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Global analysis of an exponential model of cell proliferation for estimation of cell cycle duration in the root apical meristem of angiosperms

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• **Background and Aims** Information on cell cycle duration (*T*) in the root apical meristem (RAM) provides insight into root growth, development and evolution. We have previously proposed a simple method for evaluating *T* based on the dynamics of root growth (*V*), the number of cells in the RAM (*Nm*) and the length of fully elongated cells (*l*), which we named the rate-of-cell-production (RCP) method. Here, a global analysis was performed to confirm the reliability of this method in a range of angiosperm species and to assess the advantages of this approach.
• **Methods** We measured *V*, *Nm* and *l* from live or fixed cleared primary roots of seedlings or adventitious roots of bulbs and used this information to estimate the average *T* values in 73 angiosperm species via the RCP method. The results were then compared with published data obtained using the classical but laborious and time-consuming ^{3}H-thymidine method.
• **Key Results** In most species examined, the *T* values obtained by the RCP method were nearly identical to those obtained by the ^{3}H-thymidine method.
• **Conclusions** The global analysis demonstrated that the relationship between the variables *V*, *Nm* and *l* in roots in the steady state of growth is correctly described by the equation $T = (\ln 2\ Nm\ l)V^{-1}$. Thus, the RCP method enables cell cycle duration in the RAM to be rapidly and accurately determined. This method can be performed using live or fixed roots for each individual cell type. The simplicity of the approach suggests that it will be widely used in phenomics, evolutionary ecology and other plant biology studies.

Key words: Angiosperms, cell cycle, cell cycle duration, cell proliferation, longitudinal zonation pattern, root phenotyping, root apical meristem, root development, root growth.

INTRODUCTION

To fulfil their functions, roots must grow continuously throughout the plant's life cycle. This is possible due to root apical meristem (RAM) activity and subsequent rapid cell elongation. The RAM is the source of all cells from which the root is built. Therefore, cell patterning in the RAM and the dynamics of cell division determine the architecture of the root system and are thus of paramount importance for the plant. Understanding these processes was one of the main interests of the late Peter W. Barlow. He and the senior author of this article proposed two exponential models for cell multiplication in the RAM that are essential for understanding root growth and RAM maintenance (Ivanov, 1974; Barlow, 1976*b*; Shishkova *et al.*, 2008). Many aspects of root meristem patterning and organization were studied by Barlow, including the quiescent centre of the RAM and its stem cell properties (Barlow, 1973, 1976*c*, 1987, 1994, 1997, 2015*a*, *b*, 2016; Dubrovsky and Barlow, 2015), cell cycle duration within the RAM (Barlow and MacDonald, 1973; Francis and Barlow, 1988; Barlow and Woodiwiss, 1992), the role of plant hormones in cell proliferation in the RAM (Barlow, 1976*a*, 1992; Barlow and Pilet, 1981, 1984; Barlow *et al.*, 1991; Müller *et al.*, 1993, 1994; Ponce *et al.*, 2005), and the relationship between cell cycle duration (*T*) and haploid DNA content (Francis *et al.*, 2008).

The root is an exceptionally convenient system for studying cell proliferation due to the relative simplicity of its structure, the clear distinction between the RAM and the elongation zone, and the ease of treating roots with various compounds. Howard and Pelc (1953) were the first to introduce the cell cycle concept as we know it today. The authors proposed a fundamentally new approach for studying cell proliferation based on short-term (pulse) labelling of cells with a radioactive DNA precursor (^{32}P at that time) and subsequent analysis of the labelled cells. The authors used *Vicia faba* roots, an appropriate system for obtaining numerous labelled cells (see Dubrovsky and Ivanov, 2003). Pulse labelling has since been performed in many studies based on this approach. Quastler and Sherman (1959), who analysed cell population kinetics in the mouse intestinal epithelium, further improved the method used to determine the duration of the cell cycle and its phases. By applying certain assumptions about cell behaviour and examining the relative proportions of labelled (mitotic) and unlabelled (interphase) cells after administering a DNA precursor, tritiated thymidine, the authors developed a graphical method for estimating the duration of the cell cycle and its phases. This approach became

popular for analysing plant roots. Short-term incubation of roots in a nutrient solution supplemented with ^{3}H-thymidine and their subsequent fixation at different time intervals after transfer into a solution without ^{3}H-thymidine allows the percentage of labelled cells undergoing mitosis to be estimated. The labelled mitoses values are then plotted against time, and the resulting curves can be used to determine the duration of the cell cycle and its periods. This method, known as the labelled mitoses or ^{3}H-thymidine (hereafter thymidine) method, has several limitations, as analysed by Baskin (2000). One of the disadvantages is the potential effect of radioactivity on the cell cycle itself (Torre and Clowes, 1974). Furthermore, the method is laborious, prompting Francis *et al.* (2008) to acknowledge the researchers 'who strove through sleepless nights to obtain the cell cycle times'. Nonetheless, the thymidine method has been widely used in both animal and plant studies, and has yielded much data. Furthermore, some other methods have been developed to determine cell cycle duration, including the colchicine (Van't Hof *et al.*, 1960; Van't Hof and Sparrow, 1963) and caffeine (Giménez-Martín *et al.*, 1965) methods. However, these approaches are not always practical. Overall, cell cycle duration has been determined in 170 plant species from 53 genera belonging to 38 families (Grif *et al.*, 2002). However, in recent years, this method has fallen out of favour. During the period of its greatest popularity (1970–1975), 107 papers were published in which this method was implemented, whereas in 1995–2000, only nine such studies were published (Grif *et al.*, 2002). In the past decade, this method has almost never been used.

More recently, a kinematic approach was developed based on analyses of the velocities of cell displacement or cell flux (Sharp *et al.*, 1988; Silk *et al.*, 1989; Beemster and Baskin, 1998; van der Weele *et al.*, 2003; Fiorani and Beemster, 2006; Yang *et al.*, 2017). Among other approaches (e.g. Cools *et al.*, 2010), the DNA precursors bromodeoxyuridine and 5-ethynyl-2′, deoxyuridine, sometimes in combination with flow cytometry, have occasionally been used instead of thymidine for determination of *T* in the RAM (Moretti *et al.*, 1992; Lucretti *et al.*, 1999; Hayashi *et al.*, 2013).

Ivanov (1968) proposed a simple method for *T* estimation based on the organization of the RAM and root growth. This method relies on data including the number of meristematic cells in files, the length of the cells that have completed their growth and root growth rate. The rationale behind this method, the assumptions and a proposed model of the relationship between the rate of root growth and cell production have been described in detail (Ivanov and Dubrovsky, 1997). Because this method is based on the analysis of cell production over time, it is referred to as the rate-of-cell-production (RCP) method. The cell cycle duration values obtained with the RCP and thymidine methods have been shown to match using a few examples (Ivanov and Dubrovsky, 1997). This method has been repeatedly performed using various species: maize, *Zea mays* (Ivanov, 1994), wheat, *Triticum aestivum* (Demchenko, 1976), Cactaceae (Dubrovsky *et al.*, 1998) and *Arabidopsis thaliana* (Dubrovsky *et al.*, 2000; Tapia-López *et al.*, 2008; Garay-Arroyo *et al.*, 2013; López-Bucio *et al.*, 2014; Napsucialy-Mendivil *et al.*, 2014). However, no global analysis of the RCP method has thus far been performed. Therefore, in the current study, we determined *T* in 73 angiosperm species using the RCP method and compared the results with published data obtained using the thymidine method. The results of this study confirm that the RCP method is a simple, rapid and accurate approach for determining *T* in roots.

MATERIAL AND METHODS

Species analysed and growth conditions

The roots of 73 species (Tables 1 and 2) were analysed. For 68 of these species, the primary roots of seedlings were examined and for five species, the adventitious roots obtained from bulbs were examined. Seeds from many of the species were obtained from the Vavilov Research Institute of Plant Industry in Saint Petersburg, Russia (VIR), and from other research centres mentioned in the Acknowledgments. The respective varieties or lines are marked 'VIR' in Table 3, along with a number corresponding to their collection. Seeds were germinated in Petri dishes maintained in darkness on filter paper moistened with purified and filtered tap water. The bulbs were grown in dark glass bottles filled with tap water under natural illumination. The temperature during the experiments was between 20 and 25 °C. Root length was measured using a ruler with an accuracy of 1 mm. After germination, root growth increased daily. When the roots started to grow at a constant rate, root tips (1–1.5 cm in length) were excised and fixed in 70 % ethanol. Prior to fixation, thicker roots were cut lengthwise with a razor blade. Immediately before analysis, thin roots were rinsed three times (5 min each) in distilled water, transferred onto a microscope slide in 50 % glycerol, and covered with a coverslip. Thicker roots or longitudinally halved root tips with denser layers of meristematic cells were cleared using the protocol of Malamy and Benfey (1997) and mounted in 50 % glycerol. For the roots of each species, the length of the RAM (*Lm*) was measured as the length from the distal boundary of the RAM to the point where a sharp increase in cell length began.

Principles of the RCP method and practical considerations

The RCP method has been described in detail (Ivanov and Dubrovsky, 1997). Here, we briefly outline the principles of this approach. It is based on the simplest model of cell proliferation in the RAM under the following assumptions: (1) the average cell cycle duration (*T*) for all meristematic cells is the same; (2) all meristematic cells proliferate; (3) the number of cells in a meristem (or in a cell file within the meristem) is constant; and (4) the flux of cells into and out of the non-proliferating elongation zone is the same (Ivanov and Dubrovsky, 1997). Numerous studies confirm that these assumptions are valid for roots growing at a constant rate (Baskin, 2000; Fiorani and Beemster, 2006; Yang *et al.*, 2017).

In the vast majority of wild-type (non-mutant) plants of various species, the RAM cells of the growing root are in an active proliferation state. The average *T* is constant along the meristem above the quiescent centre (Balodis and Ivanov, 1970; Barlow and MacDonald, 1973; Clowes, 1976; Baskin, 2000). Detailed analysis indicates that there is no reason to assume that several cell populations exist in the meristem that differ in *T* (Ivanov, 1974, 1987, 1994; Webster and MacLeod, 1980),

TABLE 1. *Cell cycle duration (*T*) determined by the rate-of-cell-production (RCP) method, published data obtained by the ^{3}H-thymidine (thym) method and their comparison*

Species	*T* by RCP method (h)	*T* by thymidine method (h)	Reference	Difference (h)	Difference (%)
Aegilops squarrosa auct. (tauschii Coss.)	11.2 ± 1.0	11.4	Davies and Rees (1975)	−0.2	−1.8
Aegilops umbellulata Zhuk.	12.9 ± 1.2	10.7	Kidd *et al.* (1987)	2.2	17.1
Agoseris heterophylla (Nutt.) Greene	10.9 ± 1.2	8.8	Price and Bachmann (1976)	2.1	19.3
Agoseris retrorsa (Benth.) Greene	11.8 ± 1.0	9.0	Price and Bachmann (1976)	2.8	23.7
Allium carinatum L.	15.1 ± 1.2	9.2	Bösen and Nagl (1978)	5.9	39.1
Allium sativum L.	22.7 ± 2.8	21.6 ± 1.8	See Table 2	1.1	4.8
Allium tuberosum Rottler ex Spreng.	20.7 ± 3.0	20.6	Van't Hof (1965), Matagne (1968), Bryant (1969)	0.1	0.5
Allium cepa L.	15.5 ± 1.6	16.0 ± 1.6	See Table 2	−0.5	−3.2
Allum porrum L.	23.0 ± 2.5	18.0 ± 1.2	See Table 2	5.0	21.7
Anacyclus radiatus L.	11.6 ± 1.5	14.0	Nagl (1974, 1978)	−2.4	−20.7
Anthemis austriaca L.	8.0 ± 1.2	7.0	Nagl (1974, 1978)	1.0	12.5
Anthemis cota L.	8.2 ± 1.0	6.0	Nagl (1974, 1978)	2.2	26.8
Anthemis tinctoria (L.) J. Gay ex Guss.	12.0 ± 1.2	12.0	Nagl (1974, 1978)	0.0	0.0
Artemisia absinthum L.	9.3 ± 1.3	9.5	Nagl (1974, 1978)	−0.2	−2.2
Artemisia annua L.	10.7 ± 1.0	8.0	Nagl (1974, 1978)	2.7	25.2
Avena pilosa (Roem. & Schult.) Bieb.	9.2 ± 1.7	8.9	Yang and Dodson (1970)	0.3	3.3
Avena strigosa Schreb	8.7 ± 1.4	9.8 ± 0.3	Yang and Dodson (1970)	−1.1	−12.6
Beta vulgaris L.	12.7 ± 1.4	16.0	Titsu and Popovici (1970)	−3.3	−26.0
Brassica junceae (L.) Czern.	12.5 ± 1.2	12.0	Srivastava and Levania (1978)	0.5	4.0
Coriandrum sativum L.	13.7 ± 3.1	13.0	Olszewska *et al.* (1990)	0.7	5.1
Crepis capillaris L.	10.3 ± 1.3	11.0 ± 1.0	See Table 2	−0.7	−6.8
Crepis tectorum L.	12.4 ± 2.6	12.0	Langridge *et al.* (1970)	0.4	3.2
Cucurbita pepo L.	12.8 ± 3.0	18.0	Marciniak *et al.* (1978)	−5.2	−40.6
Dactylis glomerata L.	9.6 ± 1.5	12.1 ± 1.8	See Table 2	−2.5	−26.0
Daucus carota L.	8.0 ± 2.2	8.0	Bayliss (1975)	0.0	0.0
Epilobium hirsutum L.	14.0 ± 1.6	7.0	Thomas (1992)	7.0	50.0
Eragrostis tef (Zuccagni) Troffer	11.1 ± 2.1	9.7	Kidd *et al.* (1987)	1.4	12.6
Fagopyrum esculentum Moench.	7.1 ± 1.0	6.0	Seyhodjaev (1971)	1.1	15.5
Festuca rubra L.	14.7 ± 1.8	16.2 ± 0.2	See Table 2	−1.5	−10.2
Glycine max (L.) Merr.	13.0 ± 1.2	8.6 ± 1.1	See Table 2	4.4	33.8
Helianthus annuus L.	12.2 ± 1.5	12.0 ± 1.2	See Table 2	0.2	1.6
Hordeum bulbosum L.	12.7 ± 2.2	14.0	Kidd *et al.* (1987)	−1.3	−10.2
Hordeum vulgare L.	10.5 ± 0.6	12.5 ± 0.3	See Table 2	−2.0	−19.0
Hyacinthus orientalis L.	33.0 ± 3.5	24.0	Evans and Rees (1971)	9.0	27.3
Impatiens balsamina L.	10.2 ± 1.4	9.0	Van't Hof (1965)	1.2	11.8
Lactuca sativa L.	12.0 ± 1.3	10.0	Mazzuka *et al.* (2000)	2.0	16.7
Lathyrus articulatus L.	18.3 ± 1.3	14.3	Evans and Rees (1971)	4.0	21.9
Lathyrus latifolius L.	16.4 ± 0.9	24.0	Olszewska *et al.* (1990)	−7.6	−46.3
Lathyrus odoratus L.	23.3 ± 2.5	20.0	Olszewska *et al.* (1990)	3.3	14.5
Lathyrus tingitanus L.	18.5 ± 2.4	16.8	Evans *et al.* (1972)	1.7	9.2
Lilium longiflorum Thunb.	51.0 ± 5.9	24.0	Kidd *et al.* (1987)	27.0	52.9
Linum usitatissimum L.	13.7 ± 1.2	14.0	Evans *et al.* (1972)	−0.3	−2.2
Lolium perenne L.	9.2 ± 0.9	8.1	Evans *et al.* (1972)	1.1	12.0
Luzula purpurea Lowe	21.0 ± 3.6	22.0	Montezuma-de-Carvalho (1962)	−1.0	−4.8
Lycopersicum esculentum L. ssp. Cultum	9.7 ± 1.2	13.0	Van't Hof (1965), Titsu (1967)	−3.3	−34.0
Melandrium album (Mill.) Garcke	15.1 ± 1.2	15.5	Choudhury (1969)	−0.4	−2.6
Nicotiana plumbaginifolia Viv.	12.8 ± 1.5	11.0	Gupta (1969)	1.8	14.1
Nicotiana tabacum L.	11.2 ± 1.8	9.0	Gupta (1969)	2.2	19.6
Nigella damascena L.	14.0 ± 1.7	16.5	Evans *et al.* (1972)	−2.5	−17.9
Ornithogalum umbellatum L.	49.3 ± 8.9	14.0	Tagliasacchi *et al.* (1983)	35.3	71.6
Oryza sativa L.	7.9 ± 0.8	10.8	Kidd *et al.* (1987)	−2.9	−36.7
Papaver nudicale L.	12.7 ± 2.2	10.0	Olszewska *et al.* (1990)	2.7	21.3
Papaver orientale L.	12.0 ± 1.6	16.0	Olszewska *et al.* (1990)	−4.0	−33.3
Papaver somniferum L.	10.8 ± 1.2	12.0	Olszewska *et al.* (1990)	−1.2	−11.1
Pennisetum americanum (L.) Leeke	11.3 ± 0.8	12.4	Kidd *et al.* (1987)	−1.1	−9.7
Phalaris canariensis L.	14.6 ± 1.0	14.5	Prasad and Godward (1965)	0.1	0.7
Pisum sativum L.	11.7 ± 0.7	15.3 ± 1.0	See Table 2	−3.6	−30.8
Pyrrhopappus caroliniana L.	12.7 ± 3.1	12.0	Price and Bachmann (1976)	0.7	5.5
Rumex thyrsiflorus Fingerh.	17.8 ± 1.7	16.0	Zuk (1969)	1.8	10.1
Scilla sibirica Andrews	77.0 ± 10.5	67.0	Baumann (1972)	10.0	13.0
Secale cereale L.	12.7 ± 1.1	14.0 ± 1.3	See Table 2	−1.3	−10.2
Sorghum bicolor (L.) Moench	17.0 ± 1.1	13.9	Kidd *et al.* (1987)	3.1	18.2
Triticosecale Wittm. & A.Camus	13.0 ± 1.1	11.7 ± 0.4	Kaltsikes (1971), Kidd *et al.* (1987)	1.3	10.0
Triticum aestivum L.	11.6 ± 0.9	14.3 ± 1.2	See Table 2	−2.7	−23.3

TABLE 1. *Continued*

Species	*T* by RCP method (h)	*T* by thymidine method (h)	Reference	Difference (h)	Difference (%)
Triticum dicoccoides (Körn. ex Asch. & Graebn.) Schweinf.	11.1 ± 0.8	12.7	Davies and Rees (1975)	−1.6	−14.4
Triticum monococcum L.	13.7 ± 1.0	12.0	Davies and Rees (1975)	1.7	12.4
Triticum spelta L.	19.0 ± 2.9	19.7	Davies and Rees (1975)	−0.7	−3.7
Triticum timopheevi (Zhuk.) Zhuk.	14.5 ± 1.2	15.0	Davies and Rees (1975)	−0.5	−3.4
Triticum turgidum (durum) Desf.	13.7 ± 1.1	12.3 ± 0.9	Kaltsikes (1971)	1.4	10.2
Tropaeolum majus L.	25.5 ± 1.7	8.0	Olszewska *et al.* (1990)	17.5	68.6
Vicia faba L.	12.8 ± 1.1	16.3 ± 1.3	See Table 2	−3.5	−27.3
Vicia sativa L.	13.4 ± 1.2	13.2 ± 0.8	See Table 2	0.2	1.5
Zea mays L.	12.0 ± 0.7	12.5 ± 0.2	See Table 2	−0.5	−4.2

Dif, difference between the two methods in hours and relative terms. Data are means ± s.e.

although *T* may fluctuate through consecutive divisions. The values of *T* for individual cells vary within a range of approximately 15–20 % (Ivanov, 1971).

The decrease in the mitotic index in the basal half of the meristem is caused by a gradual exit of cells from the cell cycle and not by an increase in *T* (Balodis and Ivanov, 1970). The only exception is developing metaxylem cell files in some monocots, in which mitoses terminate much more closely to the root apex than in other tissues, but these cells comprise only a small fraction of all cells in the meristem. Note that at the boundary of the meristem and the elongation zone, there is the transition domain of the RAM in which

TABLE 2. *Comparison of cell cycle duration values in the root apical meristem obtained by the thymidine method for the same species reported in different studies (means ± s.e.)*

	Number of data points	Average cell cycle time, *T* (h)	Max. value (h)	Min. value (h)	References
Allium cepa L. (seeds)	3	16.0 ± 1.6	17.8	12.8	Bryant, 1969; Evans and Rees, 1971; Van't Hof, 1965
Allium cepa L. (bulbs)	8	16.6 ± 1.3	23.0	13.5	Antosiewicz, 1990; Arcara and Nuti, 1967; Gimenez-Abian *et al*, 1987; González-Fernández *et al.*, 1971; Matagne, 1968; Morcillo *et al.*, 1978; Navarrete *et al.*, 1983.
Allium porrum L.	3	18 ± 1.2	20.0	16.0	Berta *et al.*, 1991; Olszewska *et al.*, 1990.
Allium sativum L.	3	21.6 ± 1.8	23.8	18.0	Deysson and Bonaly, 1970; Benbadis, 1970; Pareyre and Deysson, 1975.
Crepis capillaris L.	2	11.1 ± 1.0	12.0	10.1	Generalova, 1969; Kaznadzei, 1971; Van't Hof, 1965.
Dactylis glomerata L.	7	12.1 ± 1.8	21.0	7.0	Creber *et al.*, 1993.
Festuca rubra L.	2	16.2 ± 0.2	16.4	16.0	Powell *et al.*, 1986.
Glycine max (L.) Merr.	2	8.6 ± 1.1	10.7	7.0	Olszewska *et al.*, 1990; Reckless, 1995.
Helianthus annuus L.	4	12.0 ± 1.2	14.0	10.0	Marciniak *et al.*, 1978; Burholt and Van't Hof, 1971; Todorova and Ronchi, 1969.
Hordeum vulgare L.	3	12.5 ± 0.3	13.0	12.0	Kidd *et al.*, 1987; Svarinskaya and Gavrilova, 1976; Bennett and Finch, 1972.
Pisum sativum L.	10	15.3 ± 1.0	22.4	12.0	Bogdanov, 1967; Bogdanov *et al.*, 1967; Gudkov and Grodzinsky, 1972, 1976; Gudkov *et al.*, 1971, 1974; Olszewska *et al.*, 1990; Van't Hof, 1963, 1966.
Secale cereale L.	6	14.0 ± 1.3	20.0	12.0	Evans and Rees, 1971; Grif and Valovich, 1973*a*, *b*; Kaltsikes, 1971; Kidd *et al.*, 1987; Olszewska *et al.*, 1990; O'Toole, 1970.
Tradescanthia paludosa E.S.Anderson & Woodson	4	19.4 ± 0.8	20.5	17.0	Van't Hof, 1965; Van't Hof and Sparrow, 1963; Wimber, 1960, 1966; Wimber and Quastler, 1963.
Triticocereale Wittm. & A.Camus	3	11.7 ± 0.4	12.1	11.0	Kaltsikes, 1971, 1972; Kidd *et al.*, 1987.
Triticum aestivum L.	6	14.3 ± 1.2	19.7	12.0	Davies and Rees, 1975; Evans and Van't Hof, 1975; Filippenko, 1983; Grif, 1981; Grif and Machs, 1996; Gudkov and Grodzinsky, 1976.
Vicia faba L.	10	17.1 ± 1.3	28.0	13.5	Dewey and Howard, 1963; Evans and Rees, 1971; Evans and Savage, 1963; Evans and Scott, 1964; Gahan *et al.*, 1986; Ganassi, 1978; Grant and Heslot, 1965; Gudkov *et al.*, 1971; Keusch, 1971; MacLeod, 1968, 1971; Olszewska *et al.*, 1989; Webster and Davidson, 1968.
Vicia sativa L.	2	13.2 ± 0.8	15.0	11.0	Essad, 1973; Olszewska *et al.*, 1990.
Zea mays L.	14	12.5 ± 0.2	13.0	11.9	Barlow, 1976*a*; Essad and Maunoury, 1979; Evans *et al.*, 1972; Gahan and Hurst, 1976; Kidd *et al.*, 1987; Olszewska *et al.*, 1990; Pachter and Mitra, 1977; Verma and Lin, 1978, 1979; Verma, 1980.

Table 3. *Cell cycle duration (*T*) in the root apical meristem of different varieties of the same species obtained by the RCP method (*n = *8, means ± s.e.)*

Species and variety or cultivar	Cell cycle duration, *T* (h)
Pisum sativum L. 'Premium'	17.2 ± 1.9
Pisum sativum L. 'Miracle'	15.6 ± 2.0
Pisum sativum L. 'Ramensky'	10.0 ± 0.7
Pisum sativum L. 'Pioneer'	14.4 ± 1.3; 16.5 ± 1.4
Pisum sativum L. 'Alpha'	10.7 ± 0.7; 12.7 ± 0.7
Pisum sativum L. VIR No. 2227	13.8 ± 0.8
Pisum sativum L. VIR No. 6892	16.6 ± 1.0
Pisum sativum L. VIR No. 993	15.9 ± 0.8
Allium porrum L. VIR No. 2078	26.0 ± 2.6
Allium porrum L. VIR No. 2389	23.0 ± 2.5
Triticum dicoccoides (Körn.ex Asch. & Graebn.) Schweinf. VIR No. 61842	11.1 ± 0.8
Triticum dicoccoides (Körn.ex Asch. & Graebn.) Schweinf. VIR No. 61833	9.9 ± 1.0
Hordeum bulbosum L. VIR No. 250	12.4 ± 2.2
Hordeum bulbosum L. VIR No. 613	10.0 ± 1.1
Daucus carota L. 'Long red'	8.0 ± 2.2
Daucus carota L. VIR No. 4	12.0 ± 1.1; 1.0 ± 1.4
Coriandrum sativum L. VIR No. 360	13.7 ± 3.1
Coriandrum sativum L. VIR No. 420	16.0 ± 2.5
Cucurbita pepo L. 'Aeronaut'	12.8 ± 3.0
Cucurbita pepo L. VIR No. 4800	16.7 ± 1.0
Cucurbita pepo L. 'Gribovsky early'	19.5 ± 3.0
Glycine max (L.) Merr. 'Killer whale'	10.1 ± 1.2
Glycine max (L.) Merr. 'Flyer'	18.0 ± 1.6
Zea mays L. 'Interkras'	9.6 ± 0.7; 14.4 ± 1.4
Zea mays VIR No. 1329	9.5 ± 0.3
Zea mays VIR No. 6634	9.2 ± 0.8
Zea mays VIR No. 6343	11.8 ± 1.2
Zea mays VIR No. 18399	12.3 ± 0.8
Zea mays L. VIR No. 14a	5.6 ± 0.4; 7.3 ± 0.5
Zea mays L. VIR No. 15b	7.2 ± 0.6; 7.1 ± 0.4
Zea mays L. VIR No. 23427	10.4 ± 1.0
Zea mays L. VIR No. 23427	8.8 ± 0.9
Zea mays L. VIR No. 19019	8.5 ± 0.6
Zea mays L. VIR No. 19019	8.2 ± 0.6
Zea mays L. VIR No. 18997	13.2 ± 1.1
Zea mays L. VIR No. G	6.9 ± 0.4
Zea mays L. VIR No. E	8.5 ± 0.6
Zea mays L. VIR No. E	8.5 ± 0.6
Tropaeolum majus L. 'Golden highlight'	24.0 ± 1.6; 27.0 ± 1.7
Tropaeolum majus L. 'American Queen'	22.8 ± 1.2
Tropaeolum majus L. 'Empress of India'	26.9 ± 2.0
Lilium longiflorum L. 'Eagle'	51.0 ± 6.0
Lilium longiflorum L. 'Nuance'	55.0 ± 7.4

cells do not divide, instead growing at almost the same relative rate as in the proliferation domain of the meristem (Verbelen *et al.*, 2006; Baluška *et al.*, 2010; Ivanov and Dubrovsky, 2013). The length of the transition domain varies among species (Ivanov and Dubrovsky, 2013). In some species, for instance *A. thaliana*, the transition domain may comprise up to 15–27 % of the meristem length (Pacheco-Escobedo *et al.*, 2016), but it is usually shorter in other species (e.g. *Z. mays*, *Allium cepa*) (Ivanov and Dubrovsky, 2013; Kirschner *et al.*, 2017). Note that in a root that obeys the above-mentioned assumptions where *T* is constant along the meristem, the cells located at a level that is about half the length of the RAM, and all of the cells above that level, transit to the elongation zone during a time period equal to one cycle. This observation suggests that cells pass through the transition domain during a short period of time, i.e. shorter than *T*. This explains why the probability of observing divisions in the transition domain is very low. Thus, if the root grows at a constant rate and the cell number in the RAM does not change over time, the root growth rate (*V*), length of fully elongated cells (*l*), number of meristematic cells in a file (*Nm*) and *T* are associated via a simple relationship (Ivanov, 1974, 1994; Ivanov and Dubrovsky, 1997):

$$T = \left(\ln 2\, Nm\, l\right) V^{-1} \quad (1)$$

Under these assumptions, not all cells from the basal half of the meristem have time to divide before exiting the meristem. If the root grows at a non-constant rate and *Nm* and *l* change over time, the average values can be used instead, although this may cause errors.

The number of meristematic cells in a file was calculated as the ratio between RAM length (*Lm*) and the average length of cortical meristematic cells (*lm*). The latter was determined for eight to ten 50-µm portions per root located along the RAM by counting the number of cells per portion and dividing the length of the portion by the number of cells. Fifty fully elongated cortical cells per root were measured with an ocular micrometer, and the average elongated cell length (*l*) was used to estimate *T*. Cell lengths in the middle cortex layers were usually measured. For most species, eight roots were examined per experiment. For some species, independent experiments were performed two to four times (Supplementary Data Table S1). The values of *T* (h) were calculated using eqn (1). For each variable of eqn (1), the average value and standard error (s.e.) were estimated. The s.e. of T was estimated using the following equation:

$$s_{\bar{z}} = \bar{z}\sqrt{\left(\frac{s_{\bar{x}}}{\bar{x}}\right)^2 + \left(\frac{s_{\bar{y}}}{\bar{y}}\right)^2} \quad (2)$$

where $\bar{x}$ and $\bar{y}$ are average variables and $S_{\bar{x}}$ and $S_{\bar{y}}$ are standard errors of the respective averages, and $\bar{Z}$ and $S_{\bar{z}}$ are the $\bar{x}$-to-$\bar{y}$ ratio (or $\bar{x}$ and $\bar{y}$ product) and the standard error of the ratio (or the product), respectively (Urbach, 1964). *T* values reported in different laboratories for a species were used to estimate the global average value; s.e. was also estimated using eqn (2). For comparison purposes, only those reported *T* values determined by the thymidine method which were obtained for plants grown at 23 ± 2 °C were used (listed in Table 1).

RESULTS

Duration of the mitotic cycle estimated by the ^{3}H-thymidine method varies little for roots of the same species

Before comparing the results obtained by the two methods, we analysed the variability of the data for *T* values reported for the same species obtained by various laboratories. We obtained data reported more than once for the same species from young seedlings of 17 species grown at 22–24 °C (Table S1). For most of these species, *T* varied within a narrow limit, with a few exceptions. This result is somewhat surprising, since the data encompass research results from several studies performed in

different countries and years on the roots of seedlings of different ages under diverse growing conditions.

We encountered several difficulties in comparing these data. *T* values can vary across different varieties or cultivars of the same species. Some reports do not mention the cultivars or varieties examined. Creber *et al.* (1993) recorded a variation of *T* in roots from different populations of *Dactylis glomerata*, which also differed slightly in terms of haploid DNA content. However, the curves of labelled mitoses in Creber *et al.* (1993) diverge significantly from the classical curves, which probably indicates significant heterogeneity of *T* in individual roots or heterogeneity in *T* within the RAM. Furthermore, it is important to consider that *T* can change in the same root during growth. Gahan and Hurst (1976) showed that in maize roots, the average *T* value changed a few times over a 20-d period. Notably, in most of the studies cited (Table 2), the roots of seedlings were sampled several days after germination, when they were growing at a constant and relatively high rate.

To date, an evaluation of the accuracy of *T* estimated by the thymidine method has not been reported. The *T* values are frequently provided at an accuracy of up to tenths of an hour, but the method used to determine the percentage of labelled mitoses is clearly less precise. For example, to measure the percentage of mitotic labelled cells, 100 mitoses are often analysed each time root cells are fixed. If the proportion of labelled mitoses equals 50 %, the standard deviation for the analysis of 100 mitoses is at least 5 %. Therefore, the actual accuracy of *T* value determinations is at least 10 %. Nevertheless, despite these shortcomings, there is a striking similarity among the estimated *T* values for roots of the same species obtained in different studies performed in different laboratories and years. This finding allowed us to compare *T* for the same species obtained by the thymidine versus RCP methods.

Estimating cell cycle duration using the RCP method

We determined *T* in the RAMs of various angiosperm species using the RCP method. Under our growth conditions, the variation of *T* within a species occurred in a relatively narrow window (Tables 1 and 3). The standard error of *T* estimated by the RCP method for a single species was approx. 10 % (Table 1). The reproducibility of repeated estimates was on the order of 10–15 % (Table S1). These results are similar to those obtained by the thymidine method (Table 1, Fig. 1).

We determined *T* in the seedling roots of different varieties of several plant species (Table 3). This question has not previously been addressed in the literature. We found that in some species, such as maize, variations between varieties were high, and *T* determined by the RCP method ranged from a minimum of 5.1 to a maximum of 14.4 h (Table 3). Nonetheless, the average *T* values determined by the RCP and thymidine methods differed by only 30 % (Table 1). Such differences are of interest, since it is unlikely that the roots of different varieties of plants differ significantly in terms of haploid DNA content, a generally recognized factor that affects *T* (e.g. Francis *et al.*, 2008). There are several possible explanations for the variation in *T*. In *Dactylis glomerata*, a significantly larger *T*, as determined by the thymidine method (Table 2), was detected in seedlings from various populations with some deviations in haploid DNA content, although these deviations were <10 % (Creber *et al.*, 1993). There might be another explanation for the variability in *T* among populations (Table 3). *T* is noticeably higher in roots before a constant growth rate is attained compared to roots growing at a constant rate, as observed for *Vicia faba* and *Pisum sativum* roots (data not shown). This difference might not have been accounted for in some studies.

Comparison of the results obtained from the thymidine versus RCP methods

We determined *T* for 73 angiosperm species using the RCP method and compared the results with published results obtained by the thymidine method. For 69 of the species, the estimates of *T* obtained by the two methods were nearly identical (Table 1, Fig. 1). The *T* values obtained differed significantly from previously published data for only a few species: *Epilobium hirsutum*, *Tropaeolum majus*, *Ornithogalum umbellatum* and *Lilium longiflorum*. Repeated *T* estimates using the RCP method obtained from different groups of seedlings grown from different seed stocks coincided (Table S1). Hence, these differences were not due to discrepancies in the methods used for these species. Overall, these results indicate that the two methods yield similar results, which confirm the previously observed similarity in *T* values reported for only a few species (Ivanov and Dubrovsky, 1997). Thus, the RCP method, which is based on a simple exponential model of cell proliferation in

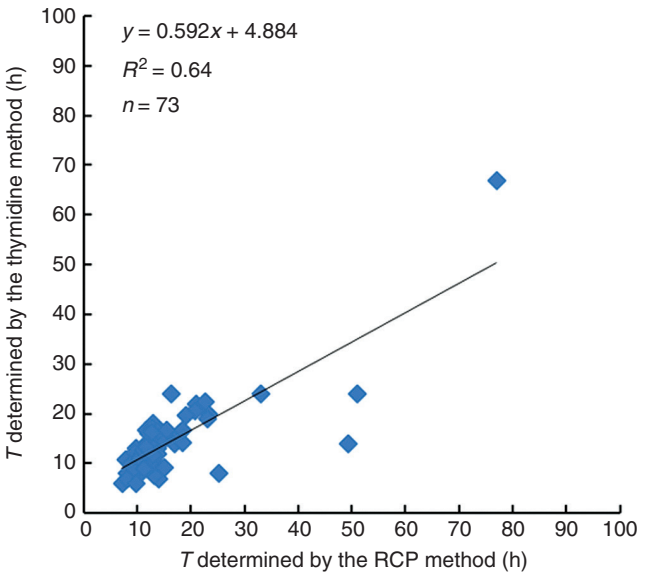

FIG. 1. Correlation between cell cycle duration determined using the rate-of-cell-production (RCP) method (obtained in this study) and the ^{3}H-thymidine method (reported in the literature) for the same species. For 73 species, the primary data are presented in Tables 1 and 2; correlation coefficient $r = 0.80$. If excluding data from the *Epilobium*, *Tropaeolum*, *Ornithogalum* and *Lilium* species ($n = 69$), $R^2 = 0.86$ and $r = 0.93$.

the RAM, confirms the results of the more complicated thymidine method.

Root growth rate calculated based on measured l *and* Nm *values and from published* T *values obtained by the thymidine method agrees with measured values*

To validate the model of the link between cell cycle duration, rate of cell production and rate of root growth (Ivanov and Dubrovsky, 1997), we compared the experimentally determined root growth rates (V) with the root growth rates (Vc) calculated based on published data, where T was determined by the thymidine method and from measured Nm and l values. From eqn (1), it follows that

$$Vc = \left(\ln 2 Nm\ l\right) T^{-1} \quad (3)$$

Nm and l were measured in the species listed in Table 1, and T values were obtained from published results (listed in Table 1). In most cases, the Vc values coincided with experimentally measured V values, except for a few species where data from the thymidine and RCP methods greatly diverged (i.e. *Epilobium hirsutum*, *Tropaeolum majus* and *Ornithogalum umbellatum*) (Fig. 2).

DISCUSSION

Our results show that T values determined by the RCP method are in good agreement with published data obtained by the thymidine method for 69 out of 73 species; it remains unclear why these values differ in the five species. Overall, this analysis demonstrates that (1) despite their different methodological approaches, the two methods yield nearly the same or similar results; (2) the model used in the RCP approach appears to be correct; and (3) despite the differences in growth conditions used in different laboratories and the different methods of analysis, not much variability was found in the distribution of T measurements within a species. In practical terms, this indicates that the RCP method, which is much simpler and less time-consuming than the thymidine method, can be routinely used for many species. Prior to the current study, the RCP approach was used in only a few laboratories.

The good agreement in the values obtained by the RCP and thymidine methods suggests that the assumptions used for the exponential model of cell proliferation to estimate cell cycle duration are correct. We are aware that any assumption represents a simplification. Nonetheless, the results obtained by the two methods differed by only an average of 0.6 ± 2.7 % for 69 of the 73 species [mean ± s.e., calculated based on data in Table 1: *Epilobium hirsutum*, *Tropaeolum majus*, *Ornithogalum umbellatum* and *Lilium longiflorum* were excluded (see below)], which is surprisingly accurate for a biological process. Nevertheless, it might appear that the main assumptions of the model (that all cells in the RAM proliferate and that the average T for all meristematic cells is the same) are weak. To explain the basis of these assumptions, we must consider the longitudinal organization of root growth zones and the behaviour of the cells

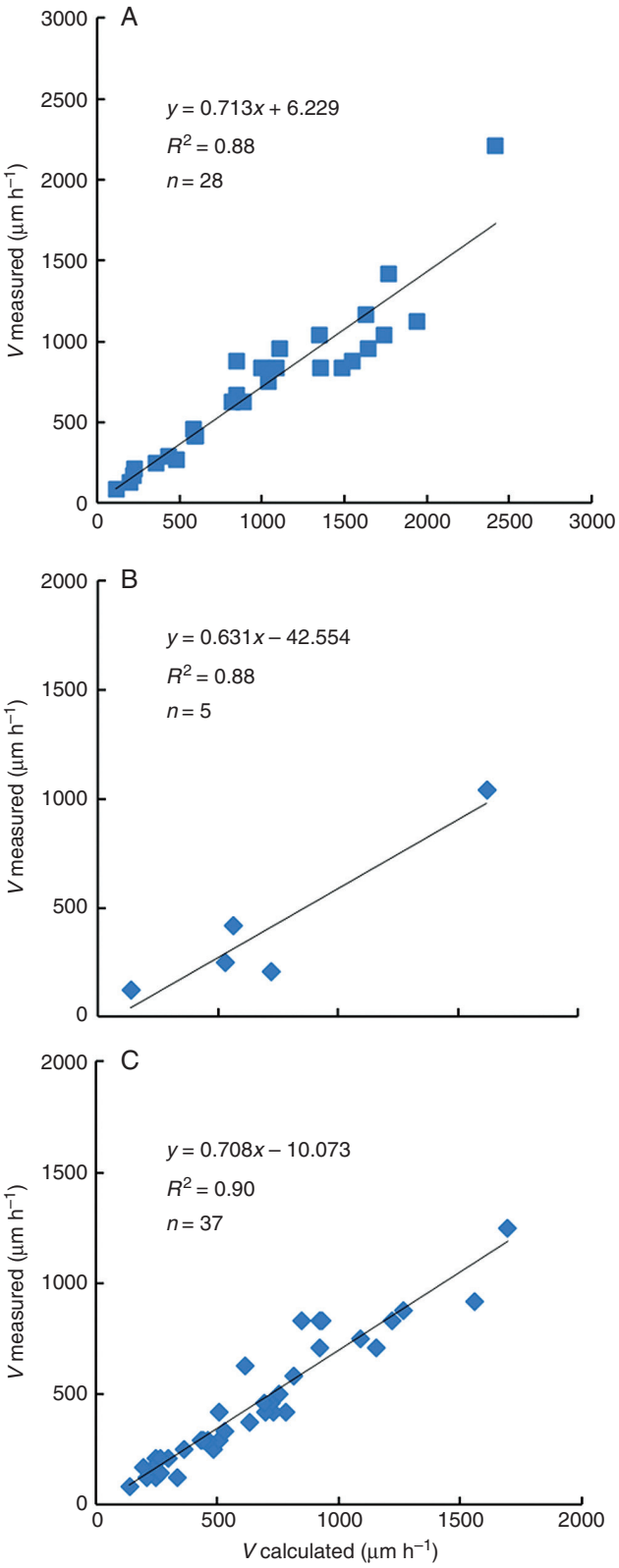

FIG. 2. Correlation between the measured rate of root growth and the predicted rate of root growth calculated based on the cell cycle duration reported in the literature (determined by the ^{3}H-thymidine method). The rate of root growth was calculated using eqn (3). (A) Primary roots of monocotyledonous species. (B) Primary roots of dicotyledonous species. (C) Adventitious roots of monocotyledonous species. Correlation coefficients $r = 0.94$ (A, B) and 0.95 (C).

within them. Some authors considered that cells in the basal portion of the RAM have a longer cycle time than in the rest of the RAM (Hejnowicz, 1959) or that not all cells in the basal RAM portion proliferate (Clowes, 1971, 1976), which contradict our assumptions. However, these conclusions were drawn based on labelled mitosis curves and did not consider the exponential age distribution of cells within the RAM (Ivanov, 1974, 1994; Webster and Davidson, 1980). Our analysis showed that the proximal portion of the RAM represents a transition domain where cells still divide (Lavrekha *et al.*, 2017), and the relative cell growth rate is the same as in the rest of the RAM (Ivanov and Dubrovsky, 2013). Also, the duration of the last cell cycle in the RAM is the same as in the proliferation domain (Balodis and Ivanov, 1970), but exit from the cell cycle at the end of the RAM is a heterogeneous process. At a given time point, cell division is less common in this domain than in the other regions, but not because cells divide less frequently or because not all cells proliferate. The lower incidence of cell division is a consequence of cell flux. With increasing distance from the quiescent centre, the cells are displaced more rapidly from the RAM (Ivanov, 1974, 1994; Ivanov and Dubrovsky, 1997, 2013). This analysis shows that during the time equal to one cell cycle, *ln2* number of cells in the meristem (~69 %) are displaced from the RAM to the elongation zone. Therefore, during the last cycle, while the cells are displaced from the RAM, not all of them have sufficient time to pass through mitosis. This explains why the mitotic index decreases sharply in the transition domain. On the other hand, cells in the quiescent centre, including initial cells, have much longer cycle times (Barlow, 1976*b*; Ivanov, 1994), but these cells commonly comprise less than 1 % of all cells in the RAM. Therefore, we assume that the average cycle time is the same for all cells. If the differences in T between individual cells were significant, the cell length within the RAM would also vary significantly, but we know this is not the case (Ivanov, 1971; Baskin, 2000). Analysis of heterogeneity of T in sister cells within the maize RAM showed they do not vary by more than 15–20 % (Ivanov, 1971). Recent time-lapse studies in arabidopsis showed that variation in sister cell T averaged 8.3 % (von Wangenheim *et al.*, 2017*b*; data extracted from their Video 3, $n = 6$ sister groups). Another source of heterogeneity is that some cell types (metaxylem in monocots) stop proliferating earlier, but the fraction of these cells is also not significant. Therefore, in the model, it is assumed that all cells have the same average T and that all cells proliferate. It is important to note that a reference thymidine method for determination of T also assumes that all cells proliferate, and only average T for all the meristem is determined. The use of live-cell imaging and high-resolution visualization of vertically grown roots (Maizel *et al.*, 2011; von Wangenheim *et al.*, 2017*a*, *b*) will reveal how close the results obtained by the two methods are to reports of T determinations based on time-lapse studies (see also Table S2).

Another important aspect of this work is related to the identification of the proximal meristem boundary. We defined the RAM as an area that includes the proliferation and transition domains, where cells proliferate more actively in the former than in the latter (Ivanov and Dubrovsky, 2013). However, dividing cells are indeed present in the transition domain and recent work on arabidopsis confirms this notion (Lavrekha *et al.*, 2017). When T is determined by the thymidine method, all RAM cells, counting those in the transition domain, are included in the analysis. As the goal of this study was to compare the RCP method with the thymidine method, the number of RAM cells was determined for both domains instead of only the proliferation domain. Clearly, this can be one of the limitations of the RCP method used here. Strictly speaking, as the method applies an exponential model, only the proliferation domain of the RAM should be considered and eqn (1) should take into account the number of cells in the proliferation domain and not the total number of meristematic cells. This corrected approach was successfully used for determination of T in arabidopsis (see below). The main result of the current study is a good overall agreement in T values determined using the RCP and thymidine methods; the difference for all the species was not statistically significant ($P > 0.05$, Student's t-test) and T determined by the RCP method was on average only 10 % greater than that determined by the ^{3}H-thymidine method. This suggests that the exponential model can indeed be applied to the entire RAM, at least in practical terms, when determining the average T in the RAM. It is important to underline that the RCP approach permits estimation of average T values and does not reflect possible differences between different cell types, cell locations within the RAM and variations in T between sister cells.

Table 1 includes a comparison of the methods for the 73 species for which determinations by the thymidine method are available. Note that some data available in the literature lack certain details (plant age, temperature, growth conditions) and the results might not be comparable. The T values obtained by the RCP method differed significantly from published data for *Epilobium hirsutum.* The T value for this species determined by the thymidine method was obtained from a review by Francis *et al.* (2008), who cite a PhD thesis that is not publicly available. Data for another outlier, *Tropaeolum majus*, were taken from Olszewska *et al.* (1990), but no details were provided in their study. Our results obtained by the RCP method for this species were consistent for three different varieties and differed by only 15.6 % (Table 3). Similarly, for adventitious roots of *Ornithogalum umbellatum*, no data were available for parameters such as bulb age and root growth dynamics (Tagliasacchi *et al.*, 1983). One possible explanation for why all outliers among T values determined by the RCP method were larger than previously reported values is that in these studies, no root growth dynamics were evaluated, and T was determined during the root growth acceleration stage.

It is also interesting to compare the results of T determinations by the RCP method with those of other methods used in the model plant *A. thaliana.* This species was not included in our current analysis because, although many reports of T determinations are available, the only data obtained by the thymidine method are for young seedlings, and the RCP method cannot be applied to plants of a similar age (Table S2). A single T determination was obtained by the thymidine method for arabidopsis roots of seedlings on the first day after germination (Van't Hof *et al.*, 1978), when no steady-state growth has been attained (Table S2). Since in the reported studies using the RCP method, T was estimated at later stages, a pertinent comparison with the thymidine method is not possible. Details of T estimation in arabidopsis by the RCP method can be found (Napsucialy-Mendivil *et al.*, 2014; López-Bucio *et al.*, 2014). In most studies, T values obtained by the RCP method

use the number of cells in the proliferation domain for eqn (1); however, some studies use the number of cells in the entire RAM (Table S2). Taking into account estimations obtained using other methods, irrespective of ecotype, the average T in roots of arabidopsis seedlings aged 6–12 d after germination (dag) grown at 20–23 °C is 16.6 ± 0.8 h, which is comparable to the average T of 15.0 ± 1.2 h (in both samples $n = 8$, mean ± s.e., $P > 0.05$ Student's t-test, Table S2) estimated by the RCP method for 5–8 dag arabidopsis seedlings grown at the same temperature when the number of cells was determined in the proliferation domain. Interestingly, the T values were very close for the same ecotype (Col-0) at the same temperature (23 °C) and age (5 dag), i.e. 14.3 h in time-lapse studies (Yin *et al.*, 2014; von Wangenheim *et al.*, 2017) and 16.4 h estimated by the RCP method (Table S2). These studies show that, similar to other species, an exponential model of cell proliferation is appropriate for estimating cell cycle duration in the arabidopsis RAM.

In summary, our results indicate that in 96 % of the species examined, T values in the RAM determined by the thymidine and RCP methods were very similar. This indicates that the RCP method is a reliable, straightforward approach that can be used to investigate numerous subjects. Notably, T is a highly stable parameter, as estimations for a single species grown under different conditions and in different countries, years and varieties produced highly similar values. Our data also indicate that T can be considered a species-specific feature. The duration of the cell cycle in the RAM is an important root trait. Analysis of this trait will provide insight into root growth mechanisms and their endogenous and exogenous control and into the general evolutionary ecology and phenomics of roots. However, this trait has not yet been used in large-scale analyses investigating these processes (Walter and Schurr, 2005; Furbank and Tester, 2011; Comas *et al.*, 2012; Maherali, 2017; Valverde-Barrantes *et al.*, 2017). The application of the RCP method may help fill this gap.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: Results of independent experiments for determinations of T by the RCP method ($n = 8$, mean ± s.e.). Table S2: Cell cycle duration in wild type *Arabidopsis thaliana* L. (Heynh) root apical meristem determined by different methods.

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