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Tools for Assessing Cell-Cycle Progression in Plants

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Estimation of cell-cycle parameters is crucial for understanding the developmental programs established during the formation of an organism. A number of complementary approaches have been developed and adapted to plants to assess the cell-cycle status in different proliferative tissues. The most classical methods relying on metabolic labeling are still very much employed and give valuable information on cell-cycle progression in fixed tissues. However, the growing knowledge of plant cell-cycle regulators with defined expression pattern together with the development of fluorescent proteins technology enabled the generation of fusion proteins that function individually or in conjunction as cell-cycle reporters. Together with the improvement of imaging techniques, in vivo live imaging to monitor plant cell-cycle progression in normal growth conditions or in response to different stimuli has been possible. Here, we review these tools and their specific outputs for plant cell-cycle analysis.

Keywords: Arabidopsis • Cell cycle • Live-imaging • Metabolic labeling • plants • Reporter genes

Introduction

The formation of an organism requires a correct balance between cell proliferation, cell differentiation and cell growth. These processes need to be tightly coordinated in time and space in the different tissues forming the organism and are genetically defined and developmentally regulated. In plants, this is particularly important because of the rigid structure of the plant body due to the presence of the cell wall to maintain tissue organization.

The cell cycle is defined by the unidirectional succession of events, including the duplication of cellular components, the replication of the genetic material during S-phase and its partition during mitosis (M) into two sister cells. S- and M-phases are preceded by two gap periods named G1 and G2, respectively, in which cells grow and prepare for the next step. During G1, cells increase the number of organelles and the protein complexes required for the activation of DNA replication origins are assembled, whereas in G2 an extensive control of genome integrity takes place, including DNA repair, and cells prepare for mitosis (Gutierrez 2009). Moreover, in plants, differentiation is often associated with one or several rounds of endoreplication, a special cell cycle that alternates DNA replication and G-phase without mitosis leading to the formation of polyploid cells.

Monitorization of mitotic cycle and endocycle during plant development is of great interest to understand how both processes are interconnected and regulated during plant development. In fact, cell division has been a topic of observation since the invention of the first microscopes, initially in fixed and stained cell preparations and later in vivo, after the development of phase contrast microscopes [reviewed in (Rieder and Khodjakov 2003)]. The continuous improvement of imaging techniques together with the specific immunodetection of key cell-cycle regulators or the use of fluorescent labeled reporters had shed light on many regulatory processes of cell proliferation. Core cell-cycle genes have been identified in plants and many of them have an expression that oscillates during the cell cycle and could potentially be used as a phase-specific marker (Vandepoele et al. 2002). Moreover, since the discovery of Green Fluorescent Protein (GFP) and its possible use as a genetically encoded fluorescent tag for expression and localization studies, multiple investigations focused on developing brighter fluorescent proteins (FPs) that work as a monomer, broadening their color range and increasing their photostability (Rodriguez et al. 2017). As a consequence, there is now a large variety of optimized FPs available for multicolor live imaging that undoubtedly benefit the development of tools needed in cell-cycle research.

Different methodologies have been developed to assess cell-cycle parameters in plants. They rely on imaging of fixed tissues after metabolic labeling or on live imaging of plant lines expressing cell-cycle-regulated fusion proteins. Here, we will review the different approaches used so far, highlighting their pros and cons, to assess cell-cycle activity in plants (**Fig. 1**).

Labeling with Nucleosides Analogs

Initial studies on plant cell cycle in the early 1950s were aimed at detecting proliferating cells in S-phase and based on the metabolic labeling of DNA by the incorporation of radioactive

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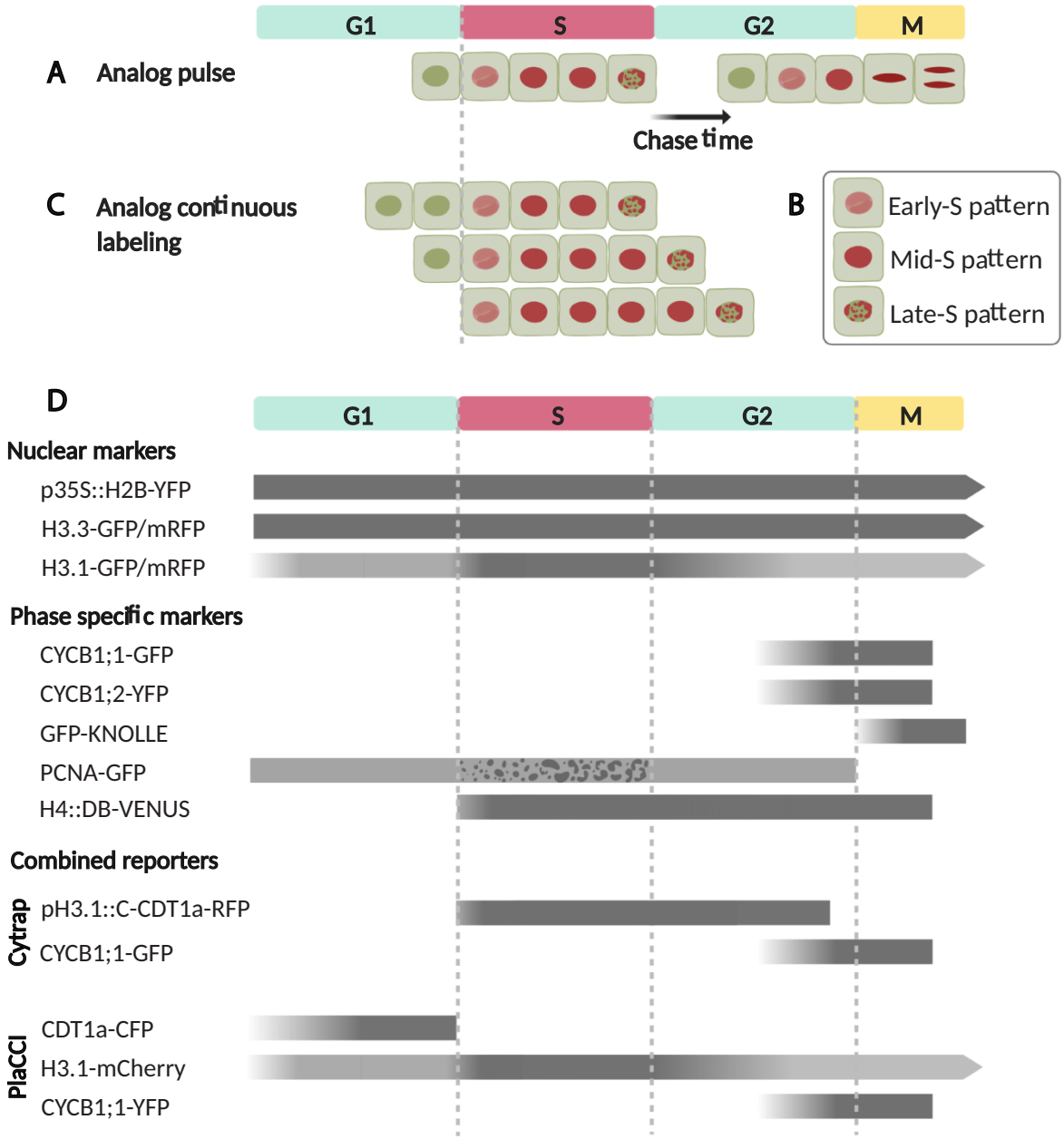

Fig. 1 Summary of the available tools to assess cell-cycle status. (A–C) Following cell-cycle progression using thymidine analogs. (A) Measure of G2 length: a cell population is treated by a short pulse of EdU. Cells undergoing S-phase are labeled and cell cycle is led to progress during different chase time periods until labeled mitoses appear. (B) Different patterns observed for early, mid and late S-phase nuclei. (C) Cells are continuously incorporating EdU as they progress through S-phase. The proportion of labeled cells during a time period is an estimation of the cell-cycle duration. (D) Distribution (bar position), fluorescence intensity (gray saturation) and expression pattern inside the nucleus (homogenous or speckled) throughout the different cell-cycle phases for the translational reporters described in this report. The arrowhead in the bar indicates that the marker remains in the next mitotic cycle, a blunt end indicates the degradation of the protein. The tag name position is indicative of a N-terminal (left) or C-terminal (right) fusion. Promoter used in the construct is only specified in case the protein is not expressed under its native regulator.

precursors (Taylor et al. 1957). The application of a pulse with H^3-thymidine to label cells undergoing S-phase allowed to monitor radioactively labeled mitoses at different chase times by autoradiography of squashed or sectioned meristems. This technique led to the estimation of average cell cycle and phase durations without any positional information [reviewed in Webster and MacLeod (1980)]. The inconvenience of using radioactivity was later circumvented by the use of non-radioactive thymidine analogs, such as 5-bromo-2′-deoxy uridine (BrdU), that could be detected with specific antibodies. Moreover, when coupled with the use of fluorescent secondary antibody and a DNA-specific dye, S-phase within a plant organ

could be identified (Xu et al. 1998). Nevertheless, immunodetection of BrdU is time-consuming. More importantly, it requires a previous chemical or enzymatic treatment to denature the DNA helix, which also affect tissue and organ structures and are incompatible with the use of DNA dyes such as DAPI (4′, 6-diamidino-2′ -phenylindole, dihydrochloride) that binds to double stranded DNA.

A remarkable improvement was achieved with the synthesis of an alkyne-modified nucleoside, the 5-ethynyl-2′-deoxy uridine (EdU), that is also incorporated into the DNA during replication. EdU is highly convenient because it can be easily detected by a copper(I)-catalyzed click coupling reaction between the ethynyl group of the nucleoside and a fluorescent dye functionalized with an azide group (Salic and Mitchison 2008). The reaction is fast and, since the reagents are small molecules, it takes place without the need of partial digestion of the plant cell wall and DNA denaturation. Therefore, this technique is compatible with the use of DNA dyes and preserves better the plant organ structure to allow gathering positional information of the S-phase cells within the tissue. Moreover, the nuclear EdU-staining pattern identifies different stages of DNA replication (**Fig. 1B**). During early S-phase, EdU is homogenously distributed and colocalizes with euchromatin marks, such as H3K4me2, whereas during late S-phase the EdU pattern is characterized by the presence of punctuated foci that colocalize with heterochromatin marks such as H3K9me2 and chromocenters (Hayashi et al. 2013). Flow cytometry sorting of EdU-labeled nuclei depending on their DNA content confirmed the previously mentioned EdU patterns. The EdU staining pattern also serves to identify intermediate profiles with a nuclear staining excluded from the nucleolus in early S that progressively increases as S-phase progresses (Dvořáčková et al. 2018). It is worth noting that the mild conditions of EdU detection make them compatible with protocols of immunofluorescence or in situ hybridization that facilitate co-localization studies, e.g. with cell-cycle-regulated proteins (Otero et al. 2016), and to assess the replication dynamics of specific loci such as the rDNA repeats (Dvořáčková et al. 2018).

EdU is linearly incorporated in the newly replicated DNA, and therefore, the frequency of EdU positive nuclei is proportional to the time of incubation (**Fig. 1**). Quantification of labeled nuclei after 1–12 h EdU treatments allowed to estimate the average duration of the cell cycle in the root meristem (∼17 h) and in the transition zone (∼30 h) (Hayashi et al. 2013). However, these estimations are valid only assuming that cell-cycle duration is constant for all cells in the defined zones of the meristem. The same approach combined with a high-resolution phenotyping platform could show local differences in cell-cycle duration between the different cell layers of the root meristem (Pasternak et al. 2021). Moreover, these imaging technologies can also be combined with flow cytometry analysis to give valuable information on the S-phase progression of a cell population (Mickelson-Young et al. 2016).

A drawback of the use of prolonged EdU treatments to assess cell-cycle parameters came from studies in mammalian cell lines revealing that EdU is a toxic compound that can provoke DNA damage, activate cell-cycle checkpoints that impair cell-cycle progression and trigger apoptosis (Zhao et al. 2013). The cytotoxicity depends on the cell line, the EdU concentration and the length of the treatment (Ligasová et al. 2015). Nowadays, no such studies have been carried out in plants, but it is likely that long-term treatments also affect cell-cycle progression, as suggested in a study describing the renewal of quiescent center cells (Cruz-Ramírez et al. 2013). Consequently, strategies using short EdU pulses followed by different chase periods in EdU-free medium should be the preferred option to avoid a prolonged exposure to the toxic molecule (**Fig. 1A**).

This experimental setting has been successfully used to determine G2-phase length of cells of the root meristem. Quantification of EdU-labeled mitotic figures at different chase times, combined with tracking the cell's position along the cell file, showed that the G2-phase of epidermal cells is shorter in the distal part of the meristem (the so-called proliferation domain) than in cells undergoing their last cell cycle before exit to differentiation (Otero et al. 2016). The same approach evidenced a delayed G2/M progression in a mutant of *SCL28* gene, encoding a plant-specific GAI-RGA-and-SCR (GRAS) transcription factor necessary for entry in mitosis and to control division planes (Goldy et al. 2021). It has been also successfully employed to label sister cells formed after mitosis, to determine the cell division plane in the leaf primordia of Arabidopsis and other plants species (Yin and Tsukaya 2016).

An alternative approach to avoid the toxic effect of EdU is the use of another nucleoside analog, the (2′S)-2′-deoxy-2′-fluoro-5-ethynyluridine (F-ara-EdU), which did not cause cell-cycle arrest or DNA synthesis inhibition in a variety of mammalian cell lines (Neef and Luedtke 2011). F-ara-EdU seems also more appropriate for long-term treatments in plants, as shown in Arabidopsis, which root growth is not affected growing in a medium containing 2 μM F-ara-EdU during 7 days (Cruz-Ramírez et al. 2013).

An additional question is not only to determine whether a cell is progressing through the S-phase but also to measure S-phase dynamics. Direct measurement of the S-phase length would require the colocalization analysis of two different analogs sequentially incorporated and separated by increasing chase times. This has been achieved by using two different halogen-modified nucleosides (CldU and IdU) recognized specifically by different antibodies (Zink et al. 1998) but also using azide-modified nucleosides that can be detected by click chemistry using ethynyl coupled fluorochrome (Neef and Luedtke 2014). Implementation of these methods in plants could give valuable information on S-phase kinetic. However, an inconvenience of these techniques based on the labeling with nucleotides analogs is that they require to fix the tissues before developing the reaction, thus precluding dynamic imaging of cell-cycle progression.

Translational Fusion Reporters

The advent of the use of translational fusions with reporter genes encoding an enzyme, luminescent or FPs, enabled monitoring cell-cycle activity in a variety of processes during plant

development. Enzymatic activities are detected by histochemical staining and give a precise picture of reporter genes expression at a fixed time point. The use of FPs has several additional advantages: first, it allows the spatiotemporal visualization of expression patterns in vivo, and second, the development of FPs with excitation-emission spectra that span over wide range of wavelengths permits the combination of various markers that can be simultaneously imaged (**Fig. 1D**).

Constitutive nuclear markers

Monitorization of cell division can be achieved in vivo by the visualization of mitotic figures in plant organs expressing a constitutive nuclear marker. Histones are the main components of chromatin and various vital reporters using histone genes have been developed. A Histone H2B-YFP has been expressed under the control of the CaMV 35S promoter or in specific tissues using the GAL4-UAS transactivation system (Boisnard-Lorig et al. 2001). Likewise, genes encoding histone variants H3.3 fused to GFP or mRFP were cloned downstream of their native promoters and expressed in plants. Fusion proteins are incorporated into the chromatin of all examined tissues including gametes (Ingouff et al. 2010, Otero et al. 2016). Despite that histones are small proteins, FP-fusions that are much bigger are functional and could be assembled in nucleosomes and efficiently immunoprecipitated in ChIP experiments (Stroud et al. 2012, Wollmann et al. 2012). In all cases, a C-terminal fusion strategy was preferred to preserve the N-terminal tail subject to most of the posttranslational modifications that affect chromatin structure and function. These reporter lines are very useful to observe the succession of the different stages of mitosis because of the easy recognition of condensed and segregating chromosomes or to identify proliferation domains in a developing organ.

The canonical H3.1 is incorporated in the chromatin during DNA replication and progressively replaced by the variant H3.3, preferentially in the euchromatin (Stroud et al. 2012, Wollmann et al. 2012). As a consequence, the presence of H3.1 is indicative of a region with high mitotic or endoreplication activity (Ingouff et al. 2010, Otero et al. 2016). Simultaneous expression of H3.3-mRFP and H3.1-GFP under their respective native promoter has been useful to identify several domains in the Arabidopsis root meristem. In the most distal part, H3.1 eviction during G2 is not complete and mitotic figures are dually labeled by both H3.1-GFP and H3.3-mRFP. On the contrary, in the proximal part of the meristem, close to the boundary with the transition zone, H3.1 is completely exchanged during G2 and mitotic figures are only labeled by H3.3mRFP, identifying cells undergoing their last mitotic cell cycle (Otero et al. 2016). Expression of the markers is not restricted to the root and observation of any proliferating/endoreplicating tissues is feasible. Moreover, since H3.1 is enriched in heterochromatin the reporter line is also useful to analyze the formation of chromocenters during normal development or in mutant background (Otero et al. 2016).

Moreover, these histone-based markers can be combined with the staining of plasma membranes by the amphiphilic dye FM4-64 that emits light in the far red, a wavelength compatible with most of FPs (Rigal et al. 2015), and is suitable for short periods of live imaging (Otero et al. 2016). Over the years, several variations have been introduced in these nuclear markers constructs, changing the FP for colocalization studies, or generating plants lines that also express a plasma membrane marker that delimit cell border (Federici et al. 2012). Live imaging experiments of nuclear marker Arabidopsis lines, for short (∼24 h) or long period (∼1 week) could inform on cell-cycle duration and provided direct evidences that in roots, stem-cells are dividing at a much lower rate than cells located in the proximal meristem (Campilho et al. 2006, Rahni and Birnbaum 2019).

Phase-specific markers

A critical point for the choice of adequate phase-specific candidate genes is that the expression window should be sharp and well defined. This is usually obtained by choosing a gene transcriptionally regulated during the cell cycle and encoding a protein that is degraded by a proteolytic system at a specific cell-cycle time. In the course of investigations of the plant cell cycle or with the purpose of developing cell-cycle indicators a variety of constructs expressing individual translational fusion reporters have been generated, characterized and showed a specific pattern during cell-cycle progression. A list of some of those that cover the different cell-cycle phases will be given below (**Fig. 1D**).

Cell-cycle progression is driven by a series of events dependent on the phosphorylation by CDK/Cyclin complexes of a myriad of substrates, by phase-specific gene transcription activation and by the controlled degradation of cell-cycle regulators (Harashima et al. 2013). The cyclin partner of CDK is important to give the substrate specificity for the phosphorylation events that unidirectionally drive the cell-cycle transitions, and it is therefore not surprising that the first fusion protein used to assess cell proliferation was a member of this family. In plants there are several genes encoding B-type cyclins that, as their mammalian counterparts, have a role during G2 and mitosis. The first member of this family characterized in Arabidopsis was CYCB1;1 that is expressed during late G2 and degraded by proteolysis during anaphase in a proteasome dependent way. Degradation is mediated by a short peptide motif, the 'destruction box' (D-box), present in the N terminal domain of CYCB1;1. Therefore, a fusion of this domain to the beta-glucuronidase (GUS) gene under the control of CYCB1;1 native promoter reflected the mitotic activity of the tissue to be analyzed (Colón-Carmona et al. 1999). This construct, or later, the equivalent using GFP as reporter that enabled in vivo imaging (Ubeda-Tomás et al. 2009), has been widely used in plant research. They were very useful to identify cell-cycle activators or repressors, e.g. members of the RBR/E2F pathway (Desvoyes et al. 2006) and to easily determine how the proliferation potential is affected in a mutant background or by a chemical treatment. The construct expressing the Arabidopsis CYCB1;1 fused to YFP is also active in other plant species, as for example in legumes, and was included in a modular tool kit together with an auxin and a cytokinin sensor to monitor cell proliferation and hormone signaling during nodulation (Nadzieja et al.

2019). The maintenance of fluorescence after clearing enables high-resolution microscopy imaging of internal tissues.

An important achievement was the discovery that CYCB1;1 and the plant-specific cyclin-dependent kinase B (CDKB) were also involved in DNA repair by homologous recombination (HR) (Weimer et al. 2016). Long before, it was observed that CYCB1;1 expression is up-regulated in response to treatment with DNA damage provoking agents or in mutants defective in chromatin organization, leading to the erroneous conclusion at that time, of a cell-cycle arrest in late G2 (Ramirez-Parra and Gutierrez 2007, Fulcher and Sablowski 2009). However, it is now proved that key components of the HR pathway such as RAD51 are targets of CYCB1;1-CDKB1 complexes during the DNA damage response (Weimer et al. 2016). Thus, since CYCB1;1 is truly up-regulated in G2, having a high level of CYCB1;1 may or may not directly reflect that a cell is cycling, unless the absence of any DNA damage response is demonstrated. However, the equivalent construct expressing the D-Box of CYCB1;2, placed under its own promoter (pCYCB1;2:D-BoxCYCB1;2-YFP), is a good alternative since it is also expressed in G2/M and degraded by the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase (Iwata et al. 2011) but is not up-regulated in response to DNA damage (Weimer et al. 2016). Despite that CYCB1;1 has been for many years 'the' cell proliferation marker in plants and, to a lesser extent but increasing lately, CYCB1;2, their windows of expression are very narrow labeling only cells that transit through G2/M.

KNOLLE, a protein of the syntaxin family, is involved in vesicle transport necessary for the formation of the new cell wall during cell division. It accumulates during mitosis first in vesicles of the trans-Golgi network and later, during telophase, in the newly formed cell plate until cytokinesis is completed. Accordingly, the construct expressing KNOLLE-GFP is thus a good choice to monitor cytokinesis (Reichardt et al. 2007).

Proliferating cell nuclear antigen (PCNA) plays a pivotal role during DNA replication and DNA repair. It was first identified as a processivity factor and a sliding clamp for DNA polymerases, but it is now known that it acts as a hub for the interaction of many proteins located at the DNA replication fork coordinating the replication of leading and lagging stands and the coupled assembly of the new nucleosomes (Boehm et al. 2016). Due to its functions, PCNA was a good candidate to be used as a S-phase marker. A construct bearing the genomic locus of Arabidopsis *PCNA1* (promoter-gene) fused to GFP has been generated to transform and image plants. Expression of PCNA-GFP was seen in most of the cells indicating that it was not restricted to S-phase but different patterns could be identified and colocalization studies with EdU could associate the observed pattern with cell status. Cells in G1 and G2 present an homogenous signal though the nucleoplasm but a dotted and speckled pattern was characteristic of early/mid and late S-phase, respectively (Yokoyama et al. 2016). Therefore, change of PCNA-GFP subnuclear localization to specific foci enables the identification of cells undergoing S-phase using microscopy techniques. However, this reporter is not compatible with the use of flow cytometry to identify the cell-cycle stage since it is expressed during the whole interphase.

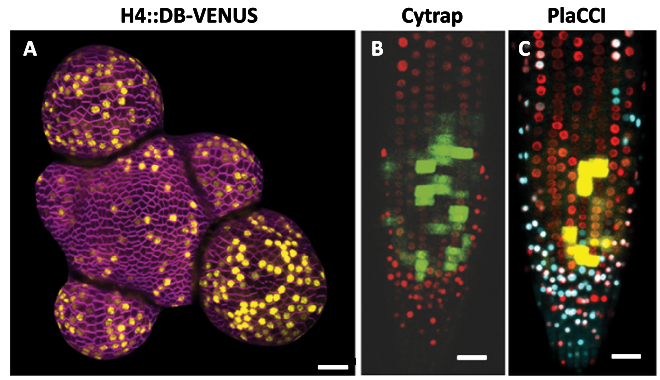

Fig. 2 Expression of a selection of cell-cycle reporters in Arabidopsis. (A) H4::DB-VENUS expression in the shoot meristem labels cells in S-G2-M (image provided by A. Jones and J. Murray). (B) Cytrap expression in the root meristem: pHTR2::CDT1a (C3)-RFP (red) and pCYCB1;1::NCYCB1;1-GFP (green) label cells in S-G2 and G2-M, respectively (image provided by M. Umeda). (C) PlaCCI expression in the root meristem: pCDT1a::CDT1a-CFP (cyan), pHTR13::HTR13-mCherry (red) and pCYCB1;1::NCYCB1;1-YFP (yellow) label cells in G1, G1-S-G2 and G2-M, respectively. Scale bars: 20 μm (A) 25 μm (B–C).

A construct that expresses a chimeric protein containing the D-Box of Arabidopsis CYCB1;1 fused to Venus under the control of an H4 promoter (H4::DB-VENUS) constitutes another strategy for cell-cycle phase identification. The promoter of histone H4 is switched on at the initiation of S-phase and the D-Box triggers the fast degradation of the fusion protein during mitosis. Thus, cells are brightly labeled during S–G2–M-phases, whereas the absence of signal reveals cells transiting through G1 (**Fig. 2A**). A combination with EdU labeling further enables the discrimination between G1, S and G2 cells when contain none, both or only one indicator(s), respectively. This strategy has been used to link cell-cycle phase transition to cell growth in the shoot apical meristem (Jones et al. 2017). However, with all the markers discussed above, the precise monitorization of all cell-cycle transitions is possible only in fixed tissues.

Reporters with multiple markers

Expression of a single reporter is not sufficient to inform about progression through all cell-cycle phases, and therefore, a combination of various markers has been the next step towards developing more advanced tools. In the animal field this was achieved by the development of Fluorescence Ubiquitin Cell-Cycle Indicator (FUCCI) based on the specific expression and degradation of two key proteins involved in licensing of DNA replication origins, CDT1 and its inhibitor geminin (Sakaue-Sawano et al. 2008). The FUCCI system, first established in cell lines, has been adapted to a large variety of model species such as mouse, fish or flies enabling the study of in vivo cell-cycle regulation in the context of development. Later, several FUCCI variants have been engineered to respond to the needs of the scientific community [reviewed in (Zielke and Edgar 2015)]. Although plant genomes encode homologues of CDT1, they do not have a canonical geminin (Caro and Gutierrez 2007), preventing the direct transfer of the FUCCI system to plants.

Efforts to develop novel cell-cycle sensors have been carried out in Arabidopsis. Cytrap (for cell-cycle tracking in plants) is a dual-color live cell reporter based on the expression of a fragment of CDT1a fused to RFP and the well-characterized CYCB1;1-GFP that label cells transiting though S-G2 and G2-M, respectively (Yin et al. 2014). As their mammalian counterparts, Arabidopsis CDT1a participates in the formation of the pre-replication complex and is a proteasome target (Castellano et al. 2004). Analysis of a series of CDT1a deletion mutants showed that the CDT1a C-terminal domain (C-CDT1a; aa 361–571) carries a motif responsible for its proteasome-dependent degradation in late G2. The coding sequence of that domain in frame with RFP was placed under the control of the canonical H3.1 (*HTR2*) promoter, because it is active in all proliferative tissues and in order to transcriptionally restrict the expression to S-phase. Thus, cells emit red fluorescence while they are in S until late G2 and green in late G2-M with a small period in G2 where both signal coincide (Yin et al. 2014) (**Fig. 2B**). However, there is a labeling gap for cells in G1. Another limitation for using the Cytrap is that both markers are independent from each other and are inserted at different loci in the Arabidopsis genome, making difficult the segregation of the two characters in mutant backgrounds.

Both limitations were circumvented with the recently developed three-color plant cell-cycle indicator (PlaCCI), since the major novelty of this system is that it includes a marker specifically identifying cells in G1 (Desvoyes et al. 2020). The full length CDT1a protein fused to CFP, controlled by its native promoter is expressed shortly after cytokinesis, accumulates during the whole G1 phase and is degraded rapidly at the onset of S-phase (unlike the C terminal fragment used in the Cytrap that is also present during S and part of the G2-phase). This was confirmed by the absence of colocalization of CDT1a-CFP with EdU signals. The second sensor is the H3.1 histone HTR13 fused to mCherry. As mentioned before, H3.1 is incorporated during DNA replication and maintained during the next G2, M and to some extent in the following G1, in cells with a high proliferation potential but excluded from chromatin in G2 in cells about to exit the proliferation domain and undergoing their last division (Otero et al. 2016). In live imaging experiment, the increase of the mCherry fluorescent signal is indicative of S-phase entry and progression. The third, is the already described G2-M reporter CYCB1;1-YFP. Therefore, the combination of the three fluorescent sensors enables the unequivocal identification of cells in G1, labeled in cyan, independently of whether they also have some reminiscent red fluorescence. Cells from S through early G2 emit red fluorescence only and from G2 through M are revealed by both red and yellow fluorescence (**Fig. 2C**). This color code is also valid to monitor cell-cycle activity in various plant organs (Desvoyes et al. 2020). CDT1a-CFP is also expressed during the G-phase of the endocycle and starts to accumulate shortly after completion of DNA replication. (Desvoyes et al. 2019). In consequence, PlaCCI is also useful to identify endoreplicating cells where an alternation of CDT1a-CFP and H3.1-mCherry is observed. This system has been refined using synthetic biology tools to generate the construct and the transcriptional subunits for the expression of the three sensors plus the antibiotic resistance gene are in a single vector. Thus, the integration of the four gene units at a unique locus in the Arabidopsis genome facilitates enormously the selection after crossing with mutants of interest.

Both Cytrap or PlaCCI systems make use of CDT1a that overexpression was shown to increase endoreplication (Castellano et al. 2004). Obviously, a reporter system should ideally not affect the normal development of the plant and this was indeed checked to show that these reporter lines have a normal growth and ploidy levels (Yin et al. 2014, Desvoyes et al. 2020). Furthermore, expressing PlaCCI does not affect cell proliferation and root meristems are comparable to wild type. However, the PlaCCI tool will still require to be improved to increase its versatility by changing the antibiotic resistance cassette (to facilitate the segregation of the constructs in a larger collection of insertion mutants) and/or exchanging the CYCB1;1 by a CYCB1;2 cassette (to make it insensitive to DNA damage), both of which are now in progress.

Perspectives

The existing cell-cycle reporter systems are so far functional in the model plant Arabidopsis. However, it would be extremely important to evaluate if the heterologous constructs could work in other plant species or need adaptation. In addition, a marker with an expression window strictly restricted to S-phase cells is still lacking in plants. In mammalian cells, a true S–G2-phase transition marker has been added to the FUCCI system, to create the FUCCI4 (Bajar et al. 2016). It takes advantage that mammalian canonical histone transcripts are not polyadenylated but their mRNAs form a stem loop structure bound by the stem-loop binding protein, degraded when S-phase finalizes. Unfortunately, this strategy cannot be directly adapted to plants since histone transcripts are polyadenylated and, therefore, efforts to identify new makers with an S-phase-specific expression pattern should be made.

Another field of improvement will be the development of automatic segmentation protocols for live imaging of confocal or light sheet images, which is necessary for high-throughput analysis of cell-cycle dynamics during organ growth. Using deep learning algorithms, three-dimensional localization of root nuclei expressing the G2/M marker CYCB1;1-GFP has been performed and the cell volumes successfully segmented in a time series (Khan et al. 2020). Despite that, the accuracy of segmentation still needs to be considerably improved. Fluorescent marker localization and segmentation is a necessary challenge ahead that undoubtedly will open the window to the improvement of these technologies. These will allow multicolor segmentation of plants expressing various reporters, rendering an excellent tool for in vivo studies of cell-cycle dynamics during development, in mutant backgrounds or in response to environmental challenges.

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