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Extrachromosomal circular DNA: A neglected nucleic acid molecule in plants

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Abstract

Throughout the years, most plant genomic studies were focused on nuclear chromosomes. Extrachromosomal circular DNA (eccDNA) has largely been neglected for decades since its discovery in 1965. While initial research showed that eccDNAs can originate from highly repetitive sequences, recent findings show that many regions of the genome can contribute to the eccDNA pool. Currently, the biological functions of eccDNAs, if any, are a mystery but recent studies have indicated that they can be regulated by different genomic loci and contribute to stress response and adaptation. In this review, we outline current relevant technological developments facilitating eccDNA identification and the latest discoveries about eccDNAs in plants. Finally, we explore the probable functions and future research directions that could be undertaken with respect to different eccDNA sources.

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Introduction

In eukaryotic plant cells, the majority of cellular DNA is organized in chromosomes and associated with histone proteins inside the nucleus. Nevertheless, a part of the cellular DNA is not included in these chromosomes and exists as extrachromosomal circular DNA (eccDNA). The eccDNA was initially identified in boar sperm DNA in 1965, and 20 years later in plants, in wheat callus and tobacco leaf [1,2]. Here, to avoid ambiguity and inconsistency between the many terms used to describe eccDNA, we refer to the recent broad classification to describe eccDNA as all the circular DNA in a plant cell, including, but not limited to, small polydisperse circular DNA (spcDNA), extrachromosomal telomeric circles (t-circles), microDNA, double minutes, and extrachromosomal DNA (ecDNA) [3].

Owing to technological restrictions, we have yet only seen the tip of the iceberg when it comes to these molecules. Indeed, all plant species tested so far contain eccDNA showing a diversity of genomic origins, size ranges, and structures [1,2,4–8]. In recent years, genomic approaches and novel bioinformatic pipelines have led to a revival in eccDNA investigations opening new avenues of research. In this review, we present the current state of knowledge about eccDNAs in plants and their links to biological functions. We anticipate that this concise collection of papers will motivate more scientists to join the area of eccDNA biology to answer long-standing questions about these neglected nucleic acid molecules in plants.

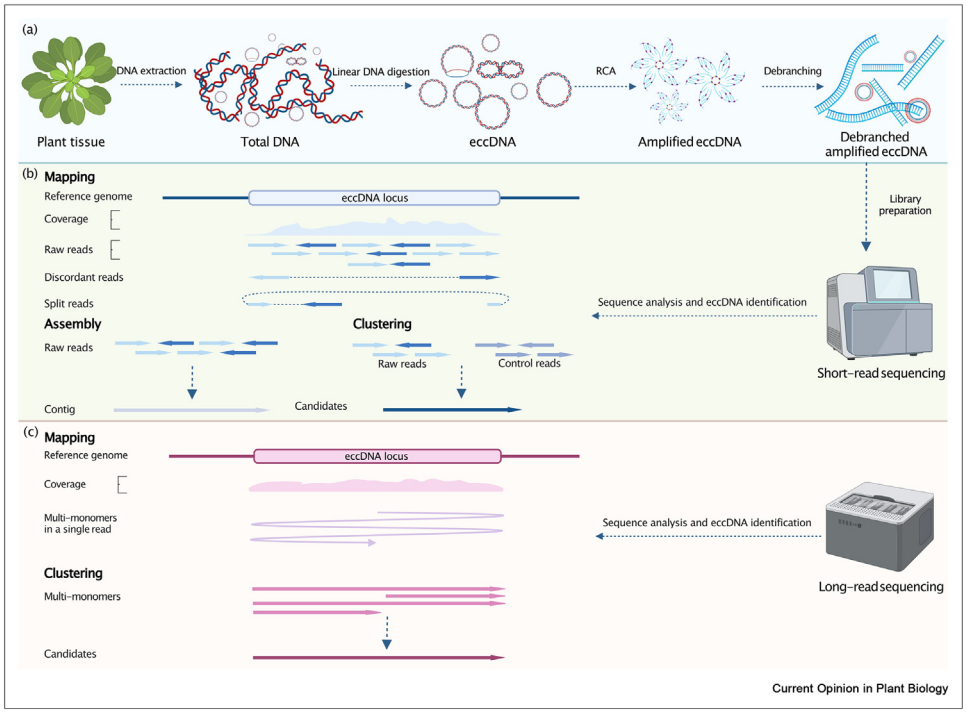
The eccDNA identification in plants: a revival thanks to genomic and bioinformatic advances

Initially, plant eccDNAs were visualized by electron microscopy, allowing their detection in wheat and beans. This technique only allowed to investigate the presence, structure, and size of eccDNA but did not allow the identification of their sequence [9,10] Nowadays, advanced electron microscopes, such as scanning electron microscope (SEM) and transmission electron microscope (TEM), combined with molecular localization techniques like fluorescence and *in situ* hybridization (FISH) allow the tracking of specific eccDNAs [11]. In plants, *in vivo* eccDNA visualization has so far not been established. For that purpose, the ANCHOR^{TM} system, which allows fluorescence-based real-time single DNA locus detection *in planta*, may provide the opportunity to track their genesis, intra-cellular and nuclear localization, and potential mobility *via* live-cell eccDNA imaging

[12]. Two-dimensional (2D) gel electrophoresis was also used for separating eccDNAs from linear molecules based on migration patterns resulting in distinct arcs. This allowed the distinction of different structural forms of eccDNA: supercoiled circles and open circles. Applying this method, many eccDNAs from a wide range of plant species (Fabaceae, Poaceae, and Brassicaceae) have been recorded indicating the ubiquity of these circular molecules in plant cells [4,8]. Although those methods contributed to the identification of numerous eccDNAs, these gel-based approaches have limitations. For instance, because they depend on the hybridization with specific probes, they only allow the detection of selected targets and do not provide information at single-base resolution.

The rapid development of high-throughput sequencing (HTS) techniques and bioinformatic pipelines has opened the door to the full landscape of plants eccDNAs. Recently, several molecular methods that allow the detection of eccDNA based on different sequencing sources have been developed. Indeed, they have successfully been applied to numerous plant species: *Arabidopsis* [5,6,13–15], wheat [6], beet [7], rice [16,17], peanut [18], potato [19], poplar [20], and carrot [21] providing a broad overview on the diverse eccDNA components in plant cells. Although differences exist between the protocols, the procedures are straightforward. Generally, linear genomic DNA is digested, and circular DNA is amplified by random-primed rolling circle amplification (RCA). This approach allows the amplification of low content eccDNA, thereby increasing sensitivity, it also leads to linearization of circles prior to sequencing. The DNA is then debranched to remove complex DNA structures. Debranched eccDNAs then go into sequencing library preparation for short- (Illumina) or long-read sequencing (*e.g.*, Oxford Nanopore or PacBio technology) (Figure 1a). An elaborate protocol that provides all the detailed wet lab steps has been described by Lanciano et al. [22]. The RCA is a linear amplification and quantification is thus possible. However, this process can induce length-dependent amplification bias so that the absolute quantification of DNA circles of very different sizes can be affected [23]. While large eccDNAs have been detected in cancer cells (for a review see Ref. [24]), there is no evidence so far that such large circles (e.g., the 400 kb eccDNAs found in *Amaranthus palmeri* [11]) would be detected using classical RCA-based eccDNA-seq in plants. Of note, a protocol without RCA but using a centrifugation in CsCl/ethidium bromide gradients and exonuclease V digestion of linear DNA has been developed (circulome-seq [25]). It has,

Figure 1



Overview of the main technological processes used for eccDNA identification by HTS in plants. (**a**) Following DNA extraction, linear DNA molecules are digested, and eccDNA molecules are amplified by random-primed rolling circle amplification (RCA). After debranching, these DNA fragments are submitted to either short-read and/or long-read sequencing. (**b**) Mapping, assembly, and clustering algorithms for detecting eccDNA loci using short-read sequencing. (**c**) Mapping and clustering algorithms for detecting eccDNA loci using long-read sequencing.

however, not yet been applied to plants. Finally, a recent preprint reports on the pulse-field gel electrophoresis enrichment for eccDNA, a technique named CRISPR-CATCH, enabled by the CRISPR-guided linearization of the eccDNA and subsequent fast migration on the gel. This technique, however, requires the design of a guide RNA corresponding to a specific eccDNA sequence [26]. New strategies aiming at sequencing eccDNA with long read without prior amplification step are likely to be developed in the near future.

To date, several bioinformatic toolkits and pipelines have been developed and applied for genome-wide identification of eccDNAs from plant species with genomes ranging from hundreds Mb to dozens Gb (Table 1) [6,7,15,17]. So far, most sequencing-based eccDNA detection approaches use short sequencing reads as an input to then assess the genomic origins of eccDNAs. To identify reads derived from circular DNA by mapping strategy, these pipelines look for reads derived from junction sites resulting from eccDNA circularization. First, reads are mapped to the reference genome, and then both discordant reads and split reads are identified. By combining these different information sources and overall read coverage over genomic regions, it is possible to identify high confidence eccDNA candidates (Figure 1b, mapping). The mapping model is the most direct way to detect eccDNAs with their start and end information when a reference genome is available. Well-assembled genomes are expected to give better results because genome gaps might prevent discordant and split reads detection, resulting in partial or lack of circle identification. If no reference genome is available, an alternative approach is to perform a *de novo* sequence assembly. The raw reads can first be assembled into long contigs and then clustered into consensus eccDNA candidates when applying ecc_finder (Figure 1b, assembly) [6]. Furthermore, ECCsplorer supports a comparative clustering module when control data are provided (Figure 1b, clustering) [7]. This comparative strategy intends to filter background noise originating from non-circular genomic DNA. When both reference genome and control data are unavailable, the conventional assembly and clustering approach are the only way to detect eccDNAs. However, if genomic regions from which eccDNAs are derived contain tandem repeat sequences or if multiple sizes of circles are derived from the same region, the results may lose accuracy. In our own analyses, we have observed that *de novo* assemblies often yield chimeric sequences which seem to be bioinformatic artifacts. This is likely caused by the highly repetitive content of eccDNA sequences. In this case, long-read sequencing has an innate advantage because a single read can cover a circle multiple times. This single read can then be split into individual monomers, allowing the generation of the circle consensus sequence (Figure 1c). Based on long-read data, around 20% of eccDNAs were found to be chimeric in the human germline, suggesting a complexity not anticipated by short-read eccDNA-seq approaches [27]. In summary, various approaches can be combined to get a very high confidence eccDNA library allowing subsequent wet-lab validation steps. We thus encourage researchers to combine different sequencing methods and detection strategies in order to obtain the most complete view of their eccDNA repertoire.

Genomic regions that spawn eccDNAs

Generally, in plants, eccDNAs are heterogeneous in size, ranging from hundreds of base pairs to several hundred-thousand base pairs, and most of them appear to be below 20 kb [15]. In *Arabidopsis*, the eccDNA size distribution shows a preferential enrichment in small regions (less than 500 bp) and in the 2 kb−4 kb size range [5,6,15]. It is likely that small eccDNAs are missed by all these sequencing approaches as the sequencing reads can be longer than the actual eccDNA. Nevertheless, these sequencing approaches provide a higher sensitivity now showing that eccDNAs can spawn from many genomic regions, including both genic and intergenic regions, and not only from satellites and repeated

Table 1

Bioinformatic toolbox to detect eccDNAs in plants

Name	CIDER-Seq2 [15]	ECCsplorer [7]	ecc_finder [6]	ecc_caller [17]
Year	2020	2022	2021	2021
Reads type	Long-read	Short-read	Short-read, long-read	Short-read
Reference free	Yes	Yes	Yes	No
Mapping aligner	MUSCLE	segemehl	segemehl, BWA, Bowtie2, minimap2	BWA
Short-read assembly tool	/	/	Unicycler	/
Long-read split tool	DeConcat	/	TideHunter	/
Clustering tool	CD-HIT	RepeatExplorer2	CD-HIT	/
Strategies	Mapping and/or c lustering	Mapping and/or clustering	Mapping or assembly and clustering	Mapping
Plant applied	*Arabidopsis*	*Arabidopsis*, Beet	*Arabidopsis*, Wheat	*Arabidopsis*, Rice

sequences [5–7] (Figure 2). In *Arabidopsis*, more than 60% of eccDNA overlap with genic or pseudogenic regions. However, most of those eccDNA boundaries start at or end within intron regions indicating that these eccDNAs do generally not code for full-length proteins [5]. Notably, the eccDNA repertoire can vary between different tissues or cell types of the same species. Tissue-specific eccDNAs can be found in *Arabidopsis* stem, leaf, flower, and roots. Similarly, differences in eccDNA accumulations were observed in rice seed, leaf, and callus [5,13]. This suggests that specific pathways and mechanisms are involved in eccDNA formation in different cell types. The eccDNA repertoire is thus dynamic throughout plant development.

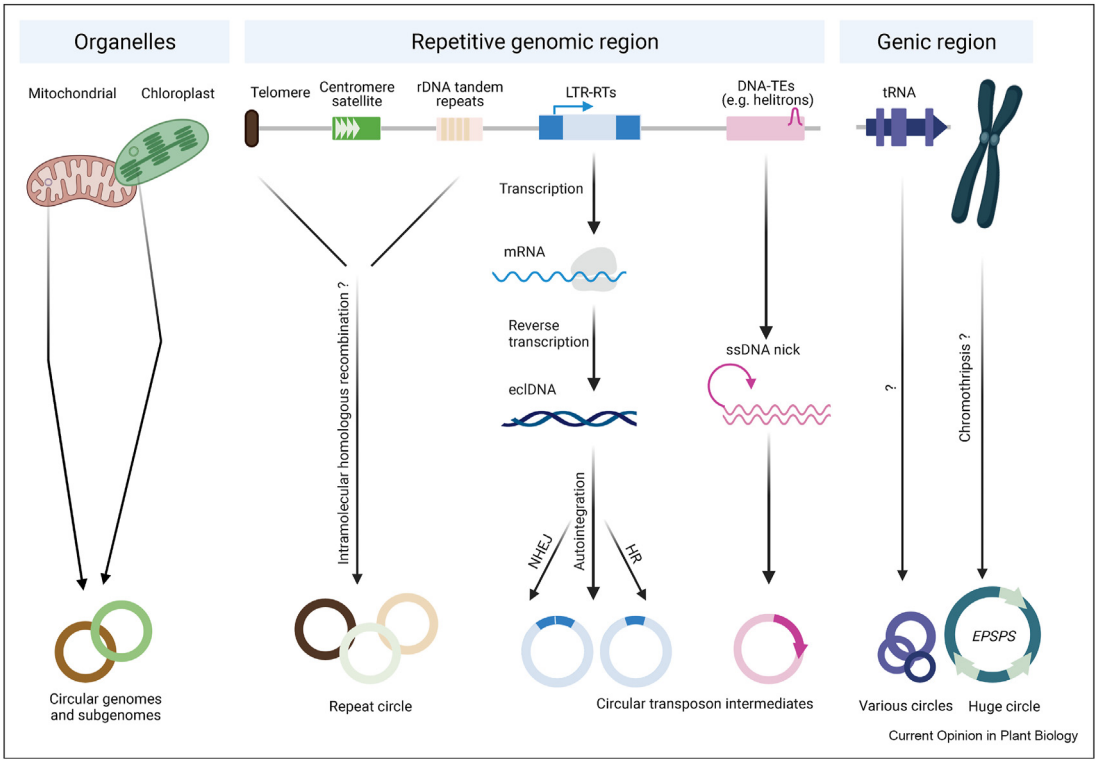
The eccDNAs from organelles

Protocols designed to enrich circular DNA also led to an accumulation of eccDNAs derived from organelles that have circular genomes and sub-genomes [28]. Therefore, it is inevitable that such DNA will also be included in the sequencing data. Indeed, approximatively 1%–10% of the reads contained mitochondrial and chloroplast-derived sequences in *Arabidopsis* when CIDER-Seq was applied [15]. Mitochondrial and plastid DNA can also be found in the nuclear genome which can complicate the identification of the true origins of these circles. For instance, *Arabidopsis* contains mitochondrial and plastid DNA on the chromosomes 2 and 4, respectively [29,30]. These insertions resulted from intracellular gene transfer (IGT) directly from mitochondrial and plastid genomes [31]. Similarly, circular chloroplast sequences of over 20 kb have been detected in wheat [6]; however, it is unclear whether these eccDNAs originate from organelles and/or from the nuclear genome.

Repetitive genomic regions

Initially, all detected eccDNAs were originated from repetitive genomic sequences, most notably derived from ribosomal DNA (rDNA), which is organized in large arrays of tandem repeats [32]. In wheat, 18S rDNA can form large circles of 40 kb in length [6]. Telomeres are also a type of highly repetitive short sequences present at the ends of linear DNA molecules which can spawn eccDNAs termed t-circles [8]. Interestingly, the DNA repair complex KU70/KU80 heterodimer specifically inhibits t-circles formation but has no effect on

Figure 2



Currently known different genomic regions that can spawn eccDNA in plants. Organelles contain circular mitochondrial and chloroplast genomes and can also contain circular sub-genomes. Telomeres, centromeres, and rDNA contain short tandem repeat sequences that can spawn repeat circles. LTR-RTs form eccDNA with single or double LTRs from ecIDNA by HR, NHEJ, or autointegration. Helitron element transposition starts with ssDNA cleavage followed by the formation of a circular intermediate. Other DNA TEs can also form eccDNA. Genic repeated regions, such as tRNAs, can spawn eccDNAs in *Arabidopsis*. Huge eccDNAs containing 59 genes have so far only been found in *Amaranthus palmeri*.

centromeric and rDNA-derived eccDNAs. Indeed, Ku defective plants accumulate an increased number of t-circles indicating that Ku specifically regulates homologous recombination (HR) at *Arabidopsis* telomeres [33]. Centromeres in plants are often composed of tandemly repeated DNA sequences termed centromeric satellites. It was shown that satellites flanked with direct repeats spawn eccDNAs in *Arabidopsis* and sugarcane [33,34]. These findings suggest that tandem repeats are susceptible to excision as extrachromosomal circles, a process also reported in the animal kingdom, including flies and humans [35,36].

Next to tandem repeats, another class of repeats that can spawn eccDNAs are transposable elements (TEs). Interestingly, different classes of TEs have their own way of contributing to the eccDNA pool. TEs are distinguished into two classes based on their transposition mode: retrotransposons and DNA transposons (DNA-TEs). During the life cycle of long terminal repeat retrotransposons (LTR-RTs), the reverse transcriptase produces extrachromosomal linear DNA (eclDNA) that can lead to the formation of eccDNA (Figure 2). It has been suggested that these eccDNAs can result from (i) self-ligation via non-homologous end joining (NHEJ) resulting in double-LTR circles; (ii) HR producing single LTR circles; or (iii) LTR-autointegration eccDNA resulting in single LTR or two separate LTR circles [37]. In plants, the mobilization of different families of LTR-RTs has been found to lead to the accumulation of eccDNAs, for example, *Évadé (EVD/ATCOPIA93)* [13] and *ONSEN* (*ATCOPIA78*) [14,16] in *Arabidopsis*; *Tos17*, *Tos5/Osr13/Houba*, and *Osr4/PopRice* in rice [13]; *Nightshade* in potato [19]; *Rider* in tomato [38]; *Tto1* in tobacco [39]; *Gagarin* in sunflower [40]; and *MIG* in spring triticale [41]. The situation is a bit less clear for DNA-TEs. Most of the DNA-TEs move *via* "cut and paste" which means they splice themselves and jump into a new position in the genome. In the case of the *Mu* and *Zuhe* DNA-TEs, it was found that they can also produce eccDNA when they are excised from the genome [18,42]. Helitrons seem to transpose through a "peel and paste" mechanism in which they can form an eccDNA intermediate by peeling off one DNA strand and copying themselves via rolling circle replication [43,44]. Finally, TE mobilization can also cause large genomic changes as they can lead to chromosome breakage which can result in the formation of eccDNAs [45,46].

In most of these cases, the eccDNA forms are thought to be dead-end by-products of the (retro)transposition cycle that are not able to reintegrate into the genome. Their detection nevertheless constitutes a diagnosis for their activity. The functions of these TE-derived eccDNAs are currently largely unknown and lead to different questions: Do they contribute to a TE's life cycle? Are they the product of the genomic defense response of the plant?

Genic regions

In contrast to human cells, eccDNAs have rarely been described to spawn from genic regions in plants [36]. In *Arabidopsis*, tRNA gene fragments can frequently be found within eccDNAs suggesting a role in protein synthesis [5]. In contrast to mammalian cancer cells or yeast, reports on eccDNAs containing full endogenous protein-coding genes are rare in plants. One notable exception is an almost 400 kb eccDNA harboring 59 genes that were discovered in glyphosate-resistant *A. palmeri*, a crop weed. One of these genes encodes a 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) which confers resistance to glyphosate. Notably, 41 of the genes encoded in this eccDNA are transcribed upon glyphosate exposure. Furthermore, these large eccDNA molecules can be inherited possibly through chromosome tethering as it has been proposed in yeast [11,47].

The eccDNAs as vehicles for gene amplification, a role in rapid adaptation?

Even though eccDNAs have been known to exist in plant genomes for quite some time, only now we started to have a more complete repertoire of genomic regions that produce eccDNA. And yet, many open questions about eccDNA biogenesis and function remain to be investigated: What are the positive and negative regulators of eccDNA biogenesis? Are there feedback loops that prevent overaccumulation? If yes, how can a plant sense a specific eccDNA quantity? To answer these questions, forward genetic mutant screens for eccDNA accumulation in *Arabidopsis* could provide valuable information. In addition, candidate gene approaches focusing on DNA repair could be helpful, albeit they showed limited success in yeast [48]. It is notable that in the case of rDNA in yeast, the genomic rDNA copy number showed a strong negative correlation to rDNA-derived eccDNA quantity [49]. In yeast, replication fork barrier protein Fob1 binding to rDNA intergenic spacer sequence can induce double-strand breaks and DNA circulation [50,51]. Cells with fewer rDNA copies have been shown to create additional eccDNA in a replication-dependent way, hinting that eccDNA might be integrated to raise the rDNA copy number [52]. Notably, genomic rDNA copy loss is concomitant with an increase of eccDNA. It remains to be tested if similar mechanisms exist in plants. Telomere rings and loops seem to be frequent telomere maintenance factors. For instance, a study in human cells has shown a strong correlation between telomere shortening and telomere eccDNA formation [53]. It seems that eccDNA is involved in regulating copy number variations. Indeed, the eccDNA harboring *EPSPS* can also be passed on to germ cells independently of the chromosomes resulting in heritable glyphosate resistance [11]. Similarly, in

cancer cells, oncogene-containing eccDNAs segregate unequally into daughter cells contributing to the dynamics of disease evolution [54,55]. Owing to the absence of centromeres, eccDNA segregation can be heterogenic resulting in progeny cells containing different eccDNA copy numbers. Cells harboring a high proportion of these eccDNAs may have a selective advantage and may thus be more adaptable to certain environments. There is currently little information on how eccDNAs are transmitted during cell division, although their inheritance most likely follows a non-Mendelian behavior. Like supernumerary B chromosomes, a balance between their influence on host fitness and their transmission rate could determine their frequency in a population [56].

Overall, because eccDNAs rarely seem to contain genes in plants, it is difficult to link eccDNAs with direct functions. Clearly, eccDNAs can represent a source of gene amplification and thus allow for rapid adaptation to environmental challenges, as this was shown for yeast leading to tolerance to copper and *A. palmeri* with a gain of herbicide tolerance [57,58]. Interestingly, in yeast, eccDNA formation can be transcription dependent. It would be of great interest to now systematically test whether stressed plants show a similar behavior providing an avenue for rapid adaptation. Furthermore, TEs provide a powerful link between stress and eccDNA biogenesis. For instance, heat stress promotes the production of eccDNA derived from the heat-stress responsive *ONSEN* retrotransposon in *Arabidopsis* [14,16], drought stress increases the *Rider* eccDNA in tomato [38], and cold stress repress the *Nightshade* eccDNA formation in potato [19]. These derivatives of (potentially) active TEs may provide substrates for DNA integration, displacement, recombination, and gene capture allowing plants to rapidly evolve novel responses to stresses.

The eccDNAs as triggers for structural variations, also in plants?

Chromothripsis describes the chromosomal breakage induced by genomic stresses that can lead to large circle formation. This chromosome aberration has been found in triticale after treatments causing strong chemical stress [59]. In mammals, chromothripsis is common in cancer cells, and there is a growing literature describing the role of eccDNA in tumor pathogenesis (for a review see Ref. [24]). In these cells, eccDNAs, notably because of their different chromatin regulations, contribute to gene amplification, drug resistance, and accelerate tumorigenesis. In neuroblastoma, eccDNAs are further considered a major source of somatic rearrangement of the genome [60]. In cattle, eccDNA is thought to have induced copy number variation at the KIT locus, giving rise to different color-sidedness phenotypes in cows [61]. In plants, such effects of eccDNA have not yet been documented, and it remains to be investigated if such events also occur under natural circumstances and at which frequency to assess the biological relevance of such findings.

The eccDNAs and epigenetic regulation

Finally, there could be an attractive role to be played for eccDNAs in plant epigenetics. Plants can be highly sensitive to gene copy number changes, often resulting in efficient gene silencing [62]. It would be surprising, if eccDNAs were not also a source for small interfering RNAs that then could target mRNAs for degradation or promote DNA methylation at homologous sequences. This could provide an attractive additional source for genomic regulation at numerous possible levels. Indeed, some studies suggest an interplay between DNA methylation and eccDNAs. For instance, treatments with DNA methylation inhibitors or defects in methyltransferases effectively reducing DNA methylation can promote rDNA eccDNA formation via recombination in human cells [63]. Similarly, plants in which DNA methylation was reduced by DNA methylation inhibitors resulted in LTR-RTs bursts and TE-derived eccDNA accumulation [16,64]. Fascinating research in mammalian cells revealed that by simulating functional eccDNA, artificial eccDNA can express small regulatory RNAs such as microRNA and small interfering RNAs. Those small eccDNAs contain miRNA genes that yield mature miRNA that suppress endogenous mRNA targets [65]. We expect that this kind of feedback could also be involved in TE repression and gene regulation. As active TEs form eccDNA containing either a copy or partial sequences of the TE, the circles may act as a silencing sensor for RNA-directed DNA methylation (RdDM). Recently, reported DNA methylation-free *Arabidopsis* lines could be an ideal resource for future studies [66]. Concerning the epigenetic status of the eccDNAs themselves, there is little evidence in plants. Indirect evidence, through methylation-sensitive Southern bot, suggests a lack of DNA methylation for the heat-induced *ONSEN* eccDNA in *Arabidopsis* [67]. In cancer cells, ATAC-seq studies indicate that the eccDNA chromatin accessibility is the highest in the cell, notably at oncogenes, supporting the fact that eccDNAs promote oncogene expression in these cells [68]. Future studies are needed to unravel the chromatin accessibility of eccDNA in plants.

Conclusion

Currently, eccDNAs in plants and other eukaryotes are undergoing a revival providing intriguing recent discoveries in plant molecular biology. Yet, even after several decades of studies, their potential biological functions remain unclear. In the last five years, thanks to the advances in HTS and bioinformatics tools, significant progresses have been made in this field. In comparison with short-read sequencing, long-read sequencing provides

significant benefits and a higher level of confidence to detect eccDNA variations originating from repetitive regions. Short reads may, on the other hand, be more suitable for quantification. We anticipate that eccDNA detection methods integrating short and long-read data will foster the characterization of the eccDNA repertoire in more species and in developmental and stress conditions. It is now clear that eccDNAs can originate from more loci than only repeated regions and can be used as markers of TEs mobilization. However, the role of eccDNA during TEs mobilization remains unknown. It seems that they may be side products of eclDNA that are ligated by plant defense mechanisms. Currently, there are no indications suggesting that any of the circles reported in plants so far can integrate back into the genome or aid in the integration of TEs. In addition, we do not know how plant cells degrade these molecular structures. We hypothesize that this kind of feedback is also involved in TE repression, acting as potential a biosensor of RdDM. Certain stresses have shown a strong correlation with the generation of eccDNAs yeast and mammalian cells. The scale at which eccDNAs may provide a selective advantage to plants now remains to be tested.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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