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Estimation of cell cycle kinetics in higher plant root meristem with cellular fate and positional resolution.

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Abstract

Root development is a complex spatial-temporal process that originates in the root apical meristem (RAM). To keep the organ's shape in harmony, the different cell files' growth must be coordinated. Thereby, diverging kinetics of cell growth in these files may be obtained not only by differential cell growth but also by local differences in cell proliferation frequency. Understanding potential local differences in cell cycle duration in the RAM requires a quantitative estimation of the underlying mitotic cell cycle phases' timing at every cell file and every position. However, so far, precise methods for such analysis are missing.

This study presents a robust and straightforward method to determine the duration of the cell cycle's key stages in all cell layers in a plant's root simultaneously. The technique combines marker-free experimental techniques based on detection of incorporation of 5-ethynyl-2′-deoxyuridine (EdU) and mitosis with a high-resolution plant phenotyping platform to analyze all key cell cycle events' kinetics.

In the *Arabidopsis thaliana* L. RAM S-phase duration was found to be as short as 18-20 minutes in all cell files. But subsequent G2-phase duration depends on the cell type/position and varies from 3,5 hours in the pericycle to more than 4,5 hours in the epidermis. Overall, S+G2+M duration in Arabidopsis under our condition is 4 hours in the pericycle and up to 5,5 hours in the epidermis.

Endocycle duration was determined as the time required to achieve 100% EdU index in the transition zone and estimated as 3-4 hours.

Besides Arabidopsis, we show that the presented technique is applicable also to root tips of other dicot and monocot plants (tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* L.) and wheat (*Triticum aestivum* L.)).

Introduction

The duration of the mitotic cell cycle and the endo-cycle, including its phases, are essential characteristics for harmonized organ growth kinetics. Investigation of cell cycle duration in plants was first done in 1951 (Brown, 1951) in pea (*Pisum sativum* L.) roots by quantification of the ratio of cells in a certain stage of the cell cycle to the total number of meristematic

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cells. The interphase duration was found to be 23 hours, prophase 2 hours, metaphase 25 minutes, anaphase 5 minutes, and telophase 22 minutes. Thereafter cell cycle durations were investigated by different methods. Clowes (1961), as well as Van't Hof and Sparrow (1963), proposed a method based on H^3-thymidine incorporation in the DNA. Van't Hof (1967) further modified this method by additional colchicine treatment in order to accumulate cells in mitosis. Using this approach, entire cell cycle duration was found to be 24 hours in pea (*Pisum sativum* L.), sunflowers (*Helianthus annuus* L.), and bean (*Vicia faba* L.). Several investigations were recently carried out to estimate cell cycle duration by using 5-Ethynyl-2'-deoxyuridine (EdU) as a non-radioactive alternative to H^3-thymidine detected by fluorescent labeling (Buck, S. B. et al., 2008). For example, Hayashi et al. (2013) investigated cell cycle duration in Arabidopsis and found it to be 17 h in the root meristem proliferation domain.
Some researchers performed estimation the duration of the different cell cycle phases: G1, S, G2, M. Using the Van't Hof method, the S-phase duration in pea (*Pisum sativum* L.), sunflowers (*Helianthus annuus* L.) and bean (*Vicia faba* L.) was found to be 4.5 hours (i.e. 30% of total cell cycle length) (Van't Hof, 1969). More recently, a combination of EdU pulse labelling with flow cytometry (Mickelson-Young, L. et al., 2016) estimated S-phase duration in Arabidopsis to be 2-3 hours.

Besides the use of labeled nucleotide analogs, several other "kinematic" methods were developed to investigate cell cycle duration in the Arabidopsis root (Beemster and Baskin, 1998; Fiorani and Beemster, 2006). Newly developed stripflow software combined with kinematic measures gave a cell cycle duration in one of the root tissues (cortex) of 14,7±0,9 hours (Yang et al., 2017). A similar Rate of Cell Production (RCP) method (Ivanov and Dubrovsky, 1997) determined a cell cycle duration of 11-13 h for cortex cells of the different lines of the *Pisum sativum*. Another recent approach to investigate cell cycle duration is to use marker lines with a dual-color marker system (Yin K., et al., 2014). With this method, cell cycle duration was found to be about 16 hours in root epidermis cells, including 3 hours S-phase. To our knowledge, these lines were developed for Arabidopsis only and the introduction of these markers to mutant lines is time but also cost consuming.

A combination of markers that simultaneously label all cell cycle stages was introduced in one construct, allowing detecting cell cycle kinetics in living roots (Desvoyes, B et al., 2020). However, similarly as in Yin K, et al., these lines are suitable for Arabidopsis only, do not distinguish between different cell types, and require a long time to introduce the mutant lines.

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The kinematic method's main drawback is that it applies mainly to the outer cell layers (epidermis/cortex) and is based on the assumption that cell cycle duration is constant throughout the proliferation zone. It is well-known since 1961 (Clowes, 1961) that cell cycle duration can be dependent on cell position and cell type (positional information). For example, it was reported that in Convolvulus arvensis cell cycle duration in the central cylinder and cortex were significantly different (21 h and 27 h, respectively), while in the quiescent center, it reaches even 420 h (Phillips Jr, H. L., & Torrey, J. G., 1972). However, these differences have not been addressed in most of the recently published methods. It was only recently shown that cell cycle duration can be regulated by H3 histone modification and was different in the proximal and distal zone of Arabidopsis root epidermis (Otero et al., 2016).

In summary, the fundamental question about differences in the duration of the cell cycle phase in functionally distinct tissues and cell files in the root meristem remains unclear. Our working hypothesis is that different cell files may exhibit different cell cycle duration and kinetics to compensate for the differences in cell growth kinetics of the different cell lines in the context of a compact organ. To resolve this issue, the precise estimation of cell cycle kinetics in each cell in the frame of an organ coordinate system is required. Here we provide a simple and robust protocol for determination of S-phase, G2-phase, and M-phase duration in roots of higher plants. The proposed method does not require marker lines and allows us to determine the duration of S, G2, and M stages in all cell files simultaneously and independently. A combination of EdU incorporation and mitosis labeling with an accurate root coordinate system (iRoCS) (Schmidt et al., 2014) allows us to create precise maps of main cell cycle events in the root of higher plants.

Results

Divergence of cellular parameters in different tissues and cell files of the Arabidopsis RAM.

The Arabidopsis root apical meristem (RAM) has a radial structure built up by functionally distinct cell layers. Detailed analysis of the cell geometry and nuclei structure demonstrates a strong divergence between and within different cell types regarding cell volume dynamics and chromatin landscape (Fig. 1). Interestingly, cortex cells exhibit a significant cell volume increase even in the proliferation domain, while for the pericycle and endodermis cells, this tendency is not visible (Fig. 1b). Moreover, nuclei landscape (chromatin structure) can be significantly different among cells even in the proliferation domain what is also reflected by a strong divergence of the average volume of the nuclei at the end of the proliferation domain

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(71,5 µm^3, 50,6 µm^3 and 38,1 µm^3 for cortex, endodermis, and pericycle cells, respectively; compare Fig. 1a and S6).

Altogether, these data led us to the hypothesis that cell cycle duration may differ in different cell files to compensate variations in cell sizes by balancing cell numbers and thereby keeping root integrity.

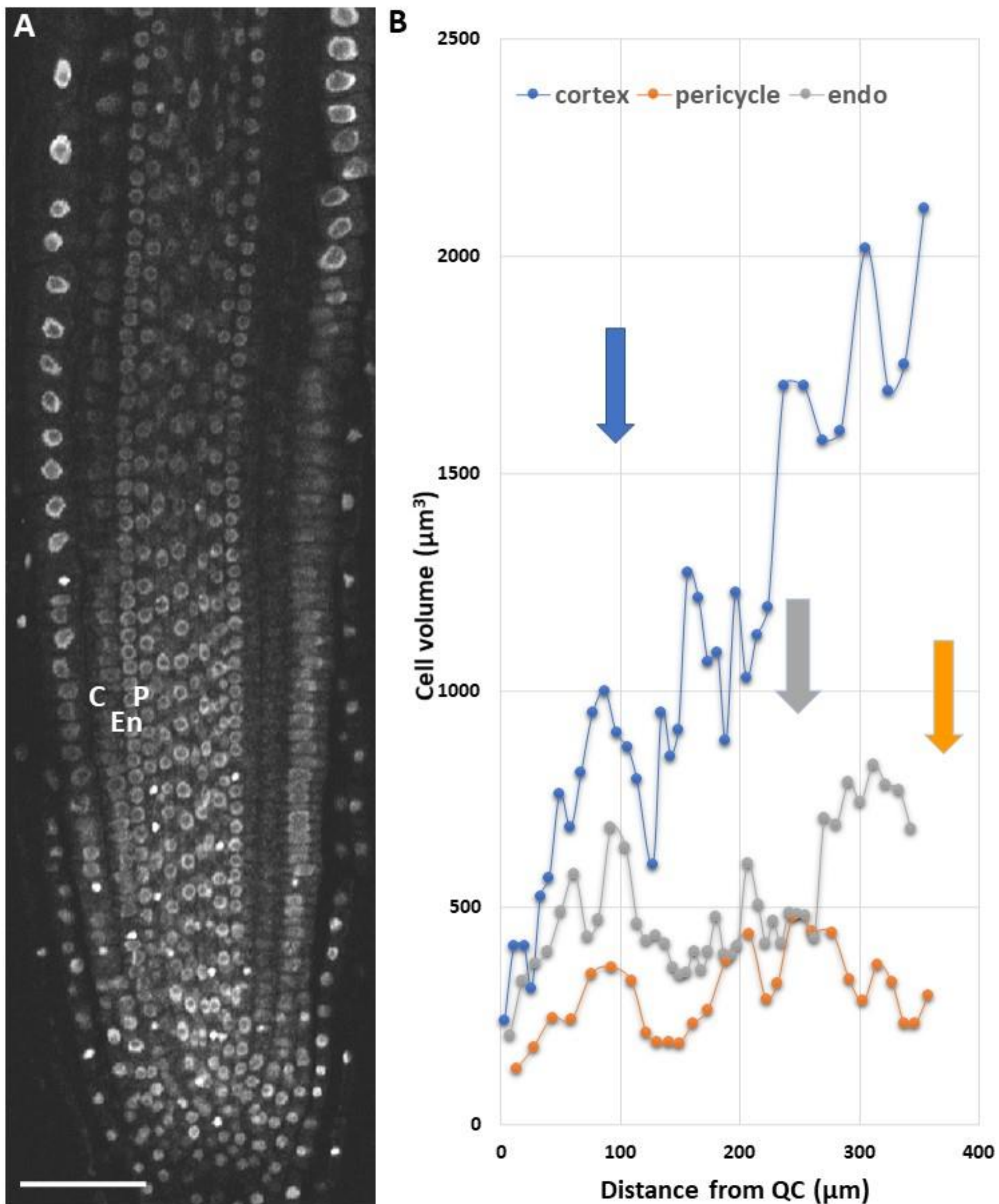

Figure 1. Divergence of cell geometries in the proliferation domain of Arabidopsis root. (A) Nuclei landscape after DAPI labelling, Five Day After Germination (DAG). C- cortex; En- endodermis; P- pericycle; (B) volume of the cells in cortex (blue), endodermis (grey) and

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pericycle (orange). Arrows mark the end of the proliferation domain, which was determined as the last mitosis position in each cell file (Lavrekha et al., 2017). Scale bar: 50 µm.

Overall work-flow to determine cell cycle parameters.

To determine the duration of the different cell cycle phases, we used the following workflow, summarizing in the figure 2. To minimize potential variations, we performed experiments by simultaneous determination of all stages in single seedling population: seedlings were distributed to 6 vials, EdU have been added at the given time points and samples were fixed in the end of the time course simultaneously.

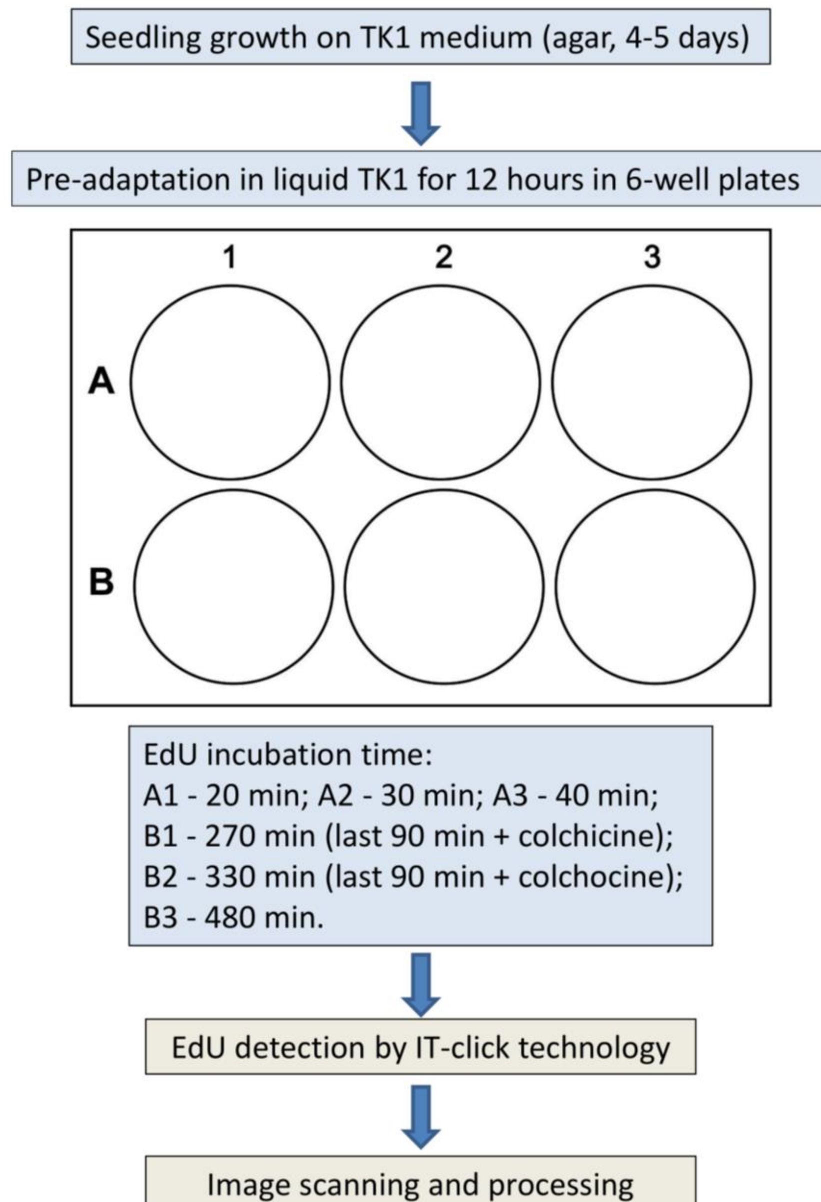

Figure 2. Workflow for estimation of cell cycle phases duration in Arabidopsis.

S-phase duration.

We determined the S-phase duration by estimating the minimal time required for full co-localization of DNA and EdU labeling. After 20 min incubation, up to 30-40% of the total

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number of the nuclei in showed complete EdU and DAPI co-localization (Figure 3). Interestingly, all cell files, even most central stele tissues in Arabidopsis, demonstrate many cells with DNA replication during 20 min incubation. From these data we conclude that the S-phase lasts about 20 min.

Besides, dense chromatin (hetero-chromatin) can also incorporate EdU (Figure 3, blue arrows), but these cells are mainly located in the transition domain when visible elongation are visible (post-mitotic domain).

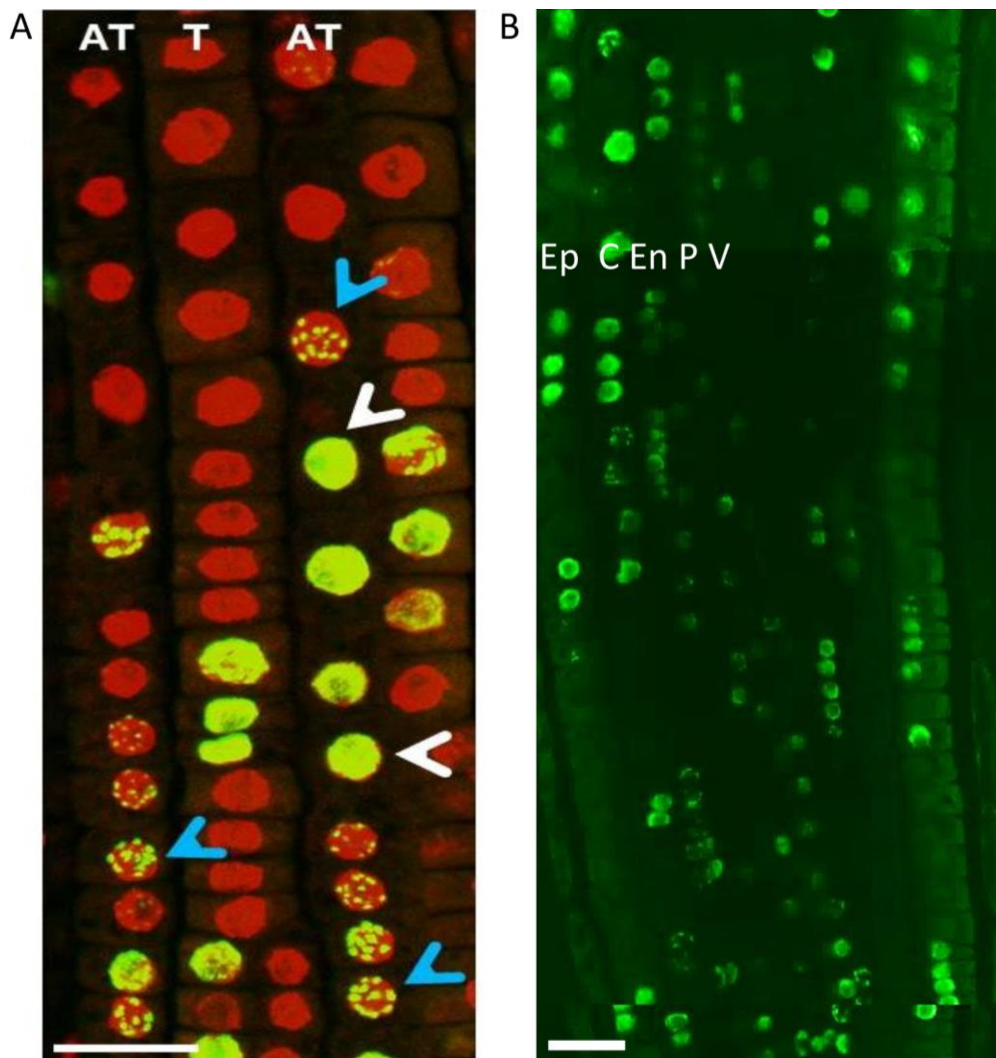

Figure 3. DNA replication in Arabidopsis root after 20 min. of the EdU incubation. A-Maximum projection of the epidermal cell layer of a representative root is shown. DAPI stain is coded in red, EdU signals in green. White arrow heads point to examples of entire labelling, blue arrow heads mark partial labelling of only condensed DNA spots. The white asterisks mark visible cell elongation in the trichoblast (T) and atrichoblast (AT) cell files (onset of transition zone). B - overview of the middle RAM. V-vasculature; P - pericycle; En-endodermis; C - Cortex; Ep - epidermis. EdU-positive nuclei are in green. Scale bar: 20 µm.

G2 duration.

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For the determination of the G2 duration, we first determined the minimal time for EdU-positive cells' appearance in the mitotic stage. For this purpose, EdU incubation was performed for 90, 180, 210, 240 and 270 minutes. EdU-positive cells passing through mitosis (i.e. pass DNA replication and G2 phase) in the pericycle and endodermis were detected after 240 minutes, but not after 210 minutes. The first EdU-positive cells in the cortex and epidermis were detected after 270 minutes. Based on this observation, we chose EdU incubation up to 270 minutes for detailed analysis and quantified EdU-positive mitotic cells' ratio in each cell file. To prevent EdU-positive cells from passing through mitosis, colchicine was added after 180 minutes of EdU incubation.

A detailed map of mitosis distribution (panel A) from a typical root and the calculated average values of about 6000 individual cells from three independent roots (panel B) are shown in figure 4. From this figure we can conclude that in the inner cell layers, cells passing the (S+G2) phase between 180 and 270 minutes are distributed equally/randomly in the whole proliferation domain of endodermis, pericycle, and vasculature. However, in the cortex and epidermis, only a few cells that are located close to the QC passed the (S+G2) phase during this time span, while more distally located cells obviously must have a longer G2 duration.

To further clarify G2 duration, we increased EdU incubation time to 330 minutes and added colchicine for the last 90 minutes. Results of a typical root analysis demonstrated that the amount of EdU-positive cells entering in mitosis after that time period increased to 80% in the cortex/epidermis, while in more inner cell layers, we still have 90-100% of EdU-positive mitosis (Figure S1).

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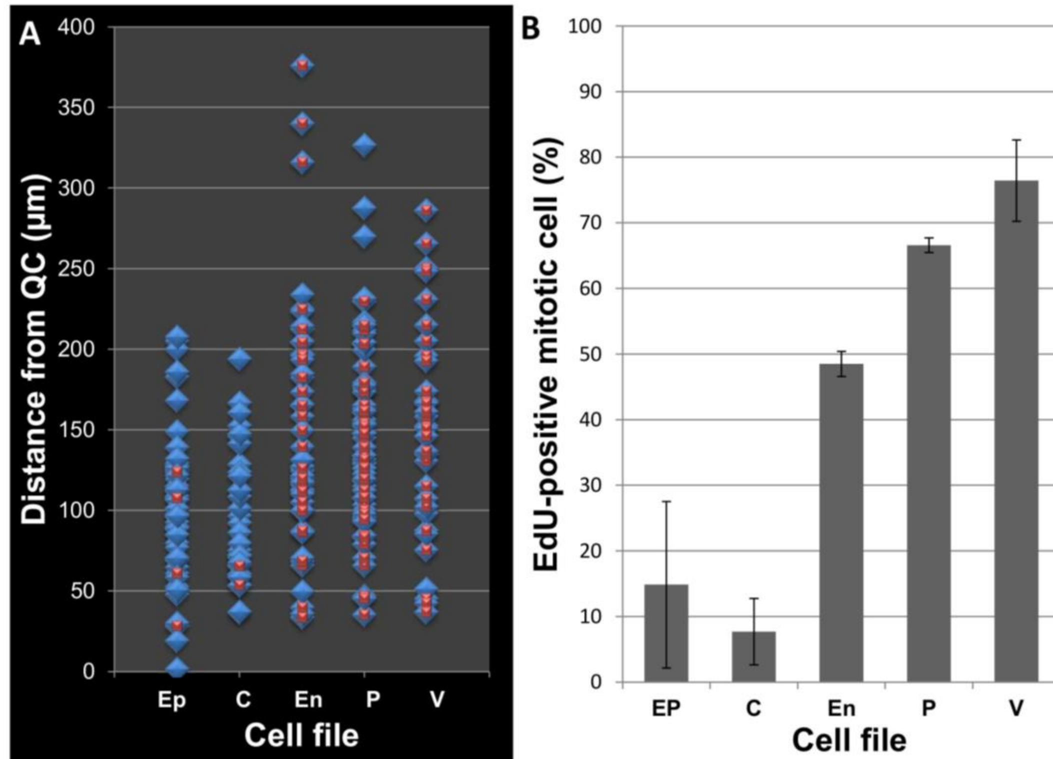

Figure 4: Vertical and horizontal gradients of G2 duration. (A) Mitosis distribution in different tissue layers in the tip of 4,5 day old Arabidopsis root. Seedlings were incubated in the presence of EdU for 180 minutes, subsequently colchicine was added for further 90 minutes. Seedlings were fixed immediately afterwards and subjected to analysis. Mitosis events only are depicted in blue; mitotic cells exhibiting EdU incorporation are marked blue plus red. (B) Average percent of EdU positive mitosis in different cell files. Ep – Epidermis, C – Cortex, En – Endodermis, P – Pericycle, V – Vasculature.

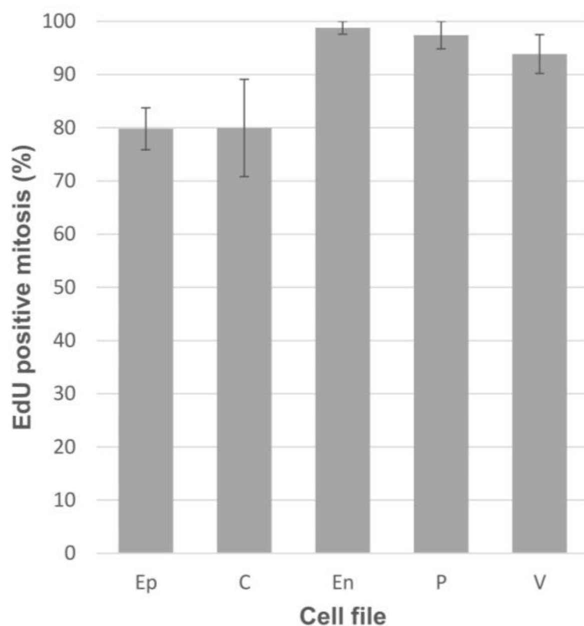

Figure S1: Average percent of EdU positive mitosis in different cell files. Five days old seedlings were incubated in the presence of EdU for 330 minutes, for the last 90 min

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colchicine was added. Seedlings were fixed immediately afterwards and subjected to analysis. Ep – Epidermis, C – Cortex, En – Endodermis, P – Pericycle, V – Vasculature.

Mitosis duration.

As an indirect estimation of mitosis duration (including all stages), we employed the value of the mitotic index, which in fact, reflects the relative duration of mitosis itself in relation to the whole cell cycle. We determined a mitotic index of 3-4% what means the mitosis duration is noticeably short (about 20 - 25 minutes) and itself has only a limited contribution to total cell cycle duration.

Determination of entire cell cycle duration.

To investigate the entire cell cycle duration, we increased EdU incubation time to 8 hours. After this incubation period, gradients of EdU-positive cell frequency from inner to the outer cell layers still exist. In the cortex cells, 85% EdU positive cell has been detected in the proliferation zone, while EdU-positive cells' rate in the pericycle reaches 98% (Figure S1 for graphical illustration). In consequence, entire cell cycle duration in the pericycle is less than 8 hours, while in the cortex and epidermis, the process lasts up to 9 - 9,5 hours. Such differences may relate to different chromatin landscapes and nuclei sizes and even related to nucleoli size, which is quite different in different cell files, even in trichoblast and atrichoblast cell files (Figure S6). Unfortunately, it is exceedingly difficult to estimate the exact duration in outer cell layers because, in these layers, the duration depends on the nuclei landscape (size) and chromatin status, which changes with QC's distance.

These data are in accordance with observed differences in G2 duration and reflect differences in whole cell cycle as well.

Interestingly, the EdU incorporation indices were significantly higher in the transition zone and reached 100% after about 5 hours of incubation (Figure S2). In consequence, we conclude that the duration of the endocycle is much shorter as the complete cell cycle in Arabidopsis.

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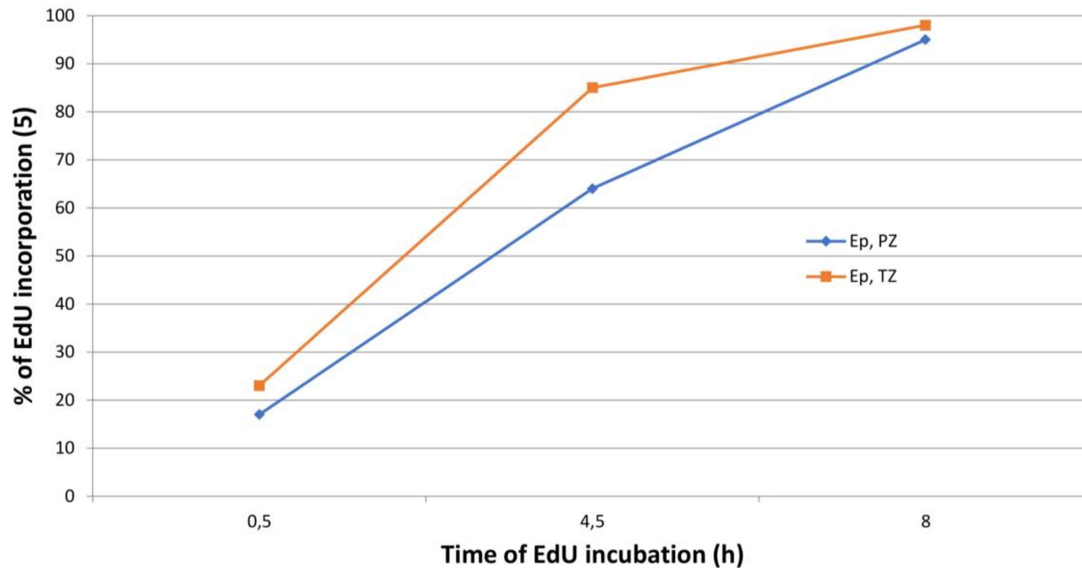

Figure S2. Ratio of EdU positive cell in the epidermis after 0.5; 4.5 and 8 h incubation in the presence of EdU. PZ- proliferation zone; TZ- transition zone (zone in which mitosis were not detected).

Divergence in cell cycle duration after formative cell divisions.

To further study potential differences in cell cycle duration in cells with shared origin but different fate, we have investigated the ratio of cell production after tangential (in epidermis) and periclinal (in endodermis) cell division what led to formation of daughter cells with different fates. In the epidermis tangential divisions lead to divergence of trichoblast and atrichoblast cells, while periclinal divisions in the endodermis generate the formation of middle cortex and endodermis cells (Baum et al., 2002).

Our results show that in both cases cell production exhibit different rates (Fig. 5), accompanied by differences in cell volume and orientation. Interestingly, divergence in cell production rates after "formative cell division" are increasing with increasing distance from the QC. For example, after periclinal cell division in the endodermis, we have observed a lower cell production (fewer cell numbers) in the more outer cell files for the same time period compared to the more inner ones. Additionally, EdU-positive mitosis was abundant more frequently (after shorter incubation time) in the cortex/epidermis cells with more proximal location (to QC), which means that the cells with less proximal location have a much longer G2 duration (Figure 3). In summary, these findings confirm that cell cycle duration can be dependent on cell fate and cell position.

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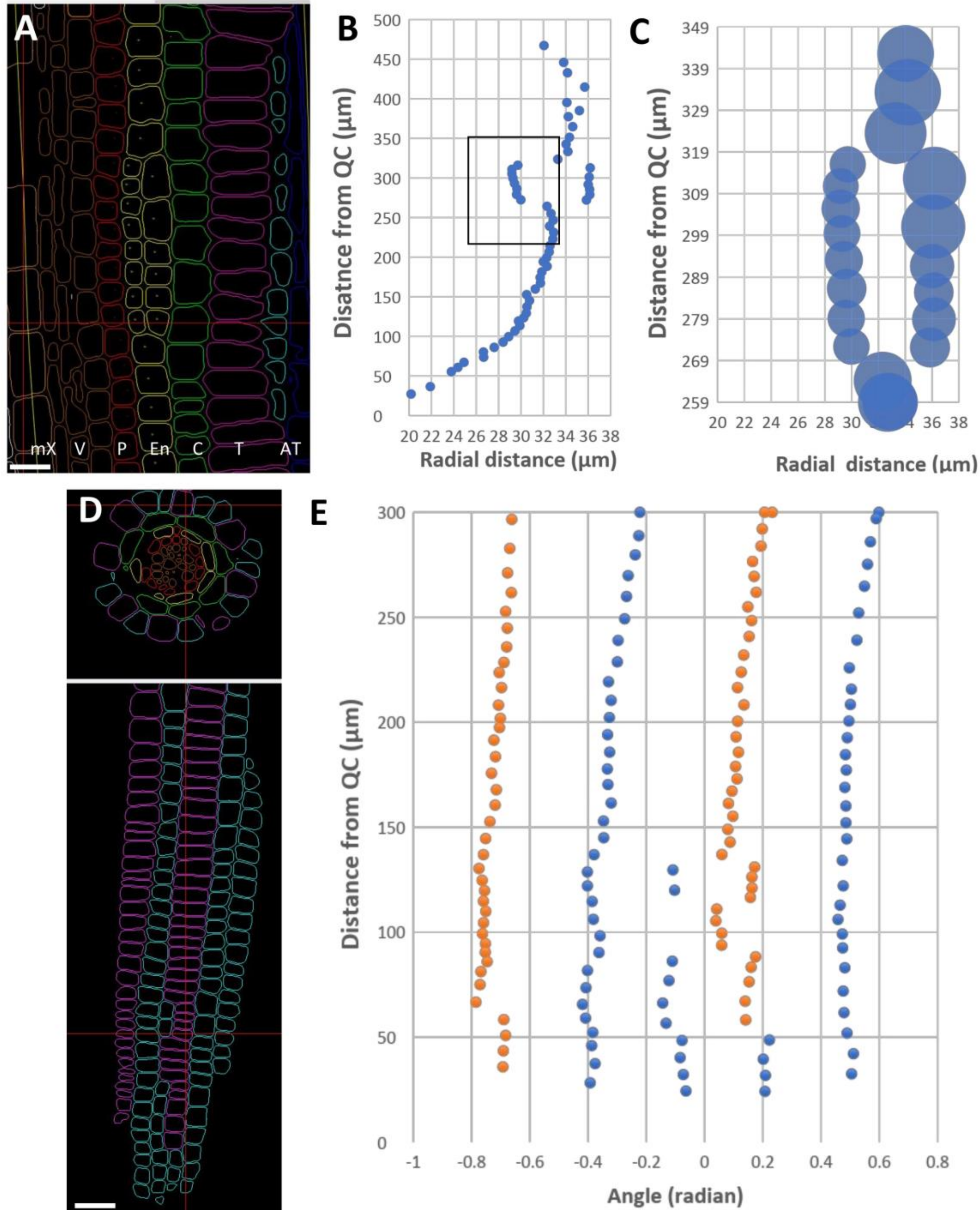

Figure 5. Differences in cell's production rate after tangential and periclinal cell divisions. Roots of 6-7 days old seedlings were fixed, labelled for cell border detection, and segmented to analyse the precise tissue structures by iRoCS (Schmidt et al., 2014). Regions of interest related to cell divisions in tangential (a, b, c; trichoblast, atrichoblast) and periclinal (d, e; middle cortex, endodermis) orientation are shown. Relative differences in cell volume are demonstrated by the size of bubbles (panel c).

Cell cycle duration in other species.

In order to test the suitability of the presented approach in other species, we investigate, while in much less detail, roots of the other species: tomato, tobacco, and wheat (Figures S3-S5).

For S-phase duration, we detected EdU incorporation with almost full DAPI co-localization after 20-35 minutes of incubation in these three species. EdU-positive mitotic plates were

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detected in tomato and tobacco after 240 minutes of incubation. Interestingly, in tobacco and tomato, no obvious differences in nucleus/chromatin organization between outer and inner cell layers were detected. In consequence, in these species, very similar G2 durations occur in all cell files analysed. Additionally, in tobacco and tomato, the duration of DNA replication and G2 phases are even shorter than Arabidopsis. Moreover, in the case of tomato, almost all cells became "EdU-positive" after 9-10 hours of incubation except in the stem cell niche where cells may be difficult to analysed due to enhanced density and attenuated stain accessibility (Figure S3).

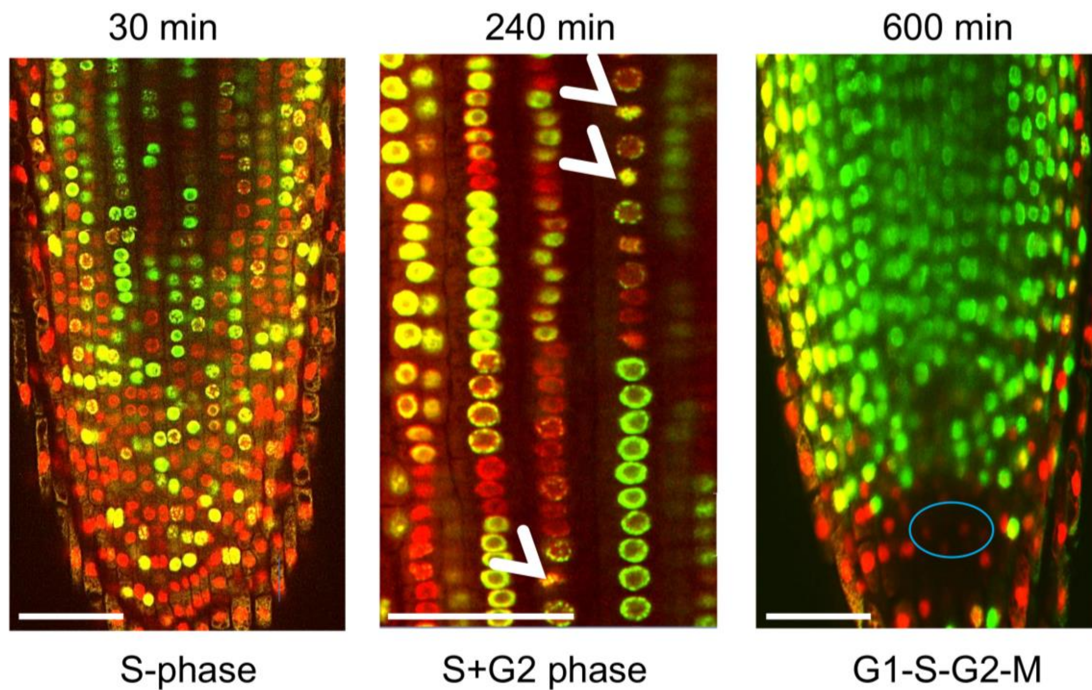

Figure S3. Cell cycle duration in tomato root. 4-5 days old seedlings were incubated with EdU for 30 min, 240 min, and 600 min. 30 minutes of incubation showed entire colocalization DAPI and EdU on cortex (2-3) focal plane; 240 min incubation led to the first appearance of EdU-positive mitosis (white arrows) and 600 min incubation to distinguish the stem cell niche. Nuclei are depicted in red; EdU positive nuclei are shown in green; the QC region (blue oval) is very dense and shows weak DAPI staining. Scale bar- 100 µm.

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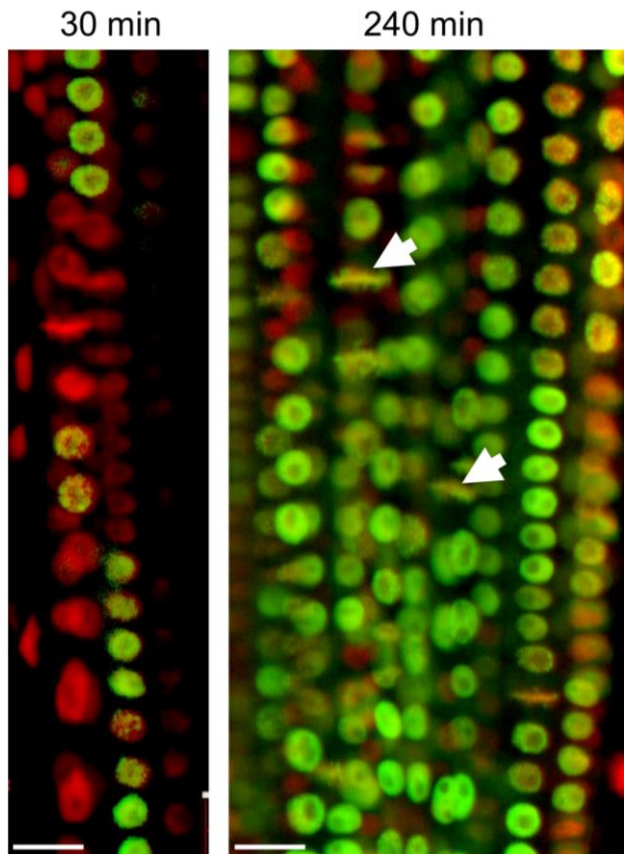

Figure S4: Cell cycle duration in tobacco. 4-5 days old seedlings were incubated with EdU for 30 min and 240 min. Arrow show selected mitotic plates. Nuclei are depicted in red; EdU positive nuclei are shown in green; Scale bar- 20 µm.

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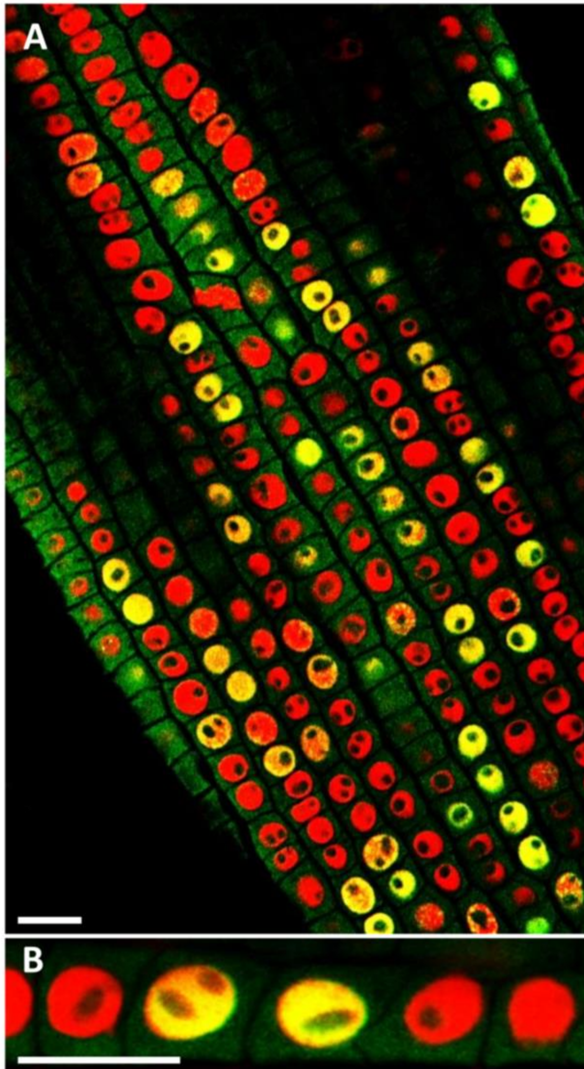

Figure S5: . EdU incorporation into wheat tissue. Four days old wheat seedlings were incubated with EdU for 35min. (A) – general view; (B) – close up view. Overlay images, DAPI stain of nuclei is red coloured, EdU signals are coded in green.Scale bar- 20 µm.

Discussion

The plant's organs' growth is a dynamic process that requires precise coordination between different cell types, individual cell position, rate of cell production, and cell expansion. Even such a simple organ as Arabidopsis root possesses significant divergence in cell geometry and chromatin status, which eventually may lead to differences in cell cycle kinetics in different files. However, in most investigations, this fact is somewhat ignored because of the lack of a simple protocol for studying the cell cycle's kinetics in each cell separately, but in the frame of whole organs. The differences in cell geometry as well as the different lengths of respective proliferation domains (Lavrekha et al., 2017), prompted us to study cell cycle characteristics in each cell file independently.

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Cell cycle duration may serve as a key regulator for coordinated root growth and may compensate e.g. cell elongation under changing temperature conditions (Yang, X. et al., 2017).

EdU-accumulation as a marker to estimate cell cycle duration.

First, we studied the distribution and timing of EdU incorporation as a marker for DNA replication events. To do this, we incubated Arabidopsis seedlings in the presence of EdU for different time periods (Figure 2) and performed an analysis of the distribution of EdU-incorporating cells in the root. After 20 min of incubation, EdU-positive cells were detected in the proliferation domain as well as the transition zone, the domain where cells start to slightly elongate and do not divide anymore (Pasternak et al., 2017; Lavrekha et al., 2017) but undergo endo-reduplication.

Longer incubation time (4 hours) allows us to detect EdU-positive mitotic cells, which means that cells undergo DNA replication, G2 stage, and enter in mitosis. The distribution of EdU-positive mitosis over EdU-incubation time allows us to quantify S+G2+M duration.

Next, after 5 hours of incubation, almost all cells which undergo elongation became EdU -positive means that all cells in this zone passed G1-S transition and the entire cycle duration is around five hours (figure S2, S6).

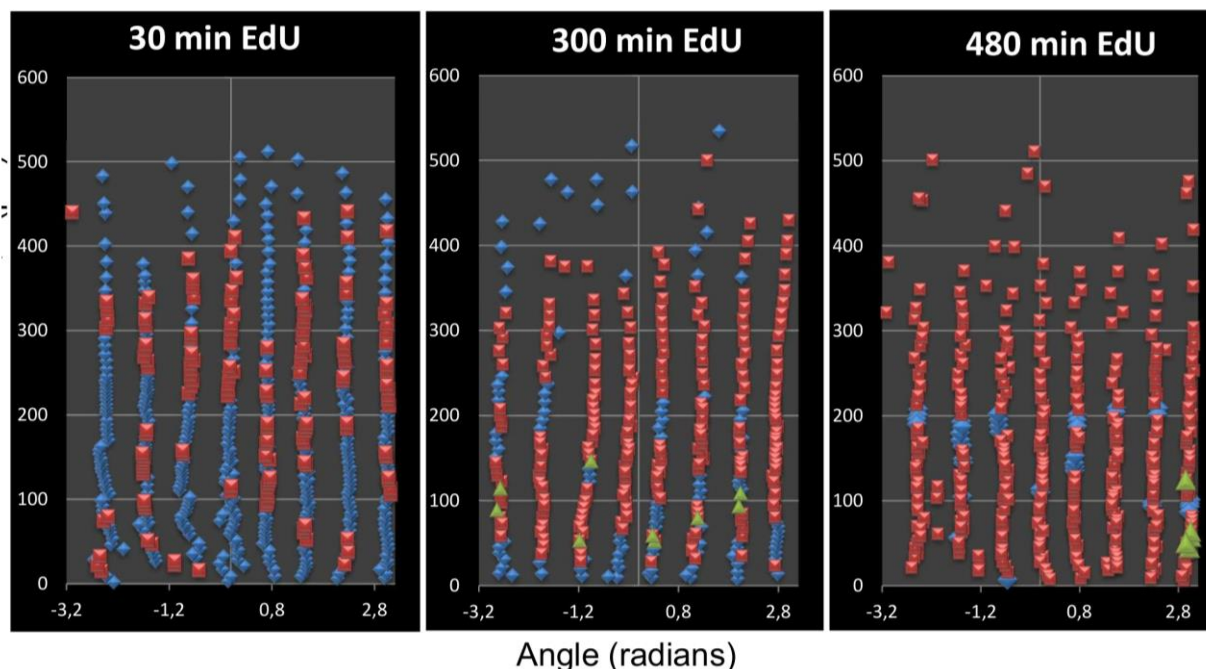

Figure S6. EdU incorporation into cortex cells after incubation for 0,5; 5 and 8h. Cells without EdU are depicted in blue, EdU-positive cells in red, and mitotic cells in green.

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Methods for determination of cell cycle duration.

Kinematic methods.

One of the most widely used is the "kinematic method," what is routinely used in several laboratories to investigate the effects of genetic, hormonal, developmental, and environmental factors on cell cycle duration in various organs (Baskin et al., 1995; Beemster and Baskin, 1998; Tardieu and Granier, 2000; Yang et al., 2017). This method used mainly cortex cells and based on several assumptions, such as the cell's constant size in the proliferation domain and constant cell cycle duration through RAM. However, there are a few points to consider regarding the accuracy of this method: 1.) Cortex cells do not have constant cell cycle duration as demonstrated by non-constant mitotic distribution (Lavrekha et al., 2017). 2.) Length of the cell proliferation zone in cortex cell and other cell files are not similar (Blein et al., 2018; Lavrekha et al., 2017). 3.) In this paper, we demonstrate that cell cycle duration can diverge in different cell files. Moreover, a detailed investigation of the "un-rolled" root maps showed that even different cortex cell lines in Arabidopsis exhibit un-even (non-symmetrical) distribution of cell cycle events with a highly heterogeneous cell cycle stage. Altogether we conclude from these results that kinematic methods should be used only with some quite precautions.

Duration of DNA replication.

Many methods for S-phase duration determination rely on investigations based on DNA replication visualized by markers (H^3-thymidine in the past; BrDU and EdU, currently). The intensity of incorporated EdU was used as a marker to calculate the duration of DNA replication (Mickelson-Young, L.et al., 2016; Pereira P. et al., 2017). These studies claim that DNA replication duration spans about 2.5-3 hours, and these calculations were based on an estimation of the time after which fluorescence intensity reached a maximum. However, there are several methodological aspects which may compromise such analysis and should be considered: 1.) Potentially, the substitution of growth media simultaneously to EdU addition, e.g., water or buffers, may significantly distort precise cell cycle progression without pre-adaptation. 2.) The pool of endogenous thymidine in the cells could be quite high. So, incorporation of externally applied EdU may represent only a few percent of a substrate during replication at the early period after addition compared with native thymidine. Therefore, thymidine continues to incorporate, and the relative EdU incorporation is increasing through time until an equilibrium of both competing compounds is achieved.

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Consequently, the transfer of plants to new mediums eventually could lead to overestimation of cell cycle duration, which has been estimated to last 17 h by Hayashi et al (2013). Similarly, the immediate medium exchange is proposed in the majority of published protocols (Van't Hof J. 1967;1969) what may lead to a temporary extension of the cell cycle itself. Consequently, in our protocol, we propose root adaptation to the new medium to prevent potential disturbances in the cell cycle.

An indirect method of estimating the duration of DNA replication is determining the number of cells in the S-phase evaluated by flow cytometry in not-synchronized suspension cells. It was found that the ratio of the cells undergo DNA replication is not exceeding 5-7% what directly means that the period of DNA replication does not exceed 5-7% of total cell cycle duration, ea. 30 minutes.

Usage of molecular marker lines for quantitative analysis.

Recently, molecular markers' usage became an extremely popular technique to investigate cell cycle dynamics in Arabidopsis (Yin K. et al., 2014). In some cases, such a method can more or less precisely reflect cell cycle kinetics. For example, cell cycle duration for cortex cells determined by Yin K. et al. (2014) is remarkably close to our results. However, there are 2 major pitfalls in this method related to this marker: 1) CyCB1.1::GFP may be detectable less accurate in inner cell layers like pericycle because of shorter G2 duration (Lavrekha et al., 2017). 2) CyCB1:1 reflected rather a G2 phase, but not mitosis itself. Besides, to our knowledge, markers for cell cycle events are available only for Arabidopsis. The introduction of such markers, e.g. into mutant lines, is a relatively time-consuming procedure that potentially also affects geno- and phenotypes. One of the latest investigations (Bhosale, R.et al., 2018) based on DNA stainability and analysis of molecular marker for DNA replication proposed an epidermis endocycle duration of only 30 minutes, which seems to be shorter as DNA replication time alone.

A new protocol was recently developed to detect the Arabidopsis root's cell cycle based on multicolor markers for each cell cycle stage (Desvoyes B. et al., 2020). It was somehow similar to Yin K et al. 2014. This protocol requires a living cell image scan, which may affect cell cycle progression (Tosheva, K. L., et al., 2020). Moreover, the Desvoyes et al (2020) method's serious disadvantage is lacking positional information (cell file, distance from QC, periclinal division). As you have seen from our results, cell cycle kinetics were dependent on cell fate and cell position and required precise 3D analysis.

Cell culture based methods.

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The majority of mechanisms involved in cell cycle regulation have been discovered employing cell cultures. This approach offers the advantage of sampling a nearly homogenous population of cells (Gould, 1984) but can't be used for real plants because of cell cycle kinetics is cell-position dependent. But what we can learn, it is a relative duration of each cell cycle phase. Flow cytometry data have shown that in rapidly cycling homogeneous cells populations, the ratio between G1:S:G2 is 50:10:40; what exactly means that S-phase represent 5-10% of cell cycle, while G1 is 20% longer as G2. This eventually led to the concept that certain cell cycle genes represent the main hub for regulating root development and misses, e.g., cell file gradients in chromatin organization or other critical regulatory mechanisms. These aspects have been pointed-out also by Desvoyes et al. (2014) who described the dependence of cell cycle duration on chromatin status as well as by Otero et al. (2016) describing more specifically cell cycle duration dependence on histone H3 status.

Positional cell cycle gradient.

Roots exhibit continuous gradients of hormones, ROS accumulation, or cells size along their axes. That's why considering positional information is very important for understanding the mechanisms of cell cycle regulation in the framework of a whole organ (Wolpert L., 1989).

Surprisingly, only a few studies quantified cell cycle duration in different cell files and zones despite early investigations mentioned such features (Clowes, 1961; Phillips Jr, H. L., & Torrey, J. G. (1972)). Recent work (Otero et al., 2016) demonstrated differences in G2 duration in different epidermis cells, depending on their distance to the QC. Differences in histone H3 dynamics explained this observation.

Our method allows performing a detailed analysis of all cell cycle events in each cell file independently and simultaneously. Previously, we demonstrated significant differences in cell geometry and sizes (Blein et al., 2018) among different cell layers what should eventually involve differences in cell cycle duration for harmonized organ growth. Here, we describe significant differences in cell cycle duration in cells with different fate. Namely, cell cycle duration is much slower in the cortex and epidermis, accompanied by a progressive slowdown of their cell cycle as a function of QC's distance. Such differences may serve to compensate faster increases in cell volume in outer cell layers (Figure 1; see Blein et al., 2018). Finally, determination of cell cycle duration after tangential or periclinal cell divisions (Figure 5) resulting in altered cell fates provides us direct evidence for dynamic regulation mechanisms of cell cycle duration.

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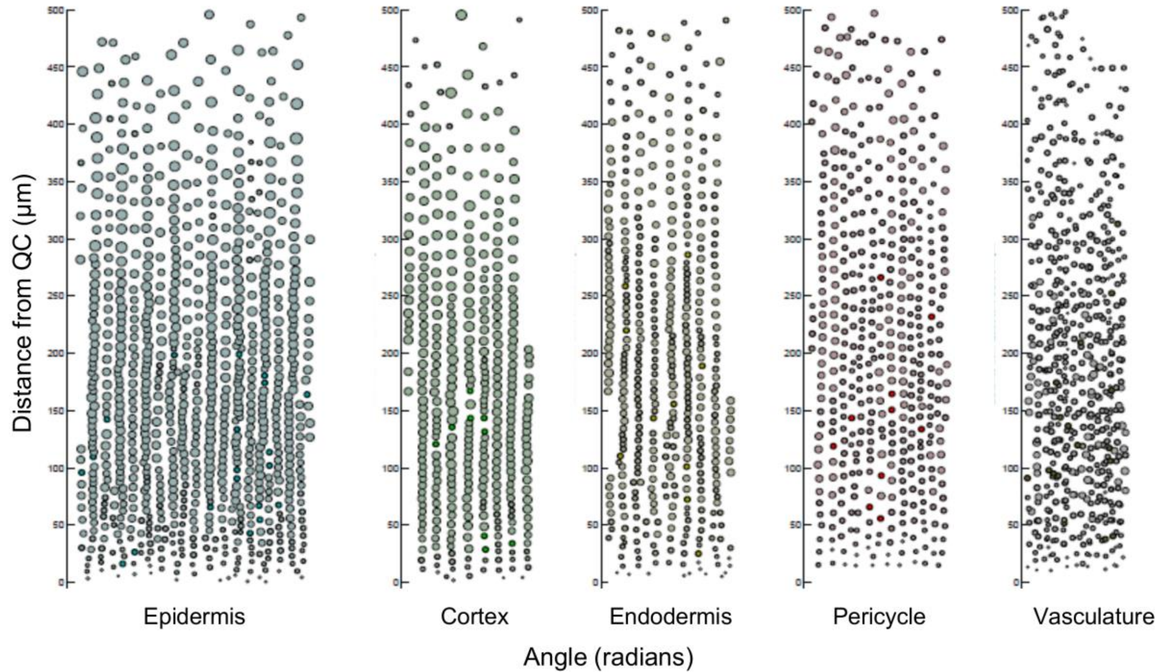

Figure S7. Distribution of the nucleoli volume along the axis in different cell files.

Volume of nucleoli in each cells of the root were extracted. Y-axis-distance from QC; X-axis-radians; Nucleoli volume were proportional to area of the circle.

Conclusions

Root growth is a dynamic process and occurs in a strictly coordinated manner. Consequently, dynamic changes in cell elongation, volume and cell proliferation of involved tissues must be strictly balanced. In Arabidopsis, significant differences in cell volume kinetics, the number of cells in distinct cell files, and their nuclear landscape were observed even in the proliferation zone (Figure 1, Figure S7). However, despite these differences, the Arabidopsis root keeps root integrity, which is partially driven by differences in cell production ratio (different cell cycle duration).

Here we propose a simple and marker-free method for rapid and simultaneous estimation of the cell cycle duration and all its stages in each cell file of plant roots. The method does not require significant mathematical calculation.

Materials and methods.

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heyhn. ecotype Col-0, *Nicotiana tabacum cv.* Samsun N.N., *Lycopersicum esculentum* L., and *Triticum aestivum* L. were used in experiments. Arabidopsis seeds sown on square Petri dishes containing TK1 medium (Pasternak et al., 2020). The plates were kept at room temperature for 4 h before being transferred to 4°C for 12 h. The plates were then transferred to 22°C under a 16 h / 8 h light/dark photoperiod with a light

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intensity of 80 μmol·m^{-2}·s^{-1} for 4.5 days. The seedlings were transferred to a 6-well plate containing liquid TK1 medium. After a 12-h incubation in similar conditions as on agar medium growth conditions, 2.5 mM colchicine and 10 µM EdU (5-ethynyl-2'-deoxyuridine) were added at defined time points (Figure 2) and seedlings from all vials were fixed 8 h after onset of first EdU addition. Seeds of the *Nicotiana tabacum* Samsun nn and *Lycopersicum esculentum* L. were surface-sterilized in 0.2% NaClO and sown on square Petri dishes containing T1 medium (supplemented with 2% sucrose) optimized for root growth (Pasternak *et al., 1999).* 4-5 days old seedlings have been used for labelling. Wheat seeds were soaked in water in Petri plates. 3-5 days old seedlings were transferred to liquid T1 medium for 16 h adaptation.

Pipeline for determination of cell cycle duration.

For determination of cell cycle duration 10 µM EdU have been added to the seedlings in the liquid medium 12, 14, 16, 18,5 and 19,65 hours after starting of adaptation for each plate separately. Seedlings have been fixed 20 h after onset of incubation. This timeline allows us to apply the following EdU-incubation time: 8 h; 6 h; 4,5 h; 1,5 h and 20 min. For determination of G2+S duration colchicine have been added to the plate after 180 and 270 min EdU incubation. These plants were fixed after further incubation for 90 minutes (see Fig.2 for experiment design).

Staining.

After EdU incubation plants were fixed in microtubule stabilizing buffer containing 4% formaldehyde (MTSB) and EdU incorporation was detected as described previously (Pasternak et al., 2015).

For volume determination, roots were fixed and labeled with the modified Truernit et al. (2009) method. Scanning and analysis procedures were done essentially as described in Schmidt et al., 2014.

Confocal imaging.

DAPI/EdU-stained samples were recorded using a confocal laser scanning microscope (LSM 510 META NLO; Carl Zeiss, Oberkochen, Germany) with a LD LCI-Plan-Apochromat 25x/0.8 DIC Imm Corr objective. For DAPI excitation, a Chameleon laser adjusted to 740 nm excitation was used and emission was detected with a band pass filter (BP 390-465 IR), Alternatively, all other suitable microscopes, including diode 405 nm laser can be used. Serial optical sections were reconstituted into 3D image stacks with in-plane (x-y) voxel extents of 0.15 µm and 0.9 µm section spacing (z). Three to five overlapping images (tiles) were recorded for each root.

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Image processing and analysis.

Images were converted to hdf5 format using the LOCI plugin for ImageJ (http://imagej.nih.gov/ij), then stitched together to obtain a root tip total length of 400 μm from the QC using xuvTools (Emmenlauer et al., 2009). 5-10 representative roots were chosen for detailed annotation. The DAPI and EdU channel images were processed with the iRoCS Toolbox (Schmidt et al., 2014) in the following way; nuclei were automatically detected using the "01-Detect Nuclei" plugin, then the epidermis was semi-automatically labelled using the "02- Label Epidermis" plugin. After the QC position was marked (Channel->New Annotation Channel), the nuclei were set in spherical coordinates using the "03-Attach iRoCS" plugin. Automatic classification of the nuclei to the corresponding cell types (epidermis, endodermis, cortex, pericycle, vasculature, root cap) was done using the "04-Assign Layers" plugin, which also enabled the automatic annotation of nuclei in mitotic state (option "Re-classify mitotic state"). All annotated roots were manually corrected for erroneous layer, mitosis, and EdU assignments.

Data analysis.

The data processed by iRoCS were exported to .csv files and analyzed with Microsoft Excel. Statistical analysis was done using Student's *t*-test. Roots were virtually divided to 50 μm sections. The DNA replication index (DRI) was calculated as the proportion of cells posing DNA replication during incubation time to all cells in the current section and was calculated for each 50 μm interval.

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Conflicts of Interest:

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Author Contributions: Conceptualization: TP, SK, KP; methodology: TP, Sample preparation, scanning, image processing: TP; resources: KP; data curation: TP and SK; writing—original draft preparation: TP; writing—review and editing: TP and SK. All authors have read and agreed to the published version of the manuscript.

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Table S1.Comparison of different methods for cell cycle duration estimation.

Method / Authors — Parameters extracted	H^3thymidine /colchicine Clowes 1961	H^3thymidine /colchicine Van't Hof 1967	EdU Hayashi et al.,2013	Kinematic Ivanov/Dybrovsky 1997	*In vivo* Method Yin et al., 2014	Stripflow Yang et al., 2017	„Plant cell cycle indicator“ Desvoyes et al., 2020	This paper
Cell type/subtype/position	yes	no	partially	only cortex	partially	only cortex	no	yes
Direct detection	yes	yes	yes	no	partially	no	yes	yes
Link to chromatin gradient	no	partially	no	no	yes	no	no	yes
Pre-adaptation	no	no	no	yes	yes	yes	yes	yes
Marker introduction to the mutant line	no	no	no	no	3-5 months	no	3-5 months	no
Cell cycle stages resolved	partially	partially	no	no	yes	no	yes	yes

Legends:

Cell type/subtype/position: information about cell type/subtype and cell position in organ.

Direct detection: detection of cell cycle stages in the living cells.

Link to chromatin status: link to histone methylation, nuclei structure in positional contents.

Marker introduction: time requires for the introduction of the marker to the mutant.

Table S2. Summary of cell cycle duration in Arabidopsis RAM.

Duration of the EdU incubation	Cell cycle stage	
20-30 min	DNA replication	Cell cycle/endocycle
3-5 hours	S-G2-M	
8-10 hours	S-G2-M-G1	Entire cycle duration
18-24 h	S-G2-M-G1	Status of the stem cell niche