripotent cells compared to differentiated cells (Adams et al., 2010; Tichy et al., 2010). Homologous recombination is divided into two main steps, the pre-synaptic and synaptic phases (Figure 3).

1.2.6 Pre-synaptic phase

The first step of DSB repair by HR is the end resection, which corresponds to the generation of a long 3-single-stranded DNA (ssDNA). The formation of the ssDNA will be later used for the strand invasion on the homologous DNA strand. The prominent actors involved in this process are the MRN complex, CtIP, and exonuclease 1 (EXO1) (Paull and Gellert, 1998; Sartori et al., 2007). The MRN complex and CtIP generate a short ssDNA overhang, allowing EXO1 recruitment and the generation of a long ssDNA overhang.

Resected 5'-end DNA strands at DSBs are coated by replication protein A (RPA), which will then be replaced by RAD51 through the action of BRCA2 (Jensen et al., 2010; Powell and Kachnic, 2003). The formation of a nucleoprotein filament composed of RAD51 is an essential step at later stages, including homology search, pairing with the template, and strand invasion (Benson et al., 1994; Sugiyama et al., 1997; Tavares et al., 2019).

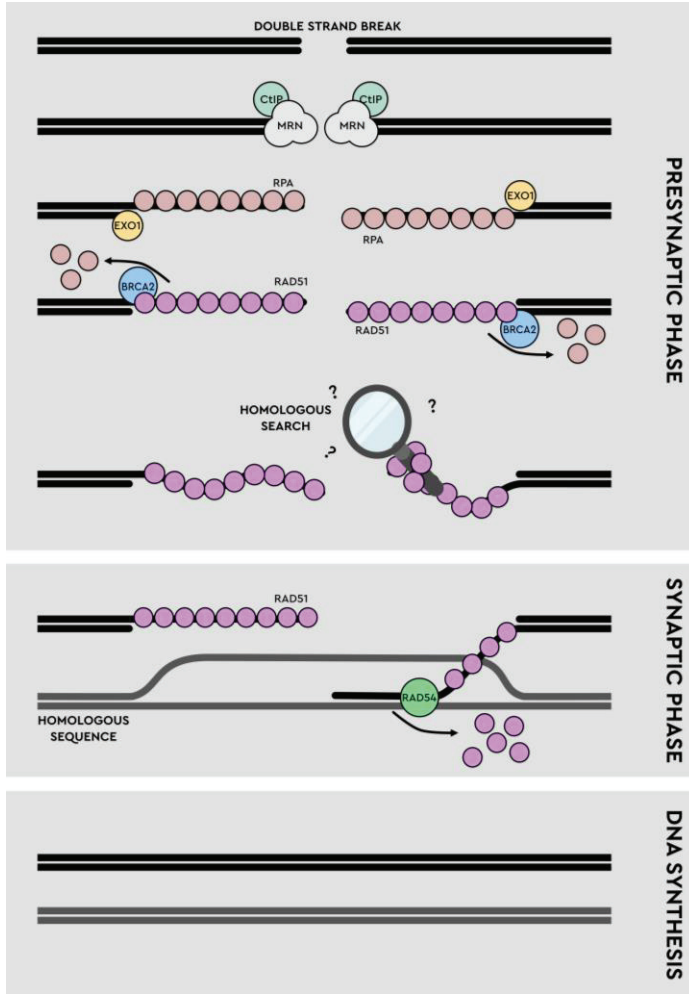

Figure 3: An overview of the homologous recombination pathway. The first step is the presynaptic phase corresponding to the DNA end resection by the EXO1 nuclease, leading to the generation of 3'overhangs at DNA double-strand break sites. 5'-terminated strands at DSBs are coated by RPA and replaced by RAD51 through BRCA2. Subsequently, RAD51 nucleoprotein filament initiates homology search, pairs with the homologous sequence (template) and enters the synaptic phase with the strand invasion via RAD54.

Once the pre-synaptic filament is formed and the template is in proximity, the strand invasion can be initiated (Bugreev et al., 2007).

1.2.7 Synaptic and DNA synthesis

During the strand invasion, the ssDNA will interact physically with the donor dsDNA and form a D-loop structure (Eggler et al., 2002; Lavery and Kowalczykowski, 1992). Rad54, a dsDNA-specific ATPase, is the motor with dsDNA translocase activity stimulated when RAD51 forms the nucleoprotein filament (Alexeev et al., 2003; Kiianitsa et al., 2002, 2006; Kowalczykowski, 2015; Swagemakers et al., 1998). RAD54 role is to remove RAD51 from the ssDNA and promotes D-loop stability, allowing DNA synthesis to occur (Mason et al., 2015). Polymerase δ or polymerase ε are recruited at the D-loop to start the DNA synthesis filling in the missing genetic information (Hicks et al., 2010; Wilson et al.,2013).

1.2.8 Homologous recombination in Arabidopsis

Similarly to animals, HR is predominantly present in the meristematic zone, where pluripotent cells are present, compared to the cells in the differentiation zone (Hirakawa et al., 2017; Da Ines et al., 2013). The main actors in HR are conserved across eukaryotes, including Arabidopsis (Heyer WD 2010; Ranjha L 2018). *RAD51* gene family is highly conserved, with seven members: *DMC1*, *RAD51*, *RAD51B*, *RAD51C*, *RAD51D*, *XRCC2*, and *XRCC3* (Bleuyard et al., 2006). Additionally, the other actors involved in HR are duplicated in Arabidopsis, such as RPA subunits RPA1 with five homologs,

RPA2 and RPA3 with two homologs each, and EXO1 with two homologs (EXO1A and EXO1B) (Eschbach and Kobbe, 2014; Kazda et al., 2012). Most of the mutants in HR, such as *atrpa1a, atrpa1b, atrad51b*, *atrad51c*, and *atxrcc2* are sensitive to DSB-induced treatments, and sterility is observed for *atrad51c and atrpa1a* (Aklilu et al., 2014; Bleuyard et al., 2004; Ishibashi et al., 2005; Osman et al., 2009; Takashi et al., 2009) suggesting their importance in somatic and meiotic tissues. Interestingly in mammals, mutations on RAD51 are embryo lethal, whereas in Arabidopsis, RAD51 is not essential for vegetative development but required for HR repair in somatic cells (Lim and Hasty, 1996; Tsuzuki et al., 1996).

In Arabidopsis, the two main HR actors, RAD51 and RAD54 are mostly present in the meristematic tissues but absent in differentiated organs (Hirakawa and Matsunaga, 2019; Hirakawa et al., 2017; Da Ines et al., 2013). Similar to other eukaryotes, RAD51 and RAD54 are restricted to the S-G2 of the cell cycle (Hirakawa et al., 2017; Weimer et al., 2016). Altogether, this suggests that HR mechanisms and regulation are mostly conserved in plants.

1.3 Homology search

One key element during homologous recombination is the need for a homologous donor template that is used for repair. This implies that HR requires the searching for a template and this process has been generally termed 'homology search'. However, the mechanisms behind the chromatin movement required during the homology search and the pairing are still not clear. Chromatin motion in interphase cells was demonstrated already 25

years ago using live imaging on GFP-tagged loci, but was disputed by researchers who argued that chromatin is static (Cremer et al., 1982). Further studies have then confirmed that chromatin moves randomly in both, *S. cerevisiae* and *D. melanogaster* (Marshall et al., 1997). Then, chromatin movement has been shown to be involved in several nuclear processes, such as gene regulation, replication and DNA repair (Soutoglou and Misteli, 2007). Studies have shown that genes can move to different nuclear compartments and that these can be associated with changes in expression. Immunoglobulin loci are shown to be either on the periphery or intranuclear position depending if their expression state is inactive or active respectively (Kosak et al., 2002). In Drosophila and Arabidopsis, particular genes were shown to cluster by moving toward each other, during silencing (Bantignies et al., 2011; Cheutin and Cavalli, 2012; Francastel et al., 2001; Rosa et al., 2013; Wani et al., 2016). Concerning DNA replication, imaging and Hi-C methods have shown changes in the reorganization of the chromatin in the nucleus to coordinate the replication timing (Dimitrova and Berezney, 2002; Ryba et al., 2010; Zhang et al., 2019). Finally, several studies in the different models have reported changes in chromatin mobility after DSB induction at the undamaged loci and damage site level (Dion et al., 2012; Hauer et al., 2017; Herbert et al., 2017; Lawrimore et al., 2017; Miné-Hattab and Rothstein, 2012; Miné-Hattab et al., 2017; Seeber et al., 2013; Strecker et al., 2016). This induction of mobility during DNA damage has been suggested as essential to finding the template during the homology search (Gehlen et al., 2011; Herbert et al., 2017; Wiktor et al., 2021). One model suggested that the DSB moves to find the template, and the increased global chromatin movement

would help by increasing the efficiency of this search (Gehlen et al., 2011; Wiktor et al., 2021).

1.4 Monitoring chromatin mobility

Technologies such as chromatin conformation capture allowed us to study changes in chromatin topology, which can be visualized by imaging methods such as DNA FISH on fixed tissues. However, those methods are snapshots of the chromatin organization, which are restricted in capturing the spatiotemporal architecture changes or chromatin dynamic. However, new technologies have emerged that allow to visualize and monitor chromatin dynamics at the locus level with a temporal resolution of seconds to sub-seconds in living cells (Barth et al., 2020). Chromatin mobility upon DNA damage can be studied at three different levels: damaged/DSB site, undamaged loci, and global level (Miné-Hattab and Chiolo, 2020; Shaban and Seeber, 2020).

To measure chromatin mobility at the locus level (undamaged loci), genetic engineering made possible the labeling of a genomic loci in real-time through gene tagging systems that allow following the movement of specific chromosome positions in living cells. Two types of gene tagging systems have been developed, bacterial-based- or CRISPRCas9- based methods (Figure 4A). Within the bacterial-based gene tagging systems, we find the *lacO*/LacI and *tetO*/TetR systems, which correspond to the insertion of a repeat binding sequence into the genome where fluorescently tagged proteins interact specifically (Marshall et al., 1997; Michaelis et al., 1997;

Robinett et al., 1996) (Figure 4B). In the case of these methods, the repeat sequence can be 10 kb long. More recently, another system (ANCHOR) based on the ParB/ParS bacterial operon has been engineered, in which the repeat sequence (*parS*) is much smaller (up to 1kb) and the ParB tagged with a fluorescent protein spreads over the surrounding chromatin (Saad et al., 2014).The introduction of the CRISPR (clustered regularly interspaced short palindromic repeat)–Cas9 (CRISPR-associated protein 9) system brought new opportunities also for gene locus labeling. The tagging of sequences with CRISPR/Cas9 where possible by fusing a deadCas9 with eGFP (dCas9-eGFP), and co-expressed guide RNAs (gRNAs) (Chen et al., 2013, 2016, 2018).

In Arabidopsis, *lacO*/LacI gene tagging was the only gene tagging system available (Kato and Lam, 2001; Matzke et al., 2003)Using those lines allows determining the locus position in the nucleus in a three-dimensional context providing information such as chromosome arrangement in interphase or gene clustering after gene repression (Matzke et al., 2008; Rosa et al., 2013). Additionally, CRISPR locus tagging system was used in *Nicotiana benthamiana* and allowed to measure the mobility of repeat sequences such as telomers (Dreissig et al., 2017). Now, this technology is available to visualize Arabidopsis telomere repeats (Němečková et al., 2019).

To measure the damage site mobility, gene tagging lines can include at proximity, HO endonuclease cut-site, CRISPR/Cas9 system or I-SceI endonuclease cut-site, to induce DSB at specific positions in the genome (Miné-Hattab and Rothstein, 2012; Roukos et al., 2014; Saad et al., 2014; Wiktor et al., 2021). Another approach is based on the use of fluorescent-

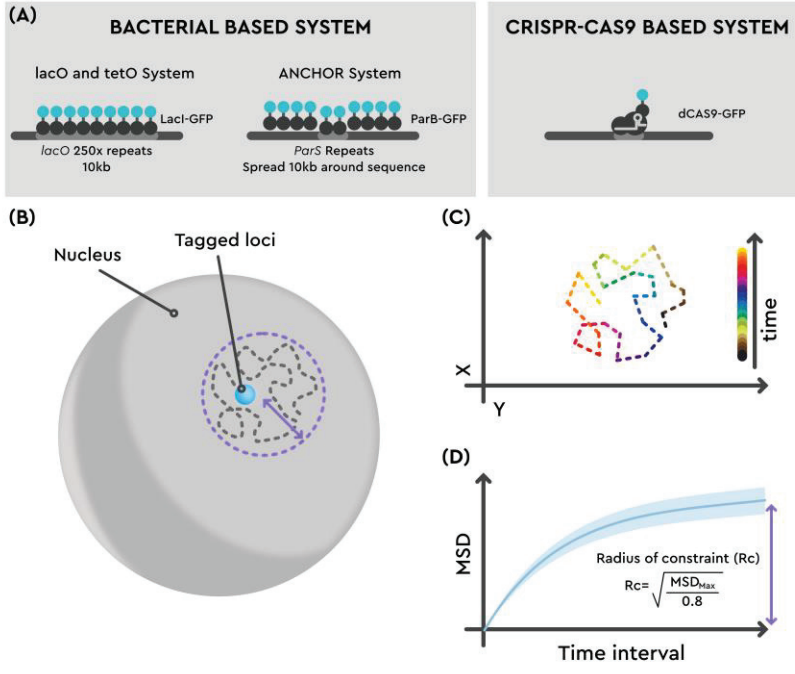

Figure 4. Different gene-tagging systems to label genomic loci in living cells. (A) Gene tags based on bacterial systems correspond to the insertion of a repeat binding sequence into the genome, where molecules fused to florescent protein will specifically interact. Schematic representation of the *lacO*/LacI system. LacI protein fused to GFP interacts with lacO repeats. In the case of ANCHOR system, the repeat sequence is smaller and ParB-GFP spreads around. CRISPR-Cas9 based system uses a catalytically dead Cas9 (dCas99 fused to a fluorescent protein. By designing specific guideRNAs, specific and several sites can be visualized. (B) Illustration of single locus mobility inside a subnuclear compartment (purple circle). (C) Representative 2D projection trajectory during time acquisition. (D) Trajectories extracted from 2D projection data are examined using the mean square displacement (MSD) analysis, which generates an MSD curve allowing to understand loci motion and to calculate the radius of constraint (Rc).

Tagged HR components which allow the detection of DSBs upon damage via radiation or genotoxic chemicals (Caridi et al., 2018; Lottersberger et al., 2015; Miné-Hattab and Rothstein, 2012).

Now, new live imaging technologies allow the study of the whole genome mobility or global mobility by measuring the movement of multiple nano genome domains simultaneously, by using fluorescent-tagged chromatin components such as histones (Lou et al., 2019; Nozaki et al., 2017; Shinkai et al., 2016).

All these imaging methods allow the monitoring of chromatin dynamics at different levels by coupling live imaging with a mean-square displacement (MSD) analysis (Caridi et al., 2018; Spichal and Fabre, 2017). MSD analysis is a common method to examine and characterize the physical space explored by a specific locus within the nucleus. It calculates the average of the distance travelled by a particle over all points of the individual trajectory by increasing the time interval (Figure 4C-D) (Michalet and Berglund, 2012; Oswald et al., 2014). Using the MSD analysis allows the generation of an MSD curve where values such as the radius of constraint (Rc) describing the nature of the chromatin movement (Figure 4D). In *Arabidopsis thaliana*, not much is known concerning chromatin mobility during DNA damage.

Plants are organisms whose genomes are constantly being challenged due to their sessile lifestyle, and which for some species corresponds to a challenge that remains for thousands of years. Because of their post-embryonic development and phototrophy, plants have to find efficient ways

to conserve genome integrity during evolution. As reported before, the homologous recombination has been extensively studied at a molecular level, but the mechanisms behind the chromatin mobility needed during homologous search in a highly ordered chromatin structure are still not clear. In this thesis, we focus on understanding the interaction between DSB repair by HR and the chromatin dynamics by using live imaging tools at a single cell analysis.

2. Research Aim

The overall aim of this thesis is to understand chromatin dynamics upon DNA damage at the global and DSB site level during DNA repair in Arabidopsis. To fulfill this objective, we have investigated four main points:

Developed methods to measure chromatin mobility in *Arabidopsis thaliana* (Paper I and II).

Investigated chromatin mobility during plant development (Paper II and III).

Investigated chromatin mobility at undamaged loci upon DNA damage (Paper III).

Investigated DSB mobility at different stages of homologous recombination (III and IV).

3. Results and discussion

3.1 Chromatin mobility quantification in *Arabidopsis thaliana*

The availability of tools to study chromatin mobility in Arabidopsis was limited at the beginning of this project. Therefore, the first objective of the thesis was to develop new tools allowing to visualize and capture chromatin mobility using live imaging methods. We have taken advantage of *lacO*/LacI lines already available (Kato and Lam, 2001; Matzke et al., 2003, 2008)to start our chromatin mobility studies in Arabidopsis. In these lines the LacI:GFP construct was put under the control of a specific promoter, pRPS5, which is active in cells of the division zone of the root tip (paper I, Figure 2A-B). The developmental structure and transparency of the Arabidopsis root are ideal for image analysis and we have therefore focused on this tissue throughout the thesis. Along the primary root, three developmental zones can be identified: the root apical meristem (RAM) with the highest proliferation rate, and cells enter a zone of elongation (EZ) and acquire their final identity at the Differentiation Zone (DZ). Therefore, by studying the Arabidopsis root, there is the possibility to additionally address differences between cell types, and developmental mechanisms.

The first step in establishing our chromatin mobility protocol was to optimize the imaging settings to capture the chromatin movement with appropriate spatial and temporal resolution in Arabidopsis root nuclei. Movies using time-lapse were needed across relatively long periods with a brief time interval between pictures. Studies measuring chromatin motion in other systems were able to capture 3D images of nuclei at short time intervals by using microscopes able to perform quick acquisitions, such as confocal microscopes coupled with spinning disc or wide-field microscopes with advanced optical configurations (e.g. SpectraX light source) (Arai et al., 2017; Dion et al., 2012; Herbert et al., 2017; Horigome et al., 2014; Seeber et al., 2013). The microscopes used in this study allowed the acquisition of Z-stack with 3 slices to conserve an optimal time resolution, limiting the analysis from 3D to 2D. Additional optimization has been implemented to allow the image acquisition on Arabidopsis roots to overcome the root movement due to growth and photobleaching due to prolonged exposure of the tissue. We found that a 5min movie with a 6sec time intervals, and 3 Z-steps was enough for capturing the locus mobility (paper I, Figure 2C). Therefore, movies from the epidermal cells in the meristematic and differentiation zones were acquired and processed to remove movement bias from root growth and xy drift (paper I, Figure 3). Trajectory data was analyzed by MSD as explained in the introduction, using Equation 1-2 from paper I, which allowed to obtain the MSD graph and the radius of constraint (Rc) (paper I, Figure 4) (paper I, Equation 3). To validate our protocol, we measured the chromatin mobility of root epidermal cells in the meristematic and differentiation zone. Interestingly, meristematic cells show higher chromatin mobility than

differentiated cells, similar to the histone exchange dynamics observed in a previous study (Rosa et al., 2014) (paper III, Figure 1B). Therefore, this method could be effective and sensitive for identifying differences in chromatin movement between cells in Arabidopsis.

To limit possible biases associated with *lacO*/LacI system, we also used other gene tagging systems in order to verify and validate our chromatin mobility measurements. In collaboration with the group of Dr Pontivianne (CNRS, France) we developed the ANCHOR system for Arabidopsis. Unlike the *lacO*/LacI, which requires many binding sites to visualize a locus, the ANCHOR system spreads over the surrounding chromatin with few repeats needed (paper II, Figure 1A). A single-copy of *parS* 1-kb-long fragment is enough to serve as a binding platform for ParB proteins (Dubarry et al., 2006) (paper II, Figure 1B). *parS* sequence is composed of four repeat sequences allowing oligomerized ParB proteins to bind and propagate over the *parS* sequence and adjacent DNA (paper II, Figure 1A). The loose interaction between ParB proteins and *parS* sequences avoids disturbances with other machineries, such as transcription or DNA repair (Saad et al., 2014).

In Arabidopsis, the ANCHOR system allowed us to visualize a single locus in different organs and cell types (paper II, Figure 3). Therefore, using our protocol, we compared the chromatin mobility between epidermis cells in the meristematic and differentiation zones. The results obtained were similar to the ones obtained with the *lacO*/LacI system, with higher chromatin mobility in the meristematic zone compared to the differentiation zone (paper II, Figure 5E).

3.2 What could influence chromatin mobility?

Using our protocol, we could measure chromatin mobility in different cellular contexts at the single cell level. As discussed previously, both gene tagging systems showed higher chromatin mobility in meristematic cells than in differentiated cells, supporting the idea that the chromatin in undifferentiated cells holds a more dynamic conformation (Arai et al., 2017; Meshorer and Misteli, 2006; Rosa et al., 2014). In Arabidopsis, histone acetyltransferase HAG1 has been shown to play a role in histone exchange increase in the meristematic zone (Rosa et al., 2014). Histone acetylation is known to decrease the affinity for DNA, causing the nucleosomes to unfold and increasing accessibility to machinery (Grunstein, 1997), which might improve chromatin mobility. This suggests that epigenetics could be one factor in regulating chromatin movement. Moreover, factors like polyploidy could also play a role in mobility. Chromatin mobility appeared to be higher in haploids than in diploid cells for particular loci (Miné-Hattab et al., 2017).

We also examined how chromatin mobility differed depending on the chromosome positions by using other lines where *lacO* sequences were localized on chromosomes 2 and 3 (chr2 and chr3) from Matzke collection (Matzke et al., 2008, 2010). *LacO* line chr2 has the same chromatin mobility as the *lacO*/LacI line used for establishing the method (paper III, Figure S5) and the ANCHOR line (paper II, Figure 5B), both localized on chromosome 5. However, the construct on chr3 shows a significant reduction in mobility compared to the other lines, which might be due to the subtelomeric localization. Interestingly, *lacO*-tagged loci localized in the subtelomeric

regions in yeast have similar mobility as other positions on the genome. This difference between yeast and Arabidopsis could be explained by the overall chromatin structure difference, where respectively (sub)telomeres are on the nuclear periphery (Gonzalez-Suarez and Gonzalo, 2008; Hozé et al., 2013; Meister et al., 2010; Zimmer and Fabre, 2011) and the nucleolus in Arabidopsis (Schubert et al., 2014). Those results suggest that chromosomal positioning could also play a role in mobility, but other subtelomeric lines have to be generated to confirm this hypothesis.

Our study has revealed the cell cycle as an important factor to take into account when studying chromatin mobility. *LacO* line (chr5) was crossed with CDT1a fused to RFP, a cell cycle marker specific to the S/G2 phase in Arabidopsis (Aki and Umeda, 2016; Yin et al., 2014) (paper III, Figure 3A-B). Cells in S/G2 show lower chromatin mobility (paper III, Figure 3D), which can be explained by the binding of the cohesin complex, which keeps sister chromatids together. Similarly to Arabidopsis, chromatin mobility is lower in the S phase than in G1 in yeast haploid cells (Dion et al., 2012; Heun et al., 2001). In mammalian cells, chromatin mobility is consistent from mid G1 until G2 phase, but increases in early G1 (Walter et al., 2003; Wiesmeijer et al., 2008).

Surprisingly, the nuclear volume does not seem to influence the overall chromatin mobility. For instance, trichoblast and atrichobalst cells that show clear differences in nuclear volume showed similar chromatin mobilities (paper III, Figure 1D-E) and increased nuclear volume due to the doubling of the DNA content during replication and endoreplication does not lead to a

proportional increases of chromatin mobility (paper III, Figure 1C) (paper II, Figure 5E). This suggests that the volume of the nucleus and the amplitude of the chromatin movement are independent.

Altogether, four main factors seem to play a role in chromatin mobility in Arabidopsis; epigenetic marks, chromosome position/nuclear organization, differentiation status/ploidy, and the cell cycle.

3.3 DSBs and chromatin mobility

To examine chromatin mobility during DSB repair, we had to choose a treatment to induce DSBs in Arabidopsis genome. Here, zeocin was used to induce DSBs, a method widely used in other models. To confirm zeocin action as a DSB inducer, an immunofluorescence staining using antibodies specific for DSB sites, γ-H2AX has been performed, revealing that the number of damage sites is proportional to the concentration of zeocin used (paper IV, Figure 2B). Similarly, the expression of DDR genes such as RAD51 and BRCA1 increases in a dose-dependent manner (paper III, Figure S1). However, is increasing the concentration of zeocin increasing the levels of response equally in all cells or does it increase the number of cells responding to DNA damage? To answer this question, we used single molecule Fluorescent In Situ hybridization (smFISH) to investigate RAD51 expression pattern at the tissue and single cell level (paper IV, Figure 1A-B). Interestingly, we observed that both the number of cells expressing RAD51 increases, and cell expression of RAD51 expression level increases (paper IV, Figure 1D).

Therefore, we can modulate both the amount and the number of cells having DSB by varying the concentration of zeocin.

We measured chromatin mobility of the *lacO* line (chr 5) in different concentrations and incubation periods of zeocin. An increase in mobility is observed only after an overnight treatment at the highest concentration (paper III, Figure 2B and S2). This suggests that chromatin mobility increases after reaching a certain level of damage, which has also been observed in yeast (Seeber et al., 2013). This threshold effect could be explained by the probability of a DSB occurring close to the tagged sequence. We then examined whether the increase in chromatin mobility depended on the chromosome location by measuring the movement of two other *lacO* lines. Each tagged locus showed a similar rise in mobility upon zeocin (paper III, Figure 2D-E). Similarly, the ANCHOR line revealed the same increase in chromatin mobility upon DNA damage (paper III, Figure 3G). Altogether, our results revealed that the induction of DSBs increases the mobility at different positions in genome, which might be the reflection of an effect at the global level.

Because DNA damage repair and the cell cycle are interconnected through the DDR pathway we thought to verify if the increased mobility induced by DNA damage is specific to a cell cycle phase. As described previously, cells in the S/G2 phase have lower chromatin mobility than G1 cells (paper III, Figure 3D). Therefore, is the mobility increase observed at 170µM zeocin due to an accumulation of G1 cells?

To test this hypothesis, we used *lacO*LacI/CDT1a line to quantify the proportion of S/G2 cells upon zeocin. Upon 10µM of zeocin, an increase of cells in S/G2 is observed, which might be due to the cell cycle arrest (paper III, Figure 3C). In the presence of 10µM of zeocin, even if the proportion of S/G2 cells increases, the increase in chromatin mobility does not occur. Additionally, upon a higher zeocin concentration (170µM), the proportion of cells in S/G2 decreases drastically (paper III, Figure 3C). However, this increase in mobility could it be explained by only the accumulation of G1, or is the chromatin mobility in S/G2 cells are also increasing? Interestingly, it is with the higher zeocin concentration in S/G2 cells where the increase in chromatin mobility has been observed (paper III, Figure 3F). Importantly, we monitored the chromatin mobility in *sog1* mutant, revealing a loss in DSB-induced chromatin mobility, suggesting that the DDR pathway controls the chromatin mobility increase upon DNA damage (paper III, Figure 5A).

Because increased chromatin mobility induced by DSBs is cell cycle and DDR dependent, the possibility of this motion resulting from extensive chromosome fragmentation can be rejected. Additionally, using propidium iodide (PI) staining, we tested if the cells monitored undergo apoptosis due to DNA damage. Once apoptosis occurs, the cell membrane integrity is lost, allowing the PI to enter, resulting in an intense signal inside cells. The absence of staining in epidermal cells from the meristematic confirmed that the increased mobility is not a consequence of cell death (paper III, Figure S3). Indeed, cells in Arabidopsis roots are still actively transcribing even at high zeocin concentrations (paper IV, Figure 2C). These results rule out increased

chromatin mobility being a by-product of cell death or DNA fragmentation, but by an active nuclear process taking place upon DNA damage.

Altogether, this work shows that chromatin mobility could be an additional factor in DNA damage repair mechanisms in plants.

3.4 DSB mobility during homologous recombination

The increased chromatin mobility upon DNA damage is observed in S/G2 cells, which could represent cells where homologous recombination is occurring. To further test this hypothesis, we used single molecule FISH (smFISH) to monitor RAD51 transcription activity combined with EdU staining to mark cells with newly replicated DNA (paper IV, Figure 4). Interestingly, cells transcribing RAD51 are mostly in the early S phase (paper IV, Figure 4), which correlates with previous results where RAD54 protein level is between S/G2 (Hirakawa et al., 2017). Therefore, homologous recombination in Arabidopsis is restricted in S/G2 in the same phase where chromatin motion increases, suggesting the mobility observed could be a component of HR.

Then, DSB sites were tracked by monitoring two essential actors of HR, which accumulate at DSBs, RAD51, and RAD54, and that had been fused with GFP and YFP, respectively (Hirakawa et al., 2017; Da Ines et al., 2013). After the resection of the broken dsDNA, RAD51 is first to be recruited forming a nucleoprotein filament (Barzel and Kupiec, 2008; Dillingham and Kowalczykowski, 2008; Li and Heyer, 2008). Once the template is found, RAD54 will interact physically with RAD51 and stimulate the DNA strand

exchange and the D-loop formation (Li and Heyer, 2008; Tavares et al., 2019). In Arabidopsis, we observed RAD51 and RAD54 foci formation follow similar order of action after DSB induction (paper III, Figure 4B). Therefore, by measuring the mobility of RAD51 and RAD54 foci, we were able to track the movement at early (pre-synaptic) and late (synaptic) phase HR (paper III, Figure 4A and C). We observed that RAD51 foci mobility is higher than RAD54, which suggests a need for mobility at an early stage of the HR (paper III, Figure 4D). Unlike Arabidopsis, in yeast RAD54 and RAD51 foci have the same mobility upon zeocin (Dion et al., 2013). However, RAD54 foci mobility varies according to their position within Arabidopsis nucleus (paper III, Figure 4E). RAD54 foci in the nucleoplasm have similar mobility as RAD51, contrary to those in the nuclear periphery, which present lower mobilities (paper III, Figure 4F).

Two hypotheses could explain the mobility of RAD54 foci similar to RAD51; RAD54 participation in the movement involved in homology search or in the mobility needed for the DSB to relocate to the periphery. The first hypothesis is supported by *in vitro* studies showing Rad51/Rad54 being a functional unit of homology search (Mazin et al., 2003; Tavares et al., 2019). However, the four hours delay between RAD51 and RAD54 recruitment would go against this hypothesis. Nevertheless, this measurement of foci formation has been performed in separate lines, limiting understanding of the recruitment kinetics at the single damage site level. Concerning the second hypothesis, RAD54 is shown to play a role in DSB relocation to the nuclear periphery in mice and in Arabidopsis, supporting the idea that high mobilities of RAD54

are associated with the relocation to the nuclear periphery (Agarwal et al., 2011; Hirakawa and Matsunaga, 2019).

Overall, our work has shown that DSB sites have higher mobility at early homologous recombination, which might correspond to the homology search during HR. Once the template is found, the DSB will relocate to the periphery, which will result in a reduction of mobility.

4. Conclusions

In this thesis, we described for the first time changes in chromatin mobility associated with DNA damage in the context of a multicellular organism. In particular, our methods and results will allow to further study chromatin mobility during different developmental contexts and in response to DNA damage in *Arabidopsis thaliana*.

A part of this thesis is based on the development of gene tagging systems and live imaging tools to capture chromatin mobility at the single cell level, to unravel the movement of the chromatin during development, DNA damage and of DSB site during HR.

One aspect investigated was how different elements might affect chromatin mobility. We found that cell cycle and cell differentiation status influence chromatin mobility, which might be through chromatin structural changes with nucleoproteins such as histones, cohesins, condensins or changes in the epigenetic marks.

This work revealed that the DDR pathway induces increased chromatin mobility upon DNA damage similarly to other models (Dimitrova et al., 2008;

Krawczyk et al., 2012; Lawrimore et al., 2017; Lottersberger et al., 2015; Miné-Hattab and Rothstein, 2012; Neumann et al., 2012; Saad et al., 2014; Schrank et al., 2018). We first found that chromatin mobility increases only after a certain level of DNA damage, where cells are still viable. This increase in mobility has been observed in different chromosome positions and by using other gene tagging systems. Importantly, we could show that the induction of chromatin mobility upon DNA damage occurs only in cells during S/G2 and requires the activation of the DDR master regulator SOG1. This result is particularly interesting because SOG1 is specific to the plant species, with no homologs in mammals or yeast where chromatin mobility changes have also been observed. Therefore, these results suggest that DNA damage-induced chromatin mobility is conserved in evolution but potentially through the action of different molecular players.

Finally, we investigated the DSB site mobility during homologous recombination. RAD51 and RAD54 were used to detect DSB sites repaired by the HR, and to track their movements in different steps, presynaptic and synaptic phases of HR respectively. We first found that RAD51 foci show a higher movement than RAD54 foci, which might correspond to the homologous search occurring at the presynaptic steps of HR. Interestingly, RAD54 foci show higher mobility in the nucleoplasm compared to the nuclear periphery, suggesting the implication of RAD54 during the homologous search and the absence of mobility once DSBs have relocated to the nucleus periphery at late HR.

Altogether the results in this thesis represent a significant advance in our understanding of how physical and molecular phenomena underlying chromatin dynamics are involved in DSB repair in higher organisms.

5. Future perspectives

From this thesis, chromatin mobility has been revealed to be part of the DNA damage response in plants, but three main questions remain to be uncovered, what are the mechanisms behind the increased mobility at the undamaged loci and DSB sites upon DNA damage (i), Does chromatin mobility happen at the global level (ii) and how the homology search happens (iii)?

5.1 Which mechanisms are behind the increased mobility at the undamaged loci and DSB sites upon DNA damage?

Our studies revealed that increased chromatin mobility upon DNA damage is SOG1-dependent. Therefore, in order to understand the mechanisms behind this increased mobility one possibility is to investigate chromatin remodelers that are SOG1 targets. Ogita *et al* transcriptomic analysis, revealed that SOG1 affects the expression of genes involved in cell cycle, DNA repair, apoptosis and chromatin remodeling (CHR8, CHR31 and RAD54)

(Ogita et al., 2018). Other chromatin remodelers are found to act independently from SOG1, and are important for HR repair efficiency, such as SWR1-C, MMS21 and RNF20 (Donà and Mittelsten Scheid, 2015; Kim, 2019). However, DNA damage-induced mobility could be controlled upstream from the DDR pathway such as ATR and ATM. Phosphoproteomic studies in response to ionizing radiation identified chromatin modifiers (FVE/MSI4), remodelers (SDG26/ASHH1/PIE1/REF6), and transcription factors (WRKY1, HON4, GTE4, GTE5, and NF-YC11) a target of ATM/ATR dependent phosphorylation (Roitinger et al., 2015). DNA repair actors have been shown to play a critical role in DSB site mobility, such as RAD51, RAD54, ATR homolog Mec1, and 53BP1 homolog Rad9 (Dion et al., 2012, 2013). In Arabidopsis, RAD54 is a key actor in the homolog sequence pairing after DNA damage (Hirakawa et al., 2015). Moreover, chromatin remodelers such as INO80 were reported to increase mobility at the DSB sites by reorganizing nucleosomes at the damage site (van Attikum et al., 2004; Morrison et al., 2004). Further studies have shown the histone degradation of 20% to 40% by INO80 upon DNA damage to relax the chromatin and increase global mobility. In Arabidopsis, INO80 has been shown to be involved in the efficiency of HR (Fritsch et al., 2004). Therefore, it will be interesting to test the chromatin mobility in these mutants as well their HR repair efficiency using GUS reporter lines (Orel et al., 2003).

5.2 Does chromatin mobility happen at the global level?

We have observed an increase in chromatin mobility upon DNA damage using different gene tagging systems at different genomic positions. However, does this mobility occur in the entire genome, in different regions with a specific epigenetic signature, or around the damage site? Studies have shown an increase in histone degradation upon DNA damage which may facilitate chromatin movement and increase recombination rate (Cheblal et al., 2020; Hauer et al., 2017), suggesting a need to change chromatin structure at a global scale. However, this chromatin change at a global scale is still unknown in plants. As observed previously, chromatin mobility induced in high levels of DNA damage might be due to a change in chromatin structure. Preliminary results from our lab have shown a significant decrease in histone content upon high levels of DNA damage. It will be interesting to confirm these global changes for instance by developing a imaging methods that allow to measure genome-wide mobility. Methods such as displacement correlation spectroscopy (DCS), SptPALM combining PALM with SPT, and Dense Flow reConstruction and Correlation (DFCC) can now be used to understand the global chromatin mobility in the genome. DCS analyze the direction of chromatin movements simultaneously across the entire nucleus through time, SptPALM localization and bleaching of photoactivatable fluorescent-proteins and Dense Flow reConstruction and Correlation (DFCC) allows for detection of chromatin motion at nanoscale resolution (Nozaki et al., 2017; Shaban et al., 2018; Zidovska et al., 2013). These new methods will now allow localizing the increase in mobility at multiple genomic positions simultaneously in the nucleus upon DNA damage.

5.3 How does the homology search happen?

In the introduction, several hypotheses concerning the homologous search have been exposed. Recent studies have tried to understand the mechanisms behind the homology search mechanisms. Two models are proposed, one based on 'chromatin tentacles' where RecA/RAD51 nucleoprotein filament explores the chromatin in 3D, allowing trans-homology search (Kim et al., 2010; Piazza et al., 2017, 2021), and the other one based on short-range 1D sliding mechanisms allowing a cis-homology search (Ragunathan et al., 2012). Both models propose that RAD51 microfilament is the functional unit operating in homology search. RecA filament in bacteria is highly dynamic, undergoes a 3D search, and reduces to 2D for a faster homology search (Wiktor et al., 2021). RAD51 HR partner, Rad54, has been proposed to participate in the homology search by interacting with RAD51 filament (Tavares et al., 2019).

Previous studies have described the formation of a lncRNA at the DSB site in a DDR-induced manner (Wei et al., 2012). This lncRNA formation at the DSB site would play a role as a scaffolding structure for molecules involved in DNA repair, or as a 'template detector' to find the homologous sequences. However, the role of those has been not described yet. By using smFISH on cuttable gene tagging lines, we would be able to detect the site of formation and visualize their dynamics during time. We would be also able to track the clustering from the damage site and homologous sequences each of them tagged with a specific ANCHOR system with different fluorescent tags.

However, further microscopy and gene tagging tools have to be developed to have a better understanding of homologous search mechanisms.

This thesis has revealed the existence of DNA damage-induced chromatin movement in plants by developing new biological and imaging tools. Understanding the role of chromatin movement in DNA repair in plants will be very important for future environmental challenges. Plants also need to find ways to resist extremely genotoxic environments induced by human activity such as Chernobyl, and chemically contaminated fields (Cui et al., 2017; Kovalchuk et al., 2004). In the case of Chernobyl, Arabidopsis progenies from this region are resisting a high genotoxic stress, possibly due to high expression levels of RAD51 (Kovalchuk et al., 2004). The constant ozone depletion by human activity will lead to a drastic increase in UV exposure and to an increase in melanoma in humans and a global decrease of crop yield of 20-25% (Barnes et al., 2019; Fiscus and Booker, 1995). Therefore, understanding DNA damage repair mechanisms in plants would be beneficial for humans and agriculture in a genetically challenging future.

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Popular science summary

Every organism has inside each cell a molecule that acts like a cookbook called DNA. This cookbook has all the recipes for elements needed for the cell to work correctly, such as proteins involved in the structure, energy, protection, and repair. However, fragments of this DNA molecule can be damaged with time or due to external factors such as radiation. Like every recipe, the consequences of the deletions of some parts could be more or less important, from no changes to drastic changes in the final product or even the total deletion of the final product. For the recipe of pancakes, forgetting the baking powder is not problematic but forgetting the flour is catastrophic! Those changes in the DNA molecule are called mutations. Those could end in three different directions: silent mutations (nothing changes), a non-silent mutation (the final protein changes, which could lead to a new function), or a deleterious mutation (leading to the absence of the final protein). The two last options could lead to cell death or pathology like cancer. Therefore, to avoid a bad ending, cells have developed ways to repair the information lost on the DNA molecule.

One of them is by glueing the remaining's of the recipe together, which fixes the page structure but does not restore the missing information. The other way is to find a piece of similar information to the broken one to restore the loss. This last repair method is called "homologous recombination", where "homologous" and "recombination" means "similarity" and "copy-paste".

The broken DNA molecule searches for another with similar information and copy-past the missing information. Researchers have found the mechanism behind the copy-paste, but the mechanism behind the search for similarities remains obscure. The DNA molecule within the cell nucleus is not only one recipe book but a recipe book divided into several volumes in a library.

The objective of my Ph.D. was to identify mechanisms allowing homologous DNA sequences to get together. The first part of my work allowed me to establish a way to track DNA molecule movement by placing fluorescent proteins, similar to placing a bookmark in a book at a specific recipe. Thanks to this method, I have observed an increase in movement of the broken DNA and the DNA surrounding it, which decreases once the damage is repaired. However, some questions remain: How do broken DNA molecules recognize the homologous DNA molecules, and what is the mechanism making DNA molecules move? Those questions are the following objectives of the lab.

Studying this subject on plants comes from the walk with my mother (a legendary florist and botanist), with whom we were amazed by the plant's ability to adapt to extreme conditions. Conditions include growing under the snow in the Alps, beneath concrete on the streets of Paris, and under the intense Mediterranean sunlight. If humans spent similar time as plants tanning under the sunlight, the number of skin burns and cancer cases would skyrocket because UV from the sunlight breaks DNA.

To avoid this, we wear cloth and hide in the shade, which is not the case for plants. Because plants do not move, they had to adapt to UV overexposure by founding ways to protect themselves from UV and repair their DNA. In a genetically challenging future for humans because of the UV exposure increase from ozone depletion, studying plants' tools to resist radiation will benefit us.

Résumé scientifique vulgarisé

Dans chacune des cellules de tout organisme vivant on trouve une molécule d'ADN. Cette molécule joue le rôle d'un livre de cuisine qui renferme toutes les recettes des éléments nécessaires au bon fonctionnement de la cellule, comme les protéines impliquées dans la structure, le métabolisme, la protection et la réparation de l'organisme. Cependant, certains fragments de cette molécule peuvent être détruits au cours du temps ou en raison de facteurs externes tels que les radiations.

Comme toute recette, les conséquences de la dégradation d'un fragment peuvent être plus ou moins importantes : ne provoquer aucun changement, changer radicalement le produit final, voire ne pas avoir de produit final. Pour la recette des pancakes, l'oubli de la levure ne serait pas critique, mais oublier la farine serait bien plus catastrophique ! Dans la molécule d'ADN, ces changements sont appelés mutations. On les classe en trois catégories : les mutations silencieuses (rien ne change), les mutations non-silencieuses (la protéine est différente voire obtient une nouvelle fonction), ou les mutations délétères (la protéine ne peut pas être produite). Les deux dernières catégories peuvent provoquer la mort de la cellule, ou des pathologies comme le cancer. Par conséquent, pour éviter une fin tragique, les cellules ont développé des moyens de réparer l'information perdue de la molécule d'ADN. L'une d'elles consiste à coller les restes de la recette ensemble, ce qui

répare la structure de la page mais ne restaure pas les informations manquantes. Une autre façon est de chercher une recette similaire à celle endommagée pour la réparer tout en rétablissant l'information perdue. Cette dernière méthode de réparation est appelée "recombinaison homologue", où "homologue" et "recombinaison" signifient respectivement "similarité" et "copier-coller". La molécule d'ADN cassée en cherche une autre avec une information similaire, puis copie-colle l'information manquante. Les chercheurs ont trouvé le mécanisme moléculaire qui permet « copier-coller ». Cependant, le mécanisme qui permet la recherche des similitudes reste obscur. L'ADN dans le noyau de la cellule n'est pas qu'un seul livre, mais plutôt un livre de cuisine divisé en plusieurs tome dans une bibliothèque. L'objectif de ma thèse était a été d'identifier les mécanismes qui permettent le rapprochement des fragments homologues.

Mes travaux on permit de trouver un moyen de suivre le mouvement de l'ADN en plaçant sur celle-ci des protéines fluorescentes, telle un petit marque page dans un livre pour repère une page avec une recette spécifique. Grace à cela, mes recherches montrent que la molécule d'ADN endommagée s'agite, ainsi que les molécules d'ADN environnantes, et ce mouvement diminue une fois la séquence homologue et le fragment endommagé réunis. Cependant, certaines questions demeurent : comment les molécules d'ADN brisées reconnaissent-elles des molécules d'ADN homologues, et quel est le mécanisme qui fait bouger les molécules d'ADN. Ce sont les nouvelles questions auxquelles mon laboratoire d'accueil essaiera de répondre.

L'idée d'étudier ce sujet sur les plantes me vient des balades avec ma mère (une très grande fleuriste et botaniste) avec qui nous nous émerveillons de l'habilité des plantes à s'adapter aux conditions extrêmes, telle que sous la neige des alpes, le béton parisien ou l'intense soleil méditerranéen. Si les gens passaient autant de temps que les plantes à bronzer au soleil,

l'incidence des coups de soleil ou des cancers de la peau exploseraient, car les UV de la lumière du soleil peuvent briser l'ADN. Pour l'éviter nous portons des vêtements et nous nous déplaçons à l'ombre, ce qui n'est pas possible pour les plantes. Parce que les plantes ne bougent pas, elles ont dû s'adapter à la surexposition aux UV en trouvant des moyens de s'en protéger et de réparer leur ADN. Dans un futur qui sera plein de défis génétiques pour l'humain, dus à la diminution de la couche d'ozone qui provoquera une augmentation de l'exposition au UV, il est important de savoir comment réparer l'ADN en l'étudiant chez les plantes.

Populärvetenskaplig sammanfattning

Varje organism har inuti varje cell en molekyl som fungerar som en kokbok som kallas DNA. Den här kokboken har alla recept på komponenter som behövs för att cellen ska fungera korrekt, såsom proteiner involverade i strukturen, energi, skydd och reparation. Men fragment av denna DNA-molekyl kan skadas med tiden eller på grund av yttre faktorer som strålning. Liksom alla recept kan konsekvenserna av utbyte av vissa delar vara mer eller mindre viktiga, från inga förändringar till drastiska förändringar i slutprodukten eller till och med total borttagning av slutprodukten. För receptet på pannkakor är det inte problematiskt att glömma bakpulvret, men att glömma mjölet är katastrofalt! Dessa förändringar i DNA-molekylen kallas mutationer. De kan sluta i tre olika riktningar: tysta mutationer (ingenting förändras), en icke-tyst mutation (det slutliga proteinet förändras, vilket kan leda till en ny funktion) eller en skadlig mutation (som leder till frånvaron av det slutliga proteinet). De två sista alternativen kan leda till celldöd eller sjukdomar som cancer. Därför har celler utvecklat sätt att reparera informationen som går förlorad på DNA-molekylen.

En av dem fungerar genom att limma ihop resterna av receptet, vilket fixar sidstrukturen men inte återställer den saknade informationen. Det andra sättet är att hitta en bit liknande information som den trasiga för att återställa förlusten. Denna sista reparationsmetod kallas "homolog rekombination", där "homolog" och "rekombination" betyder "likhet" och "kopiera-klistra".

Den trasiga DNA-molekylen söker efter en annan med liknande information och kopierar den saknade informationen. Forskare har hittat mekanismen bakom så kallad copy-paste, men mekanismen bakom sökandet efter likheter är fortfarande oklar. DNA-molekylen i cellkärnan är inte bara en receptbok utan en receptbok uppdelad i flera volymer i ett bibliotek. Målet med min doktorsavhandling var att identifiera mekanismer som tillåter homologa DNA-sekvenser att mötas. Den första delen av mitt arbete tillät mig att etablera ett sätt att spåra DNA-molekylers rörelse genom att placera fluorescerande proteiner, liknande att placera ett bokmärke i en bok vid ett specifikt recept. Tack vare denna metod har jag observerat en ökad rörelse av det trasiga DNA:t och DNA som omger det. En effekt som minskar när skadan är reparerad. Men några frågor kvarstår: Hur känner trasiga DNA-molekyler igen de homologa DNA-molekylerna, och vad är mekanismen som får DNA-molekyler att röra sig? Dessa frågor är under fortsatta studier i forskningsgruppen.

Att studera detta ämne om växter kommer ursprungligen från promenaden med min mamma (en legendarisk florist och botaniker), där vi blev förvånade över växtens förmåga att anpassa sig till extrema förhållanden. Tillstånden inkluderar att växa under snön i Alperna, under betong på gatorna i Paris och under det intensiva Medelhavets solljus. Om människor tillbringade liknande tid som växter med att sola i solljus, skulle antalet hudbrännskador och cancerfall skjuta i höjden eftersom UV från solljuset bryter DNA.

För att undvika detta bär vi tyg och gömmer oss i skuggan, vilket inte är fallet för växter. Eftersom växter inte rör sig, var de tvungna att anpassa sig till UV-överexponering genom att hitta sätt att skydda sig mot UV och reparera deras DNA. I en genetiskt utmanande framtid för människor på grund av den ökade UV-exponeringen från ozonnedbrytning, kommer det att gynna oss att studera växters verktyg för att motstå de negativa effekterna av strålning.

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Acknowledgement can mean one of two things:
First, the act of admitting the truth or existence of
Second, the act of praising or thanking for some deed or achievement.
I will use this page for doing both.

If you are a queer student, just know that your progression in Academia will extremely difficult. As queer people, we are more likely to experience intolerance, discrimination, harassment, and the threat of violence due to their sexual orientation, than those that identify themselves as heterosexual in this society. Academia is often considered as an open-minded environment, which is sadly not the case. Academia, as this society, is dominated by a heteronormative cis white men that doesn't want you, doesn't care about you, and doesn't want to change this discriminative system perfectly constructed for and by them.

The lack of openly queer people in a high position in Academia is a beautiful example of this active exclusion. Because of our identity, we will be considered as non-professional, superficial, or delusional. But just remember that we fought for our place in this world, and that has made us stronger than those cis white males will ever be.

Since my PhD, I've understood that as a nonbinary person being excellent will never be enough because of who I am. Studies have shown that queer people are less likely to publish papers, more likely to have health issues linked to work, and more likely to leave Academia (1-3).

To all heteronormative cis white males, you have 3 options:

1. Being allies through educating yourself by learning from LGBTQ+ community experiences, reading articles and attending workshops.
2. Cultivating queerphobia through jokes, insults or threats, unnecessary and degrading references to someone's sexual orientation, gender identity, or their perceived sexual orientation or gender identity, exclusion, spreading rumours or gossip including speculations about someone's sexual orientation or gender identity, or asking intrusive questions.
3. Doing nothing, which is just being complicit with 2). Doing nothing in front of any discriminative acts, makes you actively part of this discrimination.

Since starting my Ph.D. in this department, I've been outed publicly, called "instable" and "princess", a victim of discriminatory gossips, and misgendered, which made me doubt about my future in Academia. Why should I continue in Academia when I am a victim of those constantly? Because, as queer people, we ask ourselves the same question for life... and we keep being your victims just to be able to live our passions and our love. Another reason for me to continue in Academia is to be an ally to LGBTQ+, POC and women researchers to increase visibility and equality, as some researchers did for me.

My signal to Academia and this department is quite simple:
Actions are louder than words; your fecklessness is loudest.

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Merci Papi, merci beaucoup.

Bye Brenda

I

Chapter 10

Visualizing and Measuring Single Locus Dynamics in *Arabidopsis thaliana*

Anis Meschichi and Stefanie Rosa

Abstract

In eukaryotes, DNA is packed into an incredibly complex structure called chromatin. Although chromatin was often considered as a static entity, it is now clear that chromatin proteins and the chromatin fiber itself are in fact very dynamic. For instance, the packaging of the DNA into the nucleus requires an extraordinary degree of compaction but this should be achieved without compromising the accessibility to the transcription machinery and other nuclear processes. Approaches such as gene tagging have been established for living cells in order to detect, track, and analyze the mobility of single loci. In this chapter, we provide an experimental protocol for performing locus tracking in *Arabidopsis thaliana* roots and for characterizing locus mobility behavior via a Mean Square Displacement analysis.

Key words Locus tagging, Fluorescence microscopy, Green fluorescent protein, Chromatin mobility, Mean square displacement, Arabidopsis

1 Introduction

Chromatin is composed of DNA wrapped around a complex octamer of proteins called histones, forming the nucleosome. This structure is highly dynamic due to multiple nuclear processes, such as transcription, replication, or repair systems, which require a change in the accessibility of the underlying DNA sequences [1–3]. Moreover, in recent years, advances on imaging techniques have started to reveal the dynamic nature of the DNA inside the cell nucleus [4–7]. Indeed, genes can change their physical location inside the nucleus with consequences for transcriptional activity or genome integrity [8–13].

Locus-tagging systems are one approach allowing analyzing the mobility of a locus through time. There are currently several techniques allowing single locus tagging, such as *lacO* and *tetO* system, or CRISPR-based imaging [14, 15]. The *lacO*/LacI system is based on the insertion of *lacO* arrays in the genome. The *lacO* arrays typically consist of 100–256 copies of the *lacO* sequence

Jose J. Sanchez-Serrano and Julio Salinas (eds.), *Arabidopsis Protocols*, Methods in Molecular Biology, vol. 2200, https://doi.org/10.1007/978-1-0716-0880-7_10,

[16]. Additionally, this method requires the expression of the DNA-binding LacI repressor protein fused with a fluorescent protein, such as GFP or variants (CFP, YFP, RFP, etc.). The expression of these proteins should be somewhat weak in order to avoid the background fluorescence masking the specific signal at the tagged sequence. The tagged sequence is detected through the binding of the repressor protein (LacI-GFP) to the *lacO* repeats, allowing the tracking of specific chromosomal sites by in vivo fluorescence microscopy. The CRISPR imaging system is based on a catalytically inactive Cas9, fused to a fluorescent protein, where guide RNAs are designed for a specific sequence in the genome allowing the dCas9 to recognize and tag the locus of interest [17, 18]. These gene-tagging techniques have been extensively used in organisms such as yeast or mammalian cells, both to characterize chromatin mobility during DNA damage and to study mobility of actively transcribed loci [9, 13, 19–22]. In *Arabidopsis thaliana*, the *lacO*/LacI system has been used in order to study chromosome organization [23], changes in nuclear positioning of Polycomb targets [24], chromatin dynamics in endoreplicated pavement cells [25], as well as alterations of *lacO* positioning upon DNA damage [26]. One drawback of the *lacO*/LacI system is that the LacO repeats tend to form heterochromatin, which silences the locus and sometimes the neighboring genes [27]. To overcome this issue LacO repeats can be interspaced with random short sequences (~10mers), which were shown to reduce problems with repeat-induced silencing [24, 28]. Unlike *lacO*/LacI, the CRISPR imaging system has the advantage that it recognizes the endogenous sequence in the genome and is less likely to perturb the underlying chromatin structure. In plants, telomere mobility has been measured using the CRISPR imaging system in *Nicotiana benthamiana* [18]; however this method has not yet been successfully applied in *Arabidopsis thaliana*. Indeed, and despite the great progress made in the CRISPR-based imaging, many challenges still remain to be settled before this method is readily available for use in all systems, and in particular, issues associated with imaging of non-repetitive sequences and off-target binding need to yet be overcome [29].

In both systems, the tagged sequence is seen as a bright spot in the nucleus, which can be followed by in vivo time-lapse imaging. The mobility and trajectory of this bright dot can be analyzed to obtain information regarding the mechanisms underlying its movement. However, the simple tracking of the tagged loci will inform only on changes in position as a function of time, which is not enough to characterize loci mobility behavior. To this end, one method typically used to extract information about a moving particle tracking data is mean square displacement (MSD) analysis [30].

In this chapter, we will describe the protocol we use to visualize and quantify chromatin movement using the *lacO*/LacI gene-tagging system, particularly focusing in Arabidopsis roots, but a

similar approach may be taken for analysis in other tissues. Importantly, the methods described here are not specific to the *lacO*/LacI system but could be readily adapted to other locus-tagging approaches.

2 Materials

2.1 Plant Growth

1. Arabidopsis lines with a locus-tagging system (*lacO*/LacI system *see* **Note 1**).
2. Murashige & Skoog basal medium with vitamins (later referred as MS medium).
3. 5% Bleach (hypochlorite) in dH_2O.
4. Plant growth chamber (*see* **Note 2**).
5. Square Petri dishes (*see* **Note 3**).
6. Adhesive tape.
7. Laminar flow cabinet.
8. Sterile razor blades.
9. Coverslips No 1.5.
10. Microscope slides with frosted ends.
11. Secure Seal Adhesive Sheets (0.12 mm thick) (*see* **Note 4**).

2.2 Microscopy

1. Laser scanning confocal microscope (*see* **Note 5**).
2. Objective 63×/1.2 water or another high numerical aperture water immersion objective (*see* **Note 6**).
3. An argon 488 nm laser line is required for GFP.

2.3 Data Analysis

1. ImageJ or Fiji software (NIH, Bethesda, MD, http://rsb.info.nih.gov/ij/) with the plugin SpotTracker 2D (obtained from http://bigwww.epfl.ch/sage/soft/spottracker).
2. Software to perform data analysis (mean intensity values, data plotting and curve fitting with best-fit equations): Microsoft Excel, GraphPad Software (La Jolla California USA, www.graphpad.com), MATLAB, or alike.

3 Methods

3.1 Plant Growth and Sample Preparation

The objective of this part of the protocol is to grow seedlings with easy access to root tissue, which will then be transferred to a microscope slide for imaging.

1. Surface-sterilize seeds in 5% v/v sodium hypochlorite for 5 min and rinse three times in sterile distilled water.

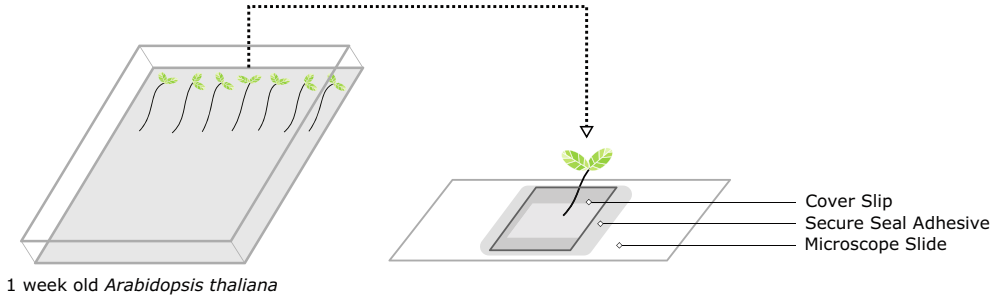

Fig. 1 A schematic representation of microscope slide preparation. Roots of 1-week-old Arabidopsis seedlings are placed between the cover slip and the microscope slide

2. Stratify the seeds by incubating for 2 days at 4 °C in darkness in 1.5 mL tubes.
3. Pour MS medium into a square petri dish.
4. When solidified, plate the seeds.
5. Seal the plate with adhesive tape.
6. Grow the seedlings in the growth chamber in vertically oriented positions for 1 week (or more) (*see* **Note 7**).
7. To avoid squashing the roots when mounting between slide and coverslip, cut small square frames of Secure Seal double adhesive sticky tape with similar dimensions to the coverslip and stick it to the slide as shown in Fig. 1. These will work as a spacer avoiding squashing the root and at the same time avoid drying of the sample during image acquisition (*see* **Note 8**).
8. Transfer one seedling to microscope slide prepared with double-sticky tape, and mount in water (Fig. 1) (*see* **Note 9**).

3.2 Image Acquisition

The objective is to produce a time-lapse series of nuclei expressing the tagged locus. The expression of LacI-GFP normally leads to background fluorescence in the nucleus due to unbound LacI-GFP (Fig. 2a,b). This background fluorescence is useful for nucleus alignment. If this background is not present, a nucleus marker (such as nucleus periphery marker or a histone fused to a different fluorescent protein) is needed to provide a reference for the alignment.

1. Acquire the images by using the 63x water objective.
2. Select an area of interest at a defined position relative to the root tip (meristematic/dividing, elongation, or differentiation zone) and a defined tissue layer (for instance, epidermis, cortex, etc.). Keep consistent throughout sampling (*see* **Note 10**).
3. Image settings: laser power should be around 10%, pinhole 61 μm, Image size 512 × 512 pixels; 3× zoom factor (pixel

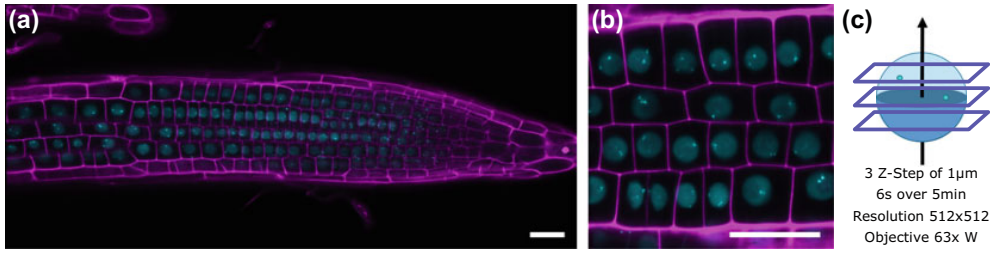

Fig. 2 Image acquisition settings for *lacO*/LacI lines. (**a**) Confocal image of an Arabidopsis root tip containing the *lacO*/LacI construct. Nuclei expressing LacI-GFP (cyan) showing spots corresponding to the tagged locus and background fluorescence due to unbound LacI-GFP. Propidium iodide (PI) staining (magenta). (**b**) Zoomed in image of the Arabidopsis root tip depicted in (**a**). Scale bar, 25 µm. (**c**) Schematic representation of a nucleus expressing the *lacI*/LacO construct and the corresponding image settings

size 0.088 µm). Z-stacks of overall 3 µm thickness (three Z-step of 1 µm). One Z-stack is collected every 6 s for 5 min (Fig. 2c) (*see* **Note 11**).

3.3 Image Processing

Image processing is an essential step that must be carried out before image analysis. These include correcting for sample movement and drift that occur during image acquisition and obtaining simpler images compatible with the algorithms used for image quantification (Fig. 3a).

1. Open the files as hyperstacks using ImageJ (Fiji distribution).
2. Apply Z-stack maximum projection to the whole image, in order to convert the 3D stack to 2D (ImageJ: image → stack → z-project → maximum intensity projection).
3. Change the bit depth to 8-bit by clicking on Image → Type → 8bit.
4. Nuclear movement, cell elongation, and sample drift will happen during the acquisition, which can add a critical bias to the locus mobility. Each nucleus must, therefore, be aligned before any locus tracking data acquisition. We use StackReg plug-in from ImageJ directly on *lacO*/LacI images since LacI-GFP background signal is sufficient to visualize the limits of the nucleus (ImageJ: PlugIn → StackReg → select: Rigid Body).

   Crop the images so that there is only one nucleus in the image.
5. Each individual nucleus must also be realigned using the option AlignNucleus in SpotTracker2D PlugIn (ImageJ: PlugIn → SpotTracker2D → AlignNucleus). To select the right threshold, the whole nucleus must be red (Fig. 3b).
6. Select the cropped image and start the Spot Tracker plugin (ImageJ: PlugIn → SpotTracker2D → SpotTracker2D).

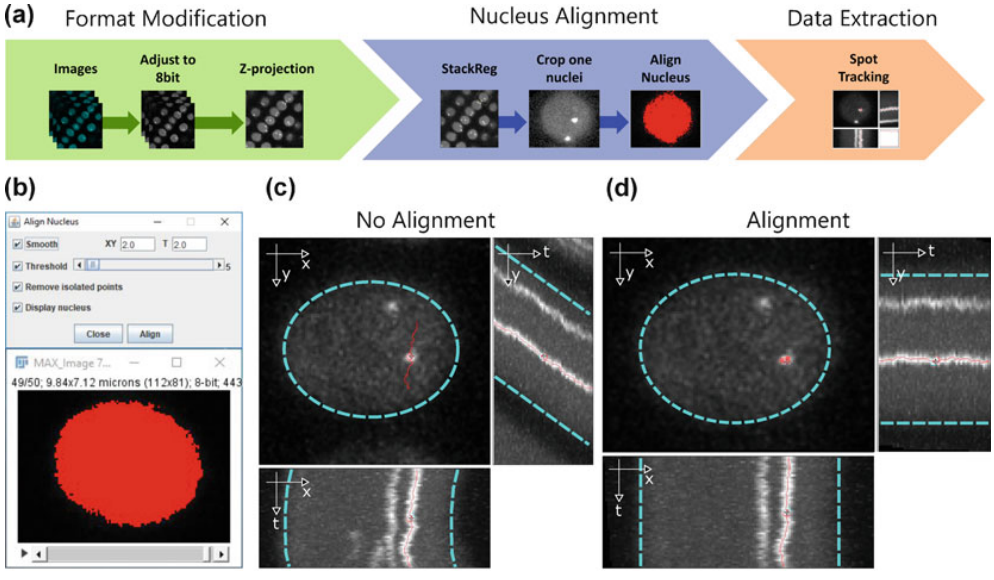

Fig. 3 Image processing workflow. (**a**) Different steps of the image processing. This processing is divided into three principal parts: the image format modification, nucleus alignment, and the tracking of the spots. (**b**) Screenshot of Align Nucleus option settings, where the threshold should be modified to isolate the nucleus fluorescence background (red) allowing the alignment of the nucleus. (**c**, **d**) Spot tracking results, showing an example of a nucleus without alignment (c) and an aligned nucleus (**d**)

7. Select Maximum intensity projection (M.I.P.). This step will improve the nucleus background and spot dection on kymographs (along the *x*- or *y*-axis), which allow visualizing the spot trajectory and the nucleus movement during the time of acquisition. This step is critical as it allows checking the nucleus alignment quality (Fig. 3c,d).
8. Select "Track." Tracking settings will be asked by the plugin such as:
   - Cone Aperture: corresponds to the size of the tracking spot.
   - Normalize factor: normalizes variations in the spot size between frames (the default value of 80% was appropriate for our samples).
   - Movement of constraint: corresponds to the theoretical maximum spot displacement between two consecutive frames, avoiding that noisy signal is considered as a new position of the spot (the default value of 25% was appropriate for our samples).
   - Center constraint: this parameter should be optimized when using a nuclear periphery marker and sets how far from the

periphery is the spot expected to be (the default value of 20% was appropriate for our samples).

- Confidence decision: represents the level at which the plugin defines the trajectory as trustworthy (default value: 10 was suitable for our samples).

9. Once settings have been set select "Track" (*see* **Note 12**).
10. When the trajectory is obtained and considered robust, export the result (SpotTracker: Result → Show as Table → Summary).
11. A table with the spot coordinates (x,y) for each time point will appear. Save the table by clicking on File/Save.
12. Label the data in a fully comprehensive manner and store them in a text file (.csv)

3.4 Data Analysis

To obtain quantitative information regarding the locus trajectory (Fig. 4a), a Mean Square Displacement (MSD) analysis should be used. The analysis of the trajectory with the MSD formula allows obtaining information about the spot trajectory, the diffusion coefficient, and the radius of constraint. This method consists in

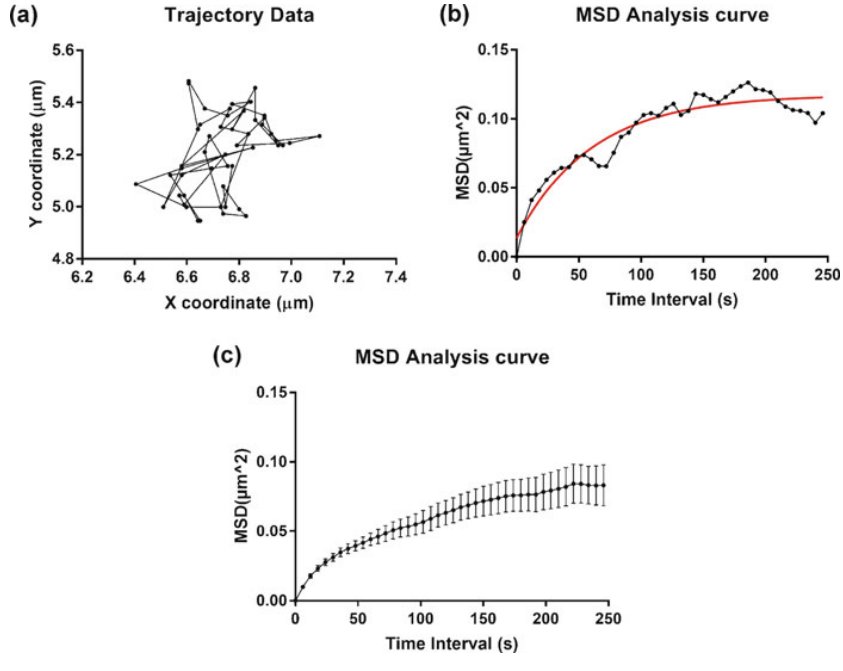

Fig. 4 Mean square displacement (MSD) analysis. (**a**) Plot of the trajectory data extracted from the plugin SpotTracking. The black line corresponds to the trajectory of a locus through time within the nucleus. (**b**) Mean of the squared displacement between two consecutive positions at a given time against increasing time intervals for one single tracked locus (black). Fitted curve (red) shows the plateau corresponding to the maximum distance that the locus has reached. (c) Example of a MSD analysis on 20 tagged loci

calculating the displacement of an object at increasing intervals of time via the Eq. (1) [31]. On this equation, $r(t)$ represents the position of the spot at a time t, and τ represents the time interval. In the case of a locus confined in a nuclear sub-compartment, the MSD curve will reach a plateau (Fig. 4b), which corresponds to the maximal displacement.

$$MSD(\tau) = \langle (r(t+\tau) - r(t))^2 \rangle = \langle \Delta r(\tau)^2 \rangle \tag{1}$$

1. Export the raw data, corresponding to the coordinates for the trajectory, to an Excel file.
2. The first step is to convert the pixel coordinates from the tracking data into microns by multiplying all the values by the pixel size.
3. To do the MSD analysis, first calculate the squared displacement $\Delta r(\tau)^2$, which is obtained by subtracting the determined position $r(t+\tau)$ to the initial position $r(t)$ for each time point and squaring the results. This operation is then repeated by increasing time intervals (τ)—Eq. (2).

$$\Delta r(\tau)^2 = (x_{t-\tau} - x_t)^2 + \left(y_{t-\tau} - y_t\right)^2 \tag{2}$$

4. Then an average of all $\Delta r(\tau)^2$ for each time interval (τ) is calculated.
5. Plot the MSD (τ) through time intervals on Excel or GraphPad Prism (Fig. 4b).
6. For each experiment, the standard error of the mean can be obtained from analyzing several nuclei (Fig. 4c).

A statistical analysis can be performed by comparing the radius of constraint in each experiment. The radius of constraint corresponds to the radius of a spherical volume, which is the confinement volume of the particle defined by the plateau of the MSD curve. In the case of 2D projected pictures the radius of constraint (R_c) has to be determined by using the maximum MSD value of the plateau (p) Eq. (3) (*see* **Note 13**).

$$R_c = \sqrt{\frac{4}{5} \times p} \tag{3}$$

4 Notes

1. In this protocol, we used lines containing 250 copies of *lacO* repeats and where the LacI protein is under the control of RPS5 promoter, which drives its expression in dividing cells [23]. The use of different promoters expressing the LacI

protein can be useful depending on which cell types are being targeted for analysis. However, and as mentioned above, the expression of the LacI-GFP should not be very strong as the background fluorescence from unbound LacI-GFP may mask the signal coming from the targeted locus.

2. Depending on the experimental setup, day-night periods, temperature, and light intensity could be changed according to the goal of the study, but these should be kept constant during experimental replicates.
3. Growing plants vertically allows roots to grow straight along the surface of the media, which facilitate the transfer of the seedlings to the microscope slide and avoid damaging the samples.
4. Mounting the roots only between a microscope slide and a cover slip without tape is possible but it increases the possibility to squash and damage the root. Adding a double-sticky tape creates a spacer, therefore avoiding squashing the roots. Additionally, it seals the slide and avoids drying of the sample during imaging. We advise this specific tape type (Grace Bio-Labs) because of its size and efficiency in sealing, as other tapes often go off in contact with water.
5. Laser scanning confocal microscopes can be limiting for 3D imaging of very dynamic processes due to a slow scan speed. A trade-off between resolution (i.e., image quality and the number of z-steps) and scanning speed is necessary to allow dynamic observations. Spinning disc confocal microscopes provide faster-imaging systems and therefore are likely to be advantageous for quantifying highly dynamic motions.
6. Using a high magnification, such as 63×, allows collecting 5–10 nuclei at the same time with good resolution, which is important for a proper nucleus alignment and spot tracking.
7. After 1 week of germination, Arabidopsis seedlings are long enough for imaging, but the time can be adjusted depending on the experimental purpose.
8. In order to limit movement of the cell imaged along the *x*–*y* axis, the seedling is immobilized by placing the upper part of the root between the double-sticky tape and the coverslip while the cotyledons are left outside the chamber (as shown in Fig. 1). Nevertheless and even though the imaging time is relatively short (approx. 5 min) the nuclei may still move along the *x*–*y* axis due to cell expansion. It is therefore necessary to limit this movement and to ensure, post-imaging, the correct alignment of the nucleus between the different frames.

9. When the root is placed on the slide there is a change of orientation (from vertical growth to horizontal); as such the root will adapt to the new gravity direction. To minimize root movement during the acquisition, the sample can be mounted and positioned horizontally for 5-10 min prior to imaging.
10. The number of nuclei per acquisition will differ in function of the root and area of interest. To keep a robust analysis, acquisitions should be taken in the same root area (i.e., using nuclei at the same stage of development).
11. The laser intensity should be kept as low as possible to avoid bleaching during the time of the acquisition. If the signal is weak, the pinhole can be slightly opened to increase the signal. The number of *Z* steps and time points can be modified depending on the samples. For instance, bigger nuclei will require larger *Z*-stacks. For our analysis, three *Z*-steps were enough to track spots in the nucleus without increasing the time interval.
12. If the trajectory proposed by the plugin does not correspond to the actual trajectory of the spot, it is possible to correct the trajectories manually. To this end, right-click on the image and click on "Add a node at" or "Remove a node at," to refine the trajectory.
13. For more details about the calculation of the radius of constraint see supplementary materials in Neumann et al. [30].

Acknowledgments

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II

frontiers in Plant Science

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ANCHOR: A Technical Approach to Monitor Single-Copy Locus Localization *in Planta*

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Together with local chromatin structure, gene accessibility, and the presence of transcription factors, gene positioning is implicated in gene expression regulation. Although the basic mechanisms are expected to be conserved in eukaryotes, less is known about the role of gene positioning in plant cells, mainly due to the lack of a highly resolutive approach. In this study, we adapted the use of the ANCHOR system to perform real-time single locus detection *in planta*. ANCHOR is a DNA-labeling tool derived from the chromosome partitioning system found in many bacterial species. We demonstrated its suitability to monitor a single locus *in planta* and used this approach to track chromatin mobility during cell differentiation in *Arabidopsis thaliana* root epidermal cells. Finally, we discussed the potential of this approach to investigate the role of gene positioning during transcription and DNA repair in plants.

Keywords: chromatin, nuclear organization, real-time imaging, microscopy, single-locus analysis, chromatin mobility

INTRODUCTION

In eukaryotes, genetic information is encoded in the chromatin, a complex structure composed of DNA packed around an octamer of histones in the nucleus. Chromosome territories form large compartments in the nucleus, themselves containing chromatin domains harboring different epigenetic signatures (Nguyen and Bosco, 2015; Pontvianne and Grob, 2020; Santos et al., 2020). In these domains, the positioning and accessibility of genes are very dynamic in response to several key biological processes that include gene transcription, genome replication, and DNA repair. Fluorescence *in situ* hybridization (FISH) approaches, such as padlock-FISH, enable to detect a single-copy locus using the fixed plant material (Feng et al., 2014). However, imaging techniques using non-living organisms are insufficient to track spatial and temporal dynamics of loci. The live-cell imaging approaches allow gene positioning visualization during these different processes, providing key elements for their understanding (Dumur et al., 2019; Shaban and Seeber, 2020).

Microscopic detection of genomic loci in plants is possible through the use of different strategies, such as zinc-finger-based imaging, transcription activator-like effectors (TALEs), and CRISPR/Cas9 (Lindhout et al., 2007; Fujimoto et al., 2016; Khosravi et al., 2020). Unfortunately, these techniques have been restricted to follow the dynamics of highly repeated regions (i.e., centromeric repeats,

telomeric sequences, and ribosomal RNA genes). Monitoring a single locus in living plants is possible due to the addition of *lacO* motifs to which the transcription factor LacI, fused to a fluorescent protein, can bind (Kato and Lam, 2003; Fang and Spector, 2007). Live-cell imaging of *Flowering Locus C* (*FLC*) alleles associated with *lacO* (*FLC-LacO*) could be performed to demonstrate that *FLC-LacO* repression during vernalization provokes their physical clustering (Rosa et al., 2013). In addition, the Tet repressor protein fused to a fluorescent protein could also be used to label a genomic region containing numerous Tet operator sequences (Matzke et al., 2005). In both cases, amplification of the signal is directly linked to the multiplicity of the targeted sequences. However, these repetitions often affect local chromatin organization and can trigger silencing of the reporter gene (Watanabe et al., 2005). Thus, a standardized and robust technique for tracking the dynamics of a single locus is still not available.

The ANCHOR system is a DNA-labeling tool derived and optimized from chromosome partitioning complex of bacteria. A single-copy of *parS*—1-kb-long fragment—serves as a binding platform for ParB proteins (Dubarry et al., 2006). Natural *parS* sequence is composed of four canonical inverted repeat sequences that are bound *via* the helix-turn-helix motif present in ParB (Funnell, 2016). Upon binding, oligomerization of ParB proteins then propagates over the *parS* sequence and adjacent DNA (**Figure 1A**). Importantly, oligomerized ParB proteins are loosely associated and can be displaced transiently and easily upon transcription or DNA repair (Saad et al., 2014). This phenomenon is also described as the caging step (Funnell, 2016). This system has been adapted successfully to monitor a unique locus in living yeast and human cells using a fluorescent-tagged ParB (Germier et al., 2017). This approach is also able to visualize DNA viruses in human cells (Komatsu et al., 2018; Mariamé et al., 2018; Blanco-Rodriguez et al., 2020; Gallardo et al., 2020; Hinsberger et al., 2020). In this study, we demonstrated that the ANCHOR system can also be used to visualize a single locus in fixed and living plant tissues. Using this approach, we also revealed that chromatin mobility is distinct in differentiated cells compared with meristematic cells of plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Col-0 was used in this study. *lacO*/LacI line used was obtained from the study by Matzke et al. (2005). To test the ANCHOR system, *A. thaliana* (Col-0) plants were transformed by agroinfiltration using the floral dip protocol (Clough and Bent, 1998), using *Agrobacterium tumefaciens* GV3101 strain. Transformants were grown on soil and sprayed with Basta herbicide for selection (10 mg/L). All the plant materials used here was grown in control growth chambers on soil at 21°C with a daylight period of 16 h/day. The transformant 2F (T2F) line was crossed to Col-0 wild-type plants expressing the histone variant H2A.W fused to a red fluorescent protein (RFP) (Yelagandula et al., 2014). The T2F line used in this study is heterozygote for the ANCHOR transgene, except in the data shown in **Figure 2**, where homozygous lines have been used.

For *in vitro* growth, seeds were surface sterilized in 5% v/v sodium hypochlorite for 5 min and rinsed three times in sterile distilled water. Seeds were stratified at 4°C for 48 h in the darkness and plated on the Murashige and Skoog (MS) medium. Seedlings were placed in a growth cabinet (16 h light, 22°C) for 1 week in a vertically oriented Petri dish before imaging.

Plasmid Construction

A cassette allowing the expression of ParB has been synthetized by GenScript (USA). The nature and sequences of the ANCHOR system and the property of NeoVirTech SAS are confidential. The cassette was cloned into the pEarleyGate302 vector (Earley et al., 2006).

Nanopore Sequencing

Genomic DNA preparation was performed as previously described by Picart-Picolo et al. (2020). Library preparation was performed using the 1D genomic DNA with ligation kit SQK-LSK109 (Oxford Nanopore Technologies, UK), following the instructions of the manufacturer. The R9.5 ONT flow-cell FLO-MIN106D (Oxford Nanopore Technologies, UK) was used. We obtained 1.93 GB of sequences (11× coverage) with an average read length of 3,675 kb for ANCHOR T2F line. ONT reads mapping the transgene were mapped, filtered, and aligned using Geneious® software (Kearse et al., 2012).

Cytogenetic Analyses

For cytogenetic analyses, nuclei were isolated from 3- or 4-week-old plants as previously described (Pontvianne et al., 2012). In brief, fresh leaves were fixed in 4% formaldehyde in Tris buffer (10 mM Tris–HCl at pH 7.5, 10 mM EDTA, and 100 mM NaCl) for 20 min and then chopped with a razor blade in 0.5 mL of LB01 buffer (15 mM Tris–HCl at pH 7.5, 2 mM NaEDTA, 0.5 mM spermine, 80 mM KCl, 20 mM NaCl, and 0.1% Triton X-100). The lysate was filtered through a 30-μm cell strainer (BD Falcon, USA), and 12 μL of sorting buffer (100 mM Tris–HCl at pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 0.05% Tween-20, and 5% sucrose) was added per 3 μL of cell/nuclei suspension (Pontvianne et al., 2012) and spread on a polylysine slide. After air-drying, the samples were postfixed in 2% formaldehyde in phosphate buffer (PBS) for 5 min and then washed twice with water before being air-dried. The slides were then mounted in Vectashield at 1 μg/mL of DAPI and sealed them with nail polish.

Nuclei with different levels of ploidy were isolated as described by Pontvianne et al. (2016), except that propidium iodide was used to stain the nuclei, together with RNase to a final concentration of 10 μg/mL. A S3 cell sorter (Biorad, USA) with 488 nm and 561 nm 100 mW dual-lasers was used to sort the nuclei. Immunolocalization experiments were performed as described previously (Durut et al., 2014) using anti-H3K27me3 or anti-H3Ac antibodies (Abcam, USA) to a 1/1,000 dilution. Zeiss LSM 700 confocal was used to generate images as shown in **Figure 1**, while Zeiss LSM 800 with an Airyscan module was used to generate images as shown in **Figures 2–4A** with a 63× objective, N.A. 1.4 and pixel size of 0.028 × 0.028 × 0.160 μm^3.

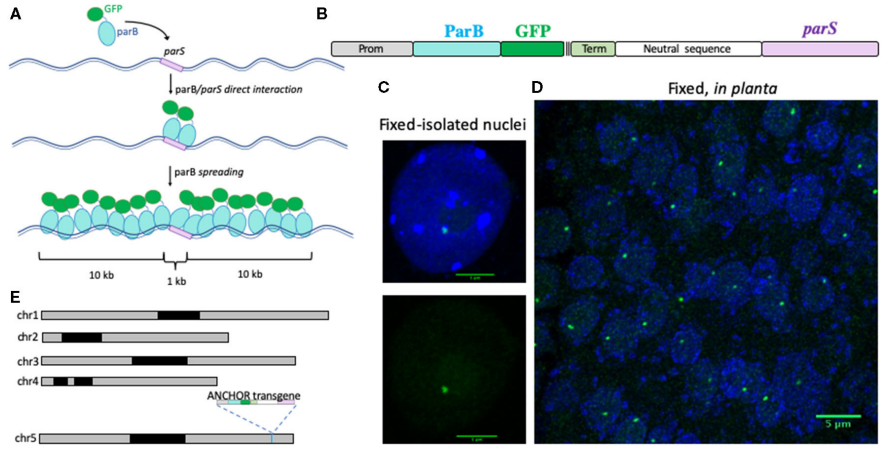

FIGURE 1 | Description of the ANCHOR system *in planta*. **(A)** Schematic representation of the ANCHOR system. ParB proteins fused to GFP can directly bind to *parS* sequence as a dimer. *parS*-ParB interactions provoke a conformational change in ParB proteins that induce their oligomerization along the flanking genomic region. **(B)** Cassette used to transform *Arabidopsis thaliana* Col-0 plants to test the ANCHOR system *in planta*. A strong and ubiquitous promoter is used to express the ParB protein fused to GFP and three FLAG tags. After a terminator sequence, a 1.5 kb-long spacer sequence has been added to separate the ParB:GFP open reading frame and the 1 kb-long *parS* sequence. Detection of a *parS*-ParB:GFP focus (green) in an isolated leaf nucleus **(C)** and in fixed root tissues **(D)** of *A. thaliana* plants containing the ANCHOR cassette described in **(B)**. Nuclear DNA is labeled with DAPI (blue). Bar = 5 µm. **(E)** Position of the transgene in the ANCHOR line T2F in the *Arabidopsis* genome using nanopore sequencing. The transgene presented in **(B)** is inserted on chromosome 5, position 23.675.998 bp.

Live-cell imaging shown in **Figure 4B** were performed using a spinning disk Zeiss Cell Observer equipped with a high-speed Yokogawa CSUX1 spinning disk confocal, an ORCA-flash 4.0 digital camera Hamammatsu (Japan) and a 40× water objective N.A. 1.2. Green fluorescent protein (GFP) was excited at 488 nm.

Live-Cell Imaging

In **Figure 5**, time-lapse imaging of *A. thaliana* roots has been carried out using a Zeiss LSM 780 confocal microscope with a 63× water immersion objective (1.20 N.A.). For visualization of root cell contours stained with propidium iodide, an excitation line of 488 nm was used, and the signal was detected at wavelengths of 580–700 nm. For the observation of GFP expression, we used a 488-nm excitation line and a band-pass filter of 505–550 nm. For all experiments, the images were acquired every 6 s, taking a series of three optical sections with a Z-step of 2 µm for 5 min. Each movie has a format of 512 × 512 pixels and a 3× zoom factor.

The 7-day-old seedlings were mounted in water, or propidium iodide, between slide and cover slip and sealed with a 0.12-mm-thick SecureSeal adhesive tape (Biorad, USA), to avoid root movements and drying during imaging.

Mean Square Displacement Analysis

All the movies have been analyzed with Fiji software (NIH, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/) and with the plugin SpotTracker 2D (obtained from www.epfl.ch/sage/soft/spottracker). The mean square displacement (MSD) analysis was performed as described by Meschichi and Rosa (2021). All quantitative measurements represent averages from at least nine cells. From the MSD plot, we calculated the radius of constraint by the square root of the plateau of the MSD curve multiplied by 5/4. Data sets were tested for normality using the Shapiro–Wilk test. The parametric analyses were done using the standard Student's *t*-test to determine the statistical significance of the results. For the statistical analysis, we used the GraphPad Prism 8.3 software.

RESULTS

Development of the ANCHOR System

Our aim was to adapt and facilitate the use of the ANCHOR system in plants. We, therefore, combined the two elements of the ANCHOR system (ParB and its target sequence *parS*) into a single transgene. A *ParB* gene, whose coding sequence has been optimized for *A. thaliana*, was fused in a frame to a GFP and triple FLAG-tag (ParB:GFP:3XFLAG) to allow detection in living and fixed nuclei (**Figure 1B**). *ParB:GFP:3XFLAG* expression was placed under the control of a promoter allowing ubiquitous expression. At the 3′ end of the ParB construct, we added the 1-kb-long ParB target sequence *parS* separated by a 1.5-kb-long spacer sequence to prevent the potential interference of *ParB* gene transcriptional activity. Such design allows a rapid selection of transgenic plants containing the two linked ANCHOR elements. In addition, detection of *parS*-ParB:GFP

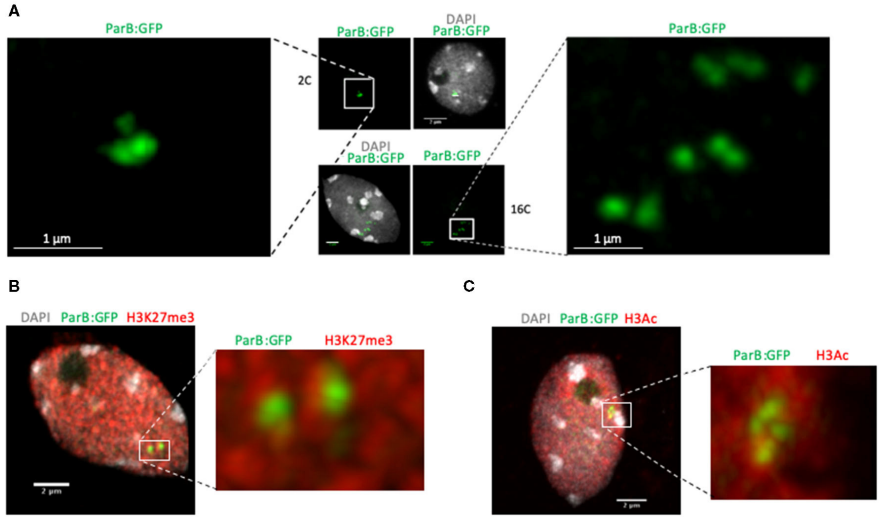

FIGURE 2 | Detection of *parS*-ParB foci in cells with different ploidy levels and after immunolocalization experiments. **(A)** Detection of *parS*-ParB:GFP foci (green) in fixed and sorted nuclei according to their ploidy levels by fluorescent-assisted cell sorting. Nuclear DNA is labeled with DAPI (gray). Enlarged view of the *parS*-ParB:GFP foci is presented to facilitate signal visualization. Bar = 1 µm. **(B,C)** Detection of *parS*-ParB:GFP foci (green) and posttranslationally modified histones (red) in fixed and isolated nuclei *from A. thaliana* Col-0 plants T2F. The image corresponds to a confocal 2D stack. Nuclear DNA is labeled with DAPI (gray). Trimethylated H3K27 signals are shown in **(B)**, while acetylated H3 are shown in **(C)**. Enlarged views of the *parS*-ParB:GFP foci are presented to facilitate signal visualization. Bar = 2 µm.

signals would suggest that *ParB:GFP* transcription is possible even in the case of local caging of ParB:GFP proteins.

Wild-type Col-0 plants were transformed with the transgene and selected using Basta herbicide by spraying. Fixed nuclei isolated from eight different T1 transformants revealed the presence of *parS*-ParB:GFP foci in five of them (**Figure 1C**). To test the robustness of the detection approach, we then analyzed the entire root-tip from one ANCHOR line comprising a single-copy insertion at generation T2 (T2F; **Figure 1D**). One *parS*-ParB:GFP signal was detectable in almost all nuclei analyzed. Importantly, the signal-to-noise ratio is high, which allows easy detection of the specific signal (**Figure 1D**).

To further characterize the ability of the ANCHOR system to follow a single-locus *in planta*, it is important to know the exact location of the transgene. We performed long-read nanopore sequencing on an ANCHOR line with one single insertion (T2F) and extracted all long reads corresponding to the transgene to map its location in the genome. The sequence analyses revealed that the transgene could be located on the lower arm of chromosome 5, at position 23.675.998 bp, in an intergenic region (**Figure 1E**). This position is flanked by a region enriched in active chromatin marks and a region enriched with histone 3 trimethylated lysine 27 (H3K27me3), a repressive mark deposit by the polycomb repressive complex 2 (PRC2) (**Supplementary Figure 1**) (Sequeira-Mendes et al., 2014).

Detection of *parS*-ParB *Foci* in Fixed Cells

As shown in **Figure 1D**, one unique focus was usually detected in root tip cells, sometimes appearing as a doublet. Since the ANCHOR system is based on protein aggregation, we checked whether analyzing ANCHOR signals in endoreplicated cells would lead to an increased number of detected foci. We isolated 2C, 4C, and 16C cells by fluorescent-assisted cell sorting after propidium iodide labeling and RNase treatment. We stained sorted nuclei with DAPI and observed *parS*-ParB:GFP signals in sorted nuclei. We could see a higher amount of *parS*-ParB:GFP signals in sorted nuclei presenting a higher endoreplication rate (**Figure 2A** and **Supplementary Figure 2A**). Although these data suggest that the ANCHOR system is suitable to detect multiple loci simultaneously, additional experiments are required to fully demonstrate that this reporting system does not lead to aberrant locus aggregation.

In the T2F line, the transgene is located on an arm of the chromosome 5, in a region enriched in H3K27me3 deposited by the PRC2 but flanked by a genomic region enriched with active chromatin marks (**Supplementary Figure 1**). Although T-DNA transgene insertion may affect this peculiar chromatin environment locally (Rajeevkumar et al., 2015), we tested the possibility to combine both immunostaining and *parS*-ParB:GFP signal detection. Immunostaining experiments were performed on isolated leaf nuclei from 3-week-old plants using either an antibody against histone 3 acetylated (H3Ac) active

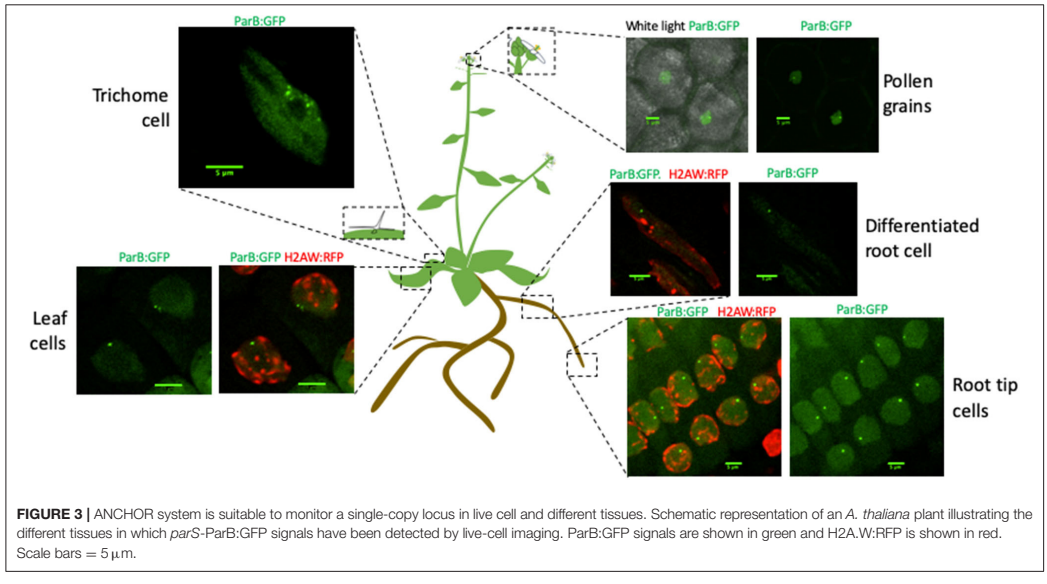

FIGURE 3 | ANCHOR system is suitable to monitor a single-copy locus in live cell and different tissues. Schematic representation of an *A. thaliana* plant illustrating the different tissues in which *parS*-ParB:GFP signals have been detected by live-cell imaging. ParB:GFP signals are shown in green and H2A.W:RFP is shown in red. Scale bars = 5 μm.

mark or H3K27me3 repressive mark. As expected, the tested histone marks and *parS*-ParB:GFP signals are excluded from heterochromatic foci stained by DAPI, corresponding to the centromeric, pericentromeric, and nucleolus organizer regions (**Figures 2B,C**). Although no clear overlap could be detected between *parS*-ParB:GFP signals and H3K27me3 marks, at least partial overlap can be seen between *parS*-ParB:GFP signals and H3Ac marks (**Figures 2B,C** and **Supplementary Figure 4**). This result is expected since active transcription is necessary to produce ParB:GFP proteins. Although we cannot conclude about the specific chromatin state surrounding the transgene insertion site in T2F, this experiment demonstrates our ability to detect *parS*-ParB:GFP signals and immunodetection approach simultaneously.

Detection of *parS*-ParB *Foci* in Live-Cell Imaging

Previous studies demonstrate that global genome organization can be cell specific and vary during plant development (Pontvianne and Liu, 2019). Therefore, we tested our ability to detect *parS*-ParB:GFP signals in different cell-types, directly *in planta*. To allow simultaneous visualization of heterochromatin and *parS*-ParB:GFP signals directly in living cells, we crossed the T2F line with another *A. thaliana* Col-0 line expressing the histone 2A variant H2A.W, fused to the RFP (Yelagandula et al., 2014). Plants were grown on MS media directly in Petri dish compatible with confocal imaging. We analyzed several tissues, including meristematic and differentiated root cells, leaf cells, and trichome cells, and also pollen grains from plants grown on soil. We were able to detect *parS*-ParB:GFP signals in all cell types tested (**Figure 3** and **Supplementary Figure 3**). As expected, *parS*-ParB:GFP signals are excluded from the heterochromatin area, labeled by H2A.W:RFP signals. It is noted that in certain cell types, the nuclear area can be seen due to non-associated ParB proteins that diffuse in the nucleoplasm.

The ANCHOR system does not require high DNA accessibility to allow the visualization of *parS*-ParB:GFP signals. In a highly condensed chromatin context, such as during mitosis, we could still detect *parS*-ParB:GFP signals in condensed chromosomes, even though the signal is usually less bright than in the neighboring cells (**Figure 4A**).

Finally, we tested our ability to perform live-cell imaging of the *parS*-ParB:GFP signals *in planta*. We analyzed *parS*-ParB:GFP dynamics in living roots using a Zeiss Cell Observer spinning disk microscope (**Figure 3B**). Although bleaching can alter the signal detection over time, we were able to detect the ParB:GFP signals at multiple time points and track its relative nuclear position, as reported earlier in human and yeast cells (Saad et al., 2014; Germier et al., 2017). Movies showing the detection of *parS*-ParB:GFP signals in live meristematic or elongated cells can be found in Supplementary Material (**Supplementary Movies 1, 2**). Altogether, our data demonstrated that the ANCHOR system is suitable for live-cell imaging *in planta*.

Studying Chromosome Mobility Using the ANCHOR System

It is now clear that higher-order organization of the chromatin exerts an important influence on genomic function during cell differentiation (Arai et al., 2017). For instance, in *A. thaliana*, histone exchange dynamics were shown to decrease

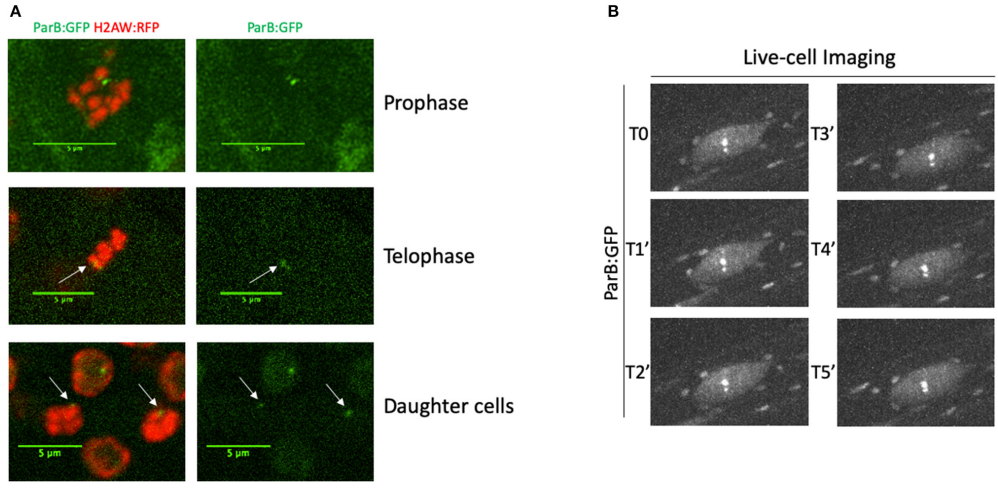

FIGURE 4 | Monitoring parS-ParB:GFP in live cell during mitosis or during a time-course. **(A)** Detection of *parS*-ParB:GFP foci (green) and H2A.W:RFP (red) in mitotic cells. Scale bars = 5 µm. **(B)** ANCHOR system enables time-lapse tracking of a single locus in live roots by confocal imaging. Time-lapse acquisition of *parS*-ParB:GFP signals (gray) in an endoreplicated root cell over 5 min.

gradually as cells progressively differentiate (Rosa et al., 2013). However, how chromosomes and the chromatin fiber move during cell differentiation is not well-studied in plants. We took advantage of our ANCHOR DNA labeling system to monitor chromatin mobility changes upon cell differentiation in the T2F line. In particular, we measured the mobility of *parS*-ParB:GFP *foci* in meristematic and differentiated cells from the root epidermis (**Figure 5A**) through live-cell imaging using confocal microscopy and quantified the mobility using the MSD analysis (Meschichi and Rosa, 2021). Interestingly, the chromatin mobility on meristematic cells was higher than in differentiated cells (**Figure 5B** and **Supplementary Movies 1, 2**). These differences were statistically significant as shown by a much higher radius of constraint (**Figure 5C**). These results may support the idea that the chromatin in undifferentiated cells holds a more dynamic conformation (Meshorer et al., 2006; Rosa et al., 2013; Arai et al., 2017). However, additional experiments would be required to further validate the biological relevance of this result.

Since single-locus dynamics in plants was mostly possible through the use of the *lacO*/LacI system (**Figure 5D**), we thought to compare chromatin mobility in meristematic cells using the ANCHOR and the *lacO*/LacI systems. Interestingly, both methods revealed a very similar MSD curve. In fact, a MSD curve, where the maximum values asymptotically reach a plateau, indicates that chromatin moves in a subdiffusive manner, which is typical for chromosomal loci tracked in interphase nuclei (Seeber et al., 2018). Additionally, the curves resulted in comparable measurements of the radius of constraint (**Figures 5E,F**), showing that the chromatin environment for these two insertion lines may be similar. While the comparison of additional lines with different chromosomal locations would be interesting, the results presented here illustrate that the ANCHOR system can be used to monitor single-locus and is suitable to study chromosome organization and dynamics in plants.

DISCUSSION AND PERSPECTIVES

In this study, we described a novel method to monitor a single-copy locus *in planta*. In comparison with existing strategies, the advantage of the ANCHOR system is the absence of repeated elements in the target sequence. This aspect is especially important in plants due to the existence of plant-specific silencing systems (Watanabe et al., 2005; Matzke et al., 2015; Grob and Grossniklaus, 2019). In fact, the *parS* sequence is only 1-kb-long and could potentially be shortened to 200 bp (NeoVirtech, personal communication). In addition, several reports in yeast and animal cells have already demonstrated the innocuity of the ANCHOR system to endogenous processes such as transcription and replication (Germier et al., 2018). This particularity makes the ANCHOR system very suitable to monitor single-copy genes in its native genomic environment. In this study, ANCHOR lines were generated by T-DNA insertion. Five out of eight independent lines showed strong ANCHOR signals. This could indicate that ANCHOR insertion site is important to be functional. However, we cannot conclude whether the ANCHOR system is suitable to monitor a genomic locus located in a heterochromatic environment. The absence of

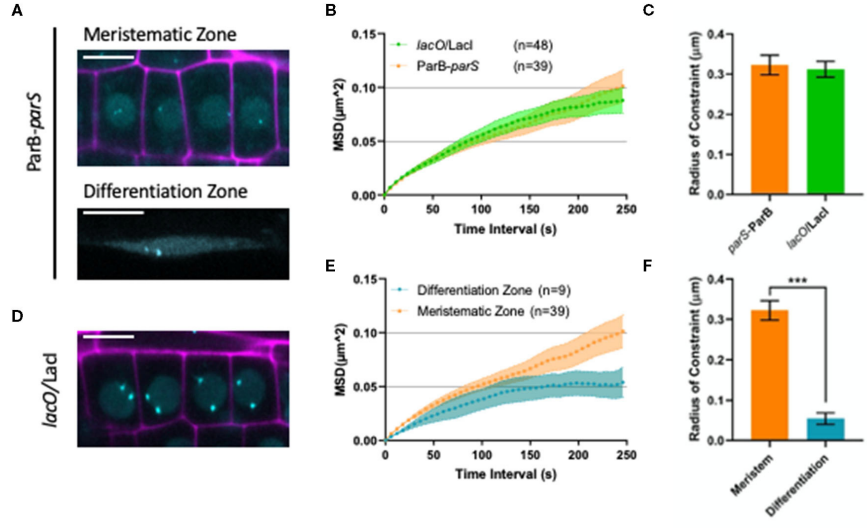

FIGURE 5 | Analyzing chromatin mobility using the ANCHOR system. **(A)** Representative images of ParB-*parS* line in meristematic (upper panel) and differentiation zone (bottom panel)showing nuclear signal with spots (cyan). Propidium iodide (PI) staining is shown in magenta. Bars = 10 µm. **(B)** The MSD analysis for *lacO*/LacI and ParB-*parS* lines based on time lapse experiment of nuclei in the meristematic zone. The values represent means ± SEM from 48 and 39 cells, respectively. **(C)** Calculated radius of constraint for MSD curves depicted in **(B)**. The values represent means ± SEM. Student's *t*-test, ***$P < 0.001$. **(D)** Representative image of *lacO*/LacI line in meristematic region showing nuclear signal with spots (cyan). PI staining is shown in magenta. Scale bar = 10 µm. **(E)** The MSD analysis for ParB-*parS* lines based on time-lapse experiments of nuclei in the meristematic and differentiated zone. 3D stacks were taken at 6 s intervals for 5 min. The values represent mean ± SEM from 39 and 9 cells, respectively. **(F)** Calculated radius of constraint for MSD curves depicted in **(E)**. The values represent means ± SEM.

parS-ParB:GFP *foci* could in fact be a consequence of a lack of ParB:GFP expression, which does not mean that *parS* accessibility is compromised. Having a separate transgene for ParB:GFP expression and *parS* detection would be necessary to address this point. In addition, T-DNA transgenes and *Agrobacterium*-directed transformation can be a source of genomic and epigenomic instability, both in *cis* and in *trans* (Rajeevkumar et al., 2015). Moreover, they can also modify the nuclear architecture of their insertion site (Grob and Grossniklaus, 2019). To specifically monitor the dynamics of selected single loci, the *parS* sequence would need to be inserted at a precise position within the desired locus. A recent approach that combine CRISPR-Cas9 technology and a homologous recombination-donor cassette can generate knock-in *A. thaliana* plants (Miki et al., 2018; Wolter et al., 2018; Merker et al., 2020). The implementation of the *parS* knock-in strategy will really improve the innocuity of this approach on the local chromatin state and should strongly reduce any bias on its nuclear positioning.

Another advantage of the ANCHOR approach is the possibility to use simultaneously different combinations of *parS*-ParB. In fact, ParB binding on *parS* sequence is species-specific, and several combinations have successfully been used separately or simultaneously so far. In this study, we used a specific *parS*-ParB, but an additional specific combination could be used. In theory, up to three combinations could be used simultaneously (Saad et al., 2014, NeoVirTech peronnal communication), although an important preliminary work would be required for plant material preparation. For instance, two alleles from the same gene could be differently labeled to monitor their potential associations while being expressed or silenced. This is an important question since previous observations suggest that allele aggregation could participate in gene transcriptional regulation (Rosa et al., 2013). These color combinations could also be used to follow the distance of two proximal regions during DNA repair, for example, as already shown in yeast (Saad et al., 2014) or to label borders of a genomic regions that can undergo different chromatin states during stress or development. This system will provide a useful tool to study the spatial organization and the dynamic behavior of chromatin at the single locus level.

DATA AVAILABILITY STATEMENT

The sequencing data presented in this study are not readily available due to proprietary restrictions. The remaining original contributions presented in the study are included in the article/**Supplementary Material**, and further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MI, FP, and SR designed the experiments. AM, MI, CP, and FP performed the experiments. AM, MI, NP, SR, and FP analyzed the data. SD, FG, KB, and MM participated in material preparation or analyzing tools. FP wrote the paper and acquired main funding. SR edited the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.677849/full#supplementary-material

Supplementary Figure 1 | Chromatin states flanking the insertion site in T2F ANCHOR line. **(A)** Snapshot of the chromatin states enriched in the region flanking the transgene insertion site in the line T2F (https://jbrowse.arabidopsis.org/). (**B**) Histogram representing the relative enrichment of each chromatin state in the 5 kb upstream and downstream region of the transgene insertion site in the line T2F.

Supplementary Figure 2 | Detection of *parS*-ParB foci in cells with different ploidy levels. Detection of *parS*-ParB:GFP foci (green) in fixed and sorted nuclei according to their ploidy levels by fluorescent-assisted cell sorting. Nuclear DNA is labeled with DAPI (gray).

Supplementary Figure 3 | Pollen grain and trichome cell. Confocal images of the *parS*-ParB:GFP signal in a trichome cell (top panels) or in pollen grains (bottom panels). Images on the right are saturated to show the trichome contour or the pollen grains.

Supplementary Figure 4 | Co-localization of *parS*-ParB foci with H3Ac and H3K27me3 marks. Detection of *parS*-ParB:GFP foci (green) and posttranslationally modified histones (red) in fixed and isolated nuclei *from A. thaliana* Col-0 plants T2F. Nuclear DNA is labeled with DAPI (gray). Trimethylated H3K27 signals are shown in the (**A**), while acetylated H3 are shown in (**B**). **(C,D)** show the relative intensity of each signal.

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Conflict of Interest: FG is an employee and FG and KB are shareholder of NeoVirTech. NeoVirTech did not have any scientific or financial contribution to this study. ANCHOR system is the property of NeoVirTech SAS, Toulouse, France.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Homologous recombination (HR) is a conservative DNA repair pathway in which intact homologous sequences are used as a template for repair. How the homology search happens in the cell nucleus is still poorly understood. Here, we described for the first time changes in chromatin mobility upon DNA damage in the context of a multicellular organism. In particular, our methods and results will allow to further study chromatin mobility during different developmental contexts and in response to DNA damage in *Arabidopsis thaliana*.

Meschichi-Duriez Anis received them graduate education at the Department of Plant Biology, SLU, Uppsala. They obtained an M.Sc. degree in Plant Biology from the University of Paris-Saclay, France, and a B.Sc. in Biology from the University of Montpellier 2, France.

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